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How to isolate a ready-to-use adipose-derived stem cells pellet for clinical application

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Abstract. – Adipose-derived stem cells (ASCs) are multipotent mesenchymal stem cells (MSCs) that show definitive stem cell characteristics such as plastic adherence in culture, ability to maintain multipotency upon *in vitro* expansion, and self-renewal capacity. ASCs are particularly promising for use in regenerative medicine because they can be harvested easily from adipose tissue by standard liposuction, with minimal donor site morbidity. Since ASCs do not necessitate ex vivo expansion to obtain clinically significant cell numbers, it is critical to identify a standardized method that maximizes the number of ASCs collected. Based on current literature, there is no standardized method to isolate ASCs for clinical application. Furthermore, clinical studies involving ASCs often show inconsistencies in the reported results. Such studies often use research-derived isolation protocols, which are complex, time-consuming, and involve the use of chemical and animal-derived reagents. In this paper, we present an in-depth review of the available data on ASC isolation protocols. Moreover, we describe our isolation protocol that allows the collection of a ready-to-use ASC pellet for clinical application.

Key Words:
Adult stem cell, Mesenchymal stem cell, Regenerative medicine, Cell- and tissue-based therapy.

Introduction

Adipose-derived stem cells (ASCs) are multipotent mesenchymal stem cells (MSCs) whose differentiation potential is similar to that of other MSCs[1]. They exhibit definitive stem cell characteristics such as plastic adherence in culture, ability to maintain multipotency upon *in vitro* expansion, and self-renewal capacity[2]. When compared to bone-marrow stem cells (BMSCs), ASCs have a greater proliferative rate as well as a higher yield upon isolation and appear to be more genetically stable in long-term culture[3,4]. ASCs are particularly promising for use in regenerative medicine and could be an attractive source of MSCs, because they can be easily harvested in large quantities from adipose tissue fragments with minimal donor site morbidity[2]. Pre-clinical and clinical studies in diverse fields followed the discovery that ASCs were not only precursors to adipocytes, but also readily underwent expansion and had the capacity to undergo adipogenic, osteogenic, chondrogenic, neurogenic, and myogenic differentiation *in vitro*[5-7]. Moreover, because ASCs do not require extensive manipulation before application, there is no requirement for "cell manufacturing" compliance in accordance with the European Good Manufacturing Practice (eGMP) Guidelines[8,9]. These restrictions are not applied in case of minimal manipulation [Regulation (EC) No 1394/2007 of the European Parliament and the Council][10]. Therefore, the development of high-yield isolation technologies with minimal handling for ASCs would be highly desirable for clinical applications[5]. In 2001, Zuk et al[11] first identified and described ASCs, which they referred to as "processed lipoaspirate cells", since they isolated these multipotent stem cells from adipose tissue obtained after liposuction procedures. Zuk et al[2] based their isolation method on existing enzymatic strategies. When enzymatically digested, adipose tissue yields a heterogeneous population of many cell types (pre-adipocytes, fibroblasts, vascular smooth muscle cells, endothelial cells, resident monocytes/macrophages, lymphocytes, and ASCs), which, upon isolation, is termed the stromal vascular fraction (SVF)[12], of which ASCs alone comprise 30%[1]. The isolation process described by Zuk et al[11] is the most

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commonly published method for ASC isolation[13]. The freshly harvested lipoaspirate was washed with sterile phosphate buffered saline (PBS), enzymatically digested, and subsequently subjected to red blood cell lysis. However, this procedure is effective, it could be complex, expensive, and particularly time-consuming for clinical application. In this work, we aim to present an in-depth review of the data available on ASC isolation protocols. Moreover, we will provide a stepwise description of our procedure for ASC isolation[14]. Our method was conceived and developed for clinical application, and is therefore, easy, safe, and fast (80 min), and yields a ready-to-use ASC pellet[14-16]. Once the adipose tissue is harvested by standard liposuction procedures, the isolation process is carried out by mechanical (centrifuge) and enzymatic (collagenase) methods[14].

Materials and Methods

The entire procedure is carried out in day surgery under assisted local anesthesia. The patient is hospitalized on the morning of the surgery and must be fasting from midnight. The discharge takes place in the afternoon. Our isolation protocol involves the use of the special sterile, single-use, 50 ml Fat Processing Unit (FPU) syringes and the Lipokit 416D (Medikhan, Seocho-gu, Seoul, South Korea), which is a CE-marked and FDA-approved device for autologous fat transfer. It is considered an all-in-one closed device, since it consists of a built-in vacuum and pressure pump with an air hose connection. The FPU syringes are designed with weight-mesh filter pistons, which press the lipoaspirate during the centrifugation and remove the oil fraction. These devices simplify and reduce the steps for collection, filtering, and isolation of ASCs. Furthermore, air contact is prevented, ensuring sterility and, therefore, safety of the procedure. Such closed processing systems are considered superior, although there are no data to suggest that air exposure increases infection rates or reduces graft viability[17].

Protocol Steps (Figure 1)

Preliminary Preparation: Setting up the Lipokit

Two 50 ml Lipokit/Adivive FPU syringes (TP-101) are assembled according to the manufacturer's instructions and connected to the Lipokit 416D. The TP-112 3 Way Valve is then inserted into the distal end of the 50 ml FPU, which is finally connected to a tumescent cannula at one end, and to the infiltration bag/bottle (containing 1000 ml of Klein solution) at the other end.

Performing the Liposuction Procedure

One milliliter of 2% lidocaine is injected into the area where access to the subcutaneous tissue for liposuction is planned. One or two accesses are usually sufficient for each area. One-centimeter cutaneous cuts are made with a No. 11 scalpel, and 150-250 ml of Klein solution is infiltrated into the area chosen for liposuction. The 3-way valve with the tumescent cannula is then removed from the distal end of the 50 ml FPU syringes and is replaced with the chosen suction cannula. Hundred milliliters of adipose tissue are harvested, filling both the 50 ml FPU syringes. The harvest area is squeezed using wet gauze to remove as much Klein solution as possible. Incisions are then sutured with 4/0 monofilament.

1st Centrifugation Step

The green FPU screw caps provided are screwed onto the distal end of the 50 ml FPU syringes. The piston screws of the mesh pistons are then unscrewed counterclockwise for 3-4 full turns, so that the two 50 ml FPU syringes can be placed symmetrically into the centrifuge buckets inside the Lipokit 416D system and centrifuged at 1260 g for 3 min. Once the centrifuge has completely stopped, the syringes are removed while being kept straight. The oil above the piston is cleared away using dry gauze, and the piston filters are closed by rotating the screw 3-4 times in a clockwise direction. The bottom tumescent/blood cell fraction is then ejected by unscrewing the cap and pushing down the piston.

Collagenase-Assisted Digestion of the Purified Adipose Tissue

The purified fat is subsequently transferred from the TP-101 FPU Syringes to two 50 ml Lipokit/Adivive FPU syringes (TP-102; previously assembled according to manufacturer's instructions) by connecting them with the provided syringe weight adapter. Next, 25 ml of a 0.1% collagenase digestion solution is transferred into each FPU syringe containing the purified adipose tissue. The FPUs are gently rotated for 30 s and then placed into the Celtibator bucket within the Celtibator, which is activated for 30 min at 37°C.

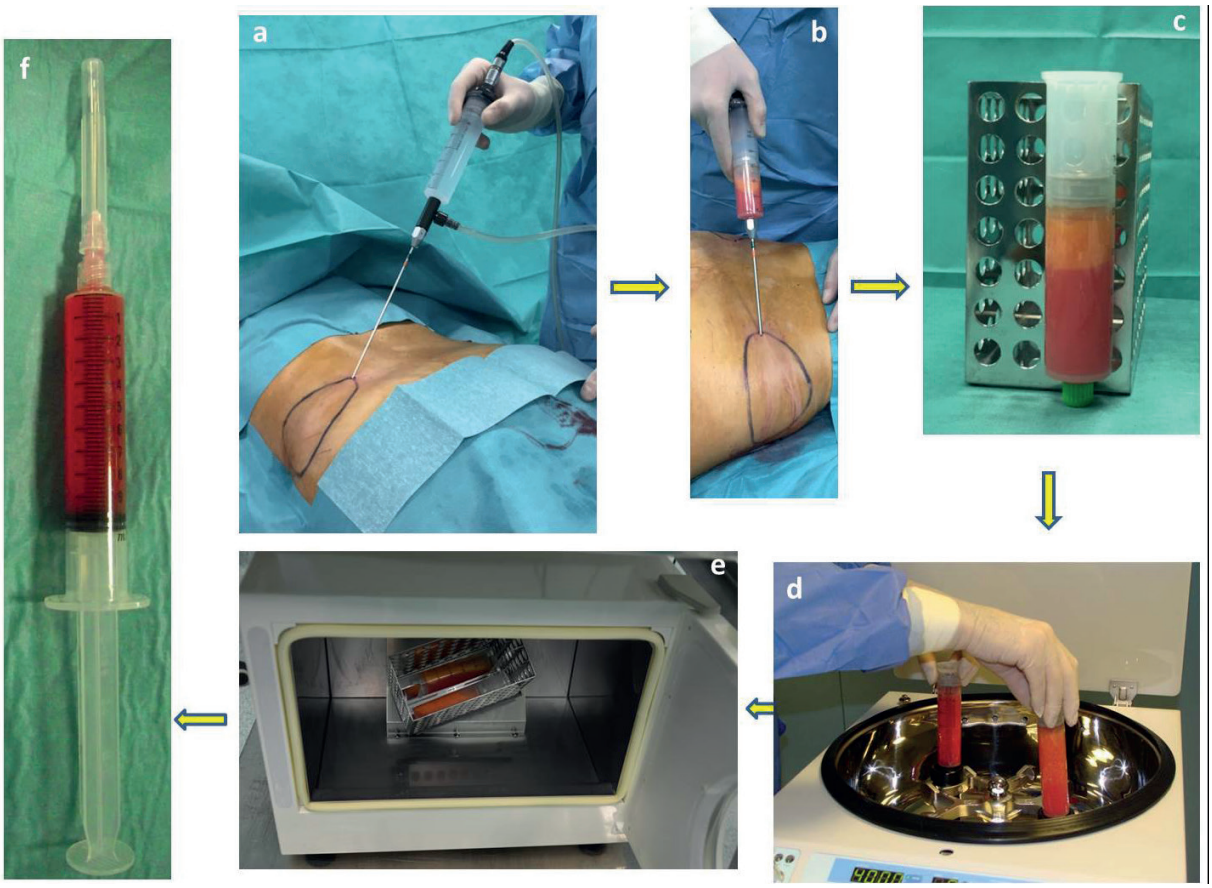

Figure 1. ASC isolation protocol. The preliminary step is the liposuction procedure. Firstly infiltrate 150-250 ml of Klein solution in the area(s) chosen for liposuction ***(A)***. Then harvest 100 ml of adipose tissue by filling two 50 ml FPU syringes ***(B,C)***. Suture each incision with 4/0 monofilament stiches and cover them with a medication. Centrifuge the harvested adipose tissue at 1260 g for 3 min ***(D)***. Remove the oil above the piston by using dry gauze, and discard the bottom tumescent/blood cell fraction to obtain purified adipose tissue ready to be blended together with the collagenase digestion solution. Incubate and toss the FPU syringes at 37°C for 30 min ***(E)***. Centrifuge the two FPU syringes at 200 RCF for 4 min. Then proceed with the rinse and spin steps. Transfer the top layer of the digested fat from the FPU syringes to empty Luer Lock syringes connected with the Luer Lock Adapter II, leaving 10 ml of clear digested fluid in the FPUs. Fill the FPU syringes with saline solution. Perform the spin, rinse, and spin cycle another time. Remove the top layer of the digested mixture from the FPU syringes into empty Luer Lock syringes connected with the Luer Lock Adapter II, leaving the 5 ml bottom fluid in which the SVF pellet is present. All the steps must be performed keeping the FPU syringes straight. Transfer the isolated SVF pellet into Luer Lock syringes of the desired size with the Luer Lock Adapter. The ASC pellet is now ready to be injected ***(F)***.

Once the Celtibator has stopped, both the 50 ml FPU syringes are placed inside the buckets of the Lipokit 416D system and centrifuged at 200 relative centrifugal force (RCF) for 4 min.

Isolating the SVF Pellet From the Digested Adipose Tissue: Spin and Rinse

Once the centrifuge has completely stopped, the FPU syringes are connected to an empty 50 ml Luer Lock syringe to transfer the top layer of digested fat while leaving 10 ml of clear digested fluid in the FPUs. The 50 ml Luer Lock syringe containing the discarded digested mixture is then replaced with a 50 ml Luer Lock containing saline solution, to fill the FPUs up to 50 ml. Both the 50 ml FPU syringes are then placed in the centrifuge, which is activated at 200 RCF for 4 min. This spin and rinse step is performed twice. The FPUs are connected to an empty 50 ml Luer Lock syringe to transfer the top layer of the digested mixture, leaving the bottom 5 ml of saline solution containing the stromal vascular fraction (SVF) pellet in the FPUs. Finally, the isolated SVF pellets from both the FPUs are pooled in a 10 ml Luer Lock syringe for future use.

Precautions

When tightening the screw in the piston, care must be taken not to strip the screw, as fluid could leak or be drawn into the system and cause contamination. The FPU Screw Caps must be securely screwed on to prevent fluid leakage and damage to the cap during centrifugation.

The FPU syringes should be placed in the centrifuge symmetrically before centrifugation and contain the same volumes so that they are balanced. Furthermore, the piston must be level with the fat before spinning, with no air gaps. During the washing steps with saline, care must be taken not to create turbulent motions in the fluid that could displace the SVF pellet from the bottom, resulting in it being discarded with the washing waste fluid. It is important to perform these steps slowly. Furthermore, one syringe must not be bent over the other through the connector, in order not to strip the screw-bed. This can easily be solved if two operators, each holding one syringe, perform all the steps. At the end of the first centrifugation cycle, the lipoaspirate should be divided into 3 phases: the oil fraction at the top (over the piston), the condensed adipose tissue in the middle, and the fluid portion of the lipoaspirate at the bottom. After the wash and spin cycles, the processed material becomes increasingly clear, and the red color gradually changes to slightly pink, indicating the removal of red blood cells. After the final spin, the cell suspension becomes almost as clear as water, with the SVF pellet at the bottom of the syringe.

Reagent and Solution Preparation

Klein Solution

Klein solution can be prepared either on the morning of the surgery or the day before. One thousand milliliters of saline solution are mixed with 50 ml of 2% lidocaine (Monico S.p.a., Venezia/Mestre, Italy), 20 ml of 8.4% sodium bicarbonate solution (1 mEq/ml), and 1 ml of adrenaline (1:1000). This solution is stored at 4°C until use.

Collagenase Digestion Solution (0.1%)

The collagenase digestion solution is composed of 1 g collagenase (Collagenase NB 6 GMP Grade 17458; Serva GmbH, Heidelberg, Germany) suspended in 10 ml sterile phosphate buffered saline (PBS). It can be stored at 0°C for up to 6 months. We defrost the solution at room temperature (24°C) during surgery. Once completely thawed, 1 ml of the 0.1% collagenase suspension is added to 49 ml phosphate buffered saline (PBS).

Isolation Yield

At the end of the isolation process, a mean of 9.06×10^5 ASCs (range: $8.4\text{-}9.72 \times 10^5$; SD $\pm$ 6.6×10^5) are regularly collected, which corresponds to 25.9% of the total number (mean of 10^6 cells; range: $3\text{-}4 \times 10^6$; SD $\pm$ 5×10^5) of isolated cells[14]. The ASC yield with this protocol is considerably higher than those reported in literature, wherein ASCs reportedly account for 2% of the nucleated SVF cells[12]. Thus, our protocol yields a highly purified ASC pellet, which is ready for clinical use. The remaining 95% cells are mostly blood-derived and endothelial cells. To characterize the isolated cells and identify ASCs, a flow cytometric assay is required, with a panel of commonly used surface antigens based on current literature. We routinely use the monoclonal antibodies CD34 APC, CD45 APC-Cy7, CD73 PE, CD31 FITC, CD 90 APC, and CD105 APC. We perform the cytometric assay by using an eight-color flow cytometer (FACSC anto II; Becton Dickinson, Franklin LaKes, NJ, USA). In order to be identified as ASCs, isolated cells have to be negative for CD31 and CD45, and express CD34, CD73, CD90, and CD105, as this is the widely accepted panel of surface antibodies characteristic of ASCs[12].

Time Considerations

The entire surgical procedure takes 2 h, from the patient's arrival in the surgery room until to the conclusion. Patient preparation, anesthesia induction, and medication at the end of the surgery usually take 40 min, while the isolation process itself routinely takes 80 min. The initial procedures could last little longer, mainly in case of technical problems. However, the whole isolation protocol rarely requires more than 2 h. As the procedure could prove complex, it is recommended to have a dedicated nurse for the procedure, who can become accustomed to the different components of the Lipokit and the FPU syringes.

Discussion

Alternative Isolation Protocols

In 1964, Rodbell[18] first presented a method for *in vitro* isolation of mature adipocytes and adipogenic progenitors from rat fat tissue. Zuk et

al[11] were the first to show that the SVF fraction isolated from human lipoaspirates contained cells with multilineage potential. Since then, interest in ASCs has grown dramatically; several groups working independently have developed and refined procedures for isolating and characterizing adipose stem cells. However, depending on the isolation process, the number of ASCs obtained from 1 g of adipose tissue is highly variable. Mizuno[19] reported a yield of approximately 5×10^3 stem cells per g of adipose tissue, which is 500-fold greater than the number of MSCs obtained from 1 g of bone marrow. Boquest et al[20] reported a yield of 1×10^7 ASCs from 300 ml of lipoaspirate. Francis et al[21] described a rapid collagenase-free isolation protocol with a yield of 25×10^4 ASCs from 250 ml of lipoaspirate. Other studies[22-24] have demonstrated that 1 g of adipose tissue can yield 2×10^6 SVF cells; 10% of these cells are likely ASCs. The effects of different harvesting techniques and harvesting sites on yield and cell proliferation have also been investigated, and contradictory reports have been published. Fraser et al[25] showed that neither the site of harvest nor the harvesting technique (liposuction, syringe-based, or pump-assisted) affected the number of ASCs obtained. Francis et al[21] showed that ultrasound- and suction-assisted lipoaspiration do not exhibit significant differences in SVF cell count. Oedayrajsingh-Varma et al[24] determined whether the yield and growth characteristics of ASCs were affected by the type of harvesting technique. They concluded that the number and viability of the isolated ASCs were not affected by the surgical procedure used (resection, ultrasound-assisted liposuction, or tumescent liposuction). However, the functional properties were affected dramatically, and lower growth capacities were observed with ultrasound-assisted liposuction. The same group also concluded that the site of harvest did not affect the yield of ASCs, and reported large variation in the number of cells obtained from different donors[26]. von Heimburg et al[27] reported that resection yielded lower numbers of viable progenitors compared to liposuction aspirates. Chung et al[28] demonstrated that laser-assisted liposuction resulted in significantly lower ASC yield, stem cell viability, and proliferative rate in culture than did traditional suction-assisted lipectomy. Rossi et al[29] showed no difference in yield, characteristics, *in vitro* proliferation, differentiation, and immunomodulatory properties of ASCs when comparing the nanofat harvesting procedure to the Coleman's technique. Donor-dependent differences in ASC populations have been widely recognized[30,31]. Fat from different patients can differ significantly in ASC content, proliferative and differentiation capacities, and the ability to secrete angiogenetic growth factors[21]. Patient characteristics such as age, body max index, and pathologies likely affect ASC content and functionality. Bailey et al[32] demonstrated that ASC count and differentiation potential depend on the anatomical location and donor's gender and age. However, because of the small number of reports published and the variations in the protocols used, it is difficult to determine the optimal harvesting technique, site of harvest, and isolation procedure. Most of scientists have broadly applied a standard protocol for the isolation of ASCs from adipose tissue by using enzymatic digestion[11]. Briefly, adipose tissue obtained from patients undergoing elective suction-assisted lipectomy procedures is washed extensively with phosphate buffered saline (PBS) in order to remove blood cells, saline, and local anesthetics. The extracellular matrix is digested with 0.075% collagenase type I at 37°C for 1 h in a water bath, with gentle agitation at 125 rpm to release the cellular fraction. Collagenase is inactivated with an equal volume of Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). The infranatant is centrifuged at 1200 g for 5 min to obtain a high-density Processed LipoAspirate (PLA) cell pellet. The supernatant is then discarded and the pellet is resuspended in Dulbecco's Modified Eagle Medium (DMEM) and 10% fetal bovine serum (FBS) and filtered through a 100-µm cell strainer to remove undigested tissue fragments. The cells are pelleted and re-suspended. It takes approximately 2 h for this process, and $2\text{-}8 \times 10^8$ PLA cells can be obtained from 300 ml of liposuction fat aspirates. Markarian et al[33] described a trypsin-based enzymatic protocol for ASC isolation that is 40 times cheaper and has a five-fold smaller yield when compared to the conventional method. Nevertheless, there is some debate regarding the likelihood of xenogenic transfer of proteins from animal-derived collagenase components to the host during cell isolation. Several alternative methods for SVF isolation have been reported that avoid enzymatic digestion completely, including direct centrifugation of lipoaspirate without processing[34-36]. Yoshimura et al[37] analyzed cells isolated from the PLA and the fluid portion of the liposuction aspirate (liposuction aspirate fluid; LAF). The PLA is the suctioned adipose tissue that has been shredded by the har-

vesting procedure; LAF is primarily composed of the saline solution preoperatively injected into the site, peripheral blood, and cells or tissue fraction derived from adipose tissue. This study showed that LAF cells and adherent PLA cells have similar characteristics with respect to growth kinetics, morphology, surface marker profiles, and capacity for differentiation. A significant, although smaller, amount of ASCs, which is sufficient to be used clinically without cell expansion, can be isolated from the LAF. In light of these findings, Francis et al[21] reported a rapid isolation method wherein SVF was centrifuged directly from lipoaspirate, and cells were washed with a red blood cell lysis buffer to produce cell capable of trilineage differentiation. Bianchi et al[10] demonstrated that a non-expanded, ready-to-use fat product may be obtained with minimal tissue manipulation by pushing aspirated fat through size reduction filters while allowing waste products to exit in a closed system. Raposio et al[5,15] described an isolation procedure from a conventional liposuction procedure performed by mechanical isolation under a laminar airflow bench. No collagenase, serum, or animal-derived reagents were needed. The entire isolation process took approximately 15 min and yielded a mean of 5×10^5 adipose-derived stem cells (rang: 4.0-6.0 $\times 10^5$; SD, $\pm 1 \times 10^5$) with a 97% viable cell rate[5].

Critical Parameters

For successful ASC isolation, one of the most critical components is the harvesting procedure. High-quality adipose tissue must be collected in order to gather the highest amount of stem cells. Care must be taken while choosing the harvesting site, so that the area of the body with the thickest subcutaneous layer is identified. No difference was found between donor sites in term of ASC yield and viability. Therefore, the hips, thighs, and abdomen areas are usually chosen, as they typically contain the most adipose tissue, and the harvesting procedure is easier and causes minimal cosmetic disadvantages. Subcutaneous adipose tissue should be properly infiltrated with Klein solution, since this is the primary step for a tumescent liposuction. We usually infiltrate 150-250 ml of Klein solution in order to harvest 100 ml of lipoaspirate depending on the donor area and adipose tissue thickness.

Troubleshooting

In Europe, ASCs are considered advanced therapy medicinal products, as defined by the European Union 1394/2007[13]. Since the clinical use of enzymatically isolated ASCs is not prohibited because it provides satisfactory results, and is not regarded as extensive manipulation, there is no requirement for "cell manufacturing" compliance in accordance with current eGMP regulations[9]. However, in the United States, enzymatically isolated ASCs are considered beyond the scope of "minimal manipulation" and are, therefore, classified as a drug, which is fully governed by the Food and Drug Administration (FDA)[38]. Thus, prior to clinical application of enzymatically isolated ASCs, a surgeon has to submit an Investigational New Drug application to the FDA, which is expensive and complex. The clinical application of mechanically isolated ASCs during the same operative session with minimal manipulation, however, is categorized as practice of medicine and thus allowed. Our previously described method could, therefore, be an effective alternative when enzymatically isolated ASCs cannot be used[5].

Conclusions

ASCs are multipotent MSCs that show definitive stem cell characteristics such as plastic-adherence in culture, ability to maintain multipotency upon *in vitro* expansion, and self-renewal capacity[11,39-42]. ASCs are particularly promising candidates for use in regenerative medicine as they can be easily harvested in large quantities from adipose tissue fragments, with minimal donor site morbidity[2]. Therefore, the development of high-yield ASC isolation technologies with minimal handling is highly desirable for clinical applications[5]. The isolation process described by Zuk et al[11] is the most commonly published method for ASC isolation[13]. Although this procedure has proven effective, it could be complex, expensive, and particularly time-consuming for clinical application. Other independent groups have published clinical reports on ASC isolation processes; however, the reported results have often shown inconsistencies. Furthermore, most of these studies[6,8,11,17,20-22,33] used research-derived isolation protocols, which shared the same drawbacks of the isolation process described by Zuk et al[2]. Thus, a number of groups have also described simpler, more economic, and quicker ASC isolation protocols than the conventional method using collagenase[5,10,15,33-36]. Although cells isolated by these methods exhibit phenotypes and differentiation potentials similar to those of

ASCs isolated by collagenase digestion, the relative yield was still significantly lower, ranging from a 3- to 19-fold decrease[13]. To the best of our knowledge, no studies have been carried out on the amount of viable ASCs required to ensure the expected therapeutic outcome. The current opinion is that the larger the amount of isolated ASCs, the more likely a favorable therapeutic outcome[43-55]. We suggest that the proposed isolation protocols should be tested in clinical settings in order to define a gold standard for ASC isolation. Once a standardized protocol is established among researchers, results of various studies can reliably be compared.

Conflict of Interest

The authors declare no conflicts of interest.

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