Optimization of Adipose-Derived Mesenchymal Stem Cells Harvest Protocol Toward Clinical Applications

Introduction

Mesenchymal Stem Cells (MSCs) are one of the most promising candidates for stem cell therapeutic modalities due to their pluripotency and immunomodulative property. Therefore, they are being used in a variety of cell therapy clinical studies and full-scale trials (http://www.wjgnet.com/1948-0210/pdf/v3/i4/25.pdf).

Adipose tissue is an abundant source of adipose-derived MSCs (ADMSCs) (1). Recently, due to the ever-increasing number of studies, development of ADMSCs-based applications have attracted interest of multiple academic and biotechnological companies. However, development of clinical applications requires standardization of method for harvest of MSCs from adipose tissue. Moreover, such method should comply with safety requirements of public health authorities. The major focus for optimization and adjustment of protocols remains on culture and expansion conditions. However, the initial steps of collecting and processing of fat tissue require thorough investigation for assuring quality of the process.

MSCs of adipose tissue are part of the stromal perivascular fraction (SVF). The most common method for SVF harvest is application of enzymatic digestion to adipose tissue. Application of collagenase is a well-known and effective method for extraction of SVF from fat. MSCs derived from collagenase-digested human adipose tissue have been demonstrated to differentiate into already classical precursors for adipocytes, chondrocytes and osteocytes as well as into cell lineages of skeletal and smooth muscles cells, endothelial cells and neuronal cells (2, 3, 4, 5).
With a few exceptions, different commercial collagenases are all made from *C. histolyticum*, or recombinant versions where *Escherichia coli* expresses a gene cloned from *C. histolyticum*. However, insufficient consistency of inter-lot activity and purity make this step of SVF separation extremely vulnerable.

The current challenge is to isolate the maximum possible number of functional progenitors without damaging the cell functionality and in conformity with standards of Good Manufacturing Process (GMP). Thorough standardization of collagenase purity and lot-to-lot variation, along with adequate processing of cell preparations, appears therefore to be mandatory before initiation of human clinical trials.

Liberase MNP-S (Roche Diagnostics Corporation) is a novel, highly purified enzyme blend and is Sterile-A according to European Pharmacopeia. The goals of the study were to evaluate the efficacy of human adipose tissue digestion by different Liberase modalities of time and concentration and to compare with our current crude collagenase protocol.

**Materials and Methods**

The protocol for collection of human fat samples was approved by the ethical board of Jointechlabs. Patient informed consents were obtained before tissue collection. Fat samples were collected in sterile containers following scheduled abdominal liposuction procedures. Seven female patients were included in the current study. The mean patient age was 37±6 years. Mean body mass index was 23±2. All patients were tested for a wide panel of viral infections. Samples arrived at the lab approximately two hours after retrieval.

Lipoaspirates were prewashed as described in details below (see “Tissue Processing”). Five prewashed lipoaspirates were divided into four samples of 100 ml of fat each and, due to limited amount of lipoaspirate, the other two lipoaspirates were divided into three samples of 100 ml each.

Divided samples were exposed to one of the following four digestion protocols:

**Protocol #1: 3X Liberase–15 min.** (Roche, Liberase MNP-S, 35 mg, Cat. No. 05578566001. We considered 3X concentration as equal to enzymatic activity of 0.85 Wünsch units/ml. In order to get final activity concentration of 0.85 Wünsch units/ml in 100 ml of lipoaspirate, we prepared 100 ml of Liberase solution with doubled concentration of 1.7 Wünsch units/ml, which was then diluted while mixing with fat. For this purpose the entire 35 mg vial of Liberase MNP-S was reconstituted with 9 ml of HBSS (Gibco-Invitrogen) and further added to 91 ml of HBSS.

**Protocol #2: 1X Collagenase–60 min.** This is standard protocol of tissue exposure to crude collagenase (Sigma, Cat. No. C0130) at an activity of 0.28 Wünsch units/ml for 60 minutes.

**Protocol #3: 1X Liberase–60 min.** As described in Protocol #1 above, an entire vial of Liberase MNP-S was reconstituted with 9 ml of HBSS, and 3 ml of this solution was then added to 97 ml of HBSS, making 100 ml of enzyme reagent. Application of prepared solution to 100 ml of lipoaspirate was calculated to give a final concentration of 0.28 Wünsch units/ml. This 1X Liberase solution was applied for a 60-minute digestion.

**Protocol #4: 1X Liberase–30 min.** 1X Liberase MNP-S, 0.28 Wünsch units/ml, was applied to tissue for 30 minutes.
**Tissue processing**

Lipoaspirates collected and delivered in hermetically sealed sterile containers were transferred aseptically into 500 ml conical tubes (Corning, Fisher Scientific Cat. No. 07-200-621). Samples were spun at room temperature at 600 x g for 10 minutes. The liquid phase with blood at the bottom was removed using a 50 ml pipette. Four milliliters of solution were separated for carrying out a bacterial test for aerobic and anaerobic contaminations. The adipose tissue fraction was thoroughly mixed with 100 ml of Hanks’ balanced salt solution supplemented with 3% antibiotic-antimycotic (Gibco-Invitrogen, Cat. No. 15240-062) and washed at 600 x g for 10 minutes. The procedure was repeated twice, so that the tissue fraction finally appeared as homogenic white fat.

At the same time, aliquots of enzymes prepared as described elsewhere above were diluted in 100 ml of PBS.

Digestion solutions and washed adipose tissue were pre-warmed in a circulating water bath at 36.6°C for 15 minutes. Thereafter, 100 ml of enzyme solution was mixed in a conical tube with 100 ml of washed adipose tissue by shaking the tube vigorously for 5 – 10 seconds. The digestion process was maintained by following with incubation in a preheated to 36.6°C shaker for the duration as by described-above protocols. The digestion process was interrupted by adding 200 ml of STOP-solution and further vigorous manual shaking for 30 seconds. STOP-solution was based on DMEM + GlutaMAX low glucose medium (Gibco-Invitrogen, Cat. No. 10567), supplemented by 10% Mesenchymal Stem Cells evaluated Fetal Bovine Serum (MSC FBS, Gibco-Invitrogen, Cat. No. 12763) and 3% antibiotic-antimycotic.

After digestion, the ability of lipid-filled adipocytes to float was used to separate them from stromal vascular fraction (SVF). This is achieved by centrifugation at room temperature at 300 x g for 10 minutes. Following centrifugation, the entire bottom fraction was collected and transferred into a 50 ml conical tube (BD-Falcon, Fisher Scientific Cat. No. 06-443-18). Most dense unbroken mucous-like pieces that gravitated to the tube’s bottom were collected and vigorously shaken in a separate 50 ml tube with STOP-medium until cells washed out, and fibroid-like pieces floated at the top of the tube and could be discarded at that point. Washed cells were returned to the original 50 ml tube with the other portion of SVF; thereafter, the entire cell fraction was pelleted by centrifugation at 300 x g for 10 minutes.

After centrifugation, the SVF pellet was collected and resuspended in 10 ml of culture medium: DMEM + GlutaMAX, low glucose, supplemented with 15% MSC FBS and 3% antibiotics. The entire solution was passed through a 100 µm disposable vacuum filtration system (Millipore, Cat. No. SCNY00100). A sample of 0.5 ml was separated and processed for hemolysis. This fraction was utilized for cell count by Scepter (Millipore) and Neubauer chamber, viability by Trypan Blue exclusion test (Invitrogen), Propidium Iodide staining and flow cytometry. Cells for flow cytometry were washed out of the protein supplement and fixed by 100% ice-cold Ethanol (Sigma), thereafter being resuspended in 1 ml of PBS.

The rest of the cellular suspension was plated in a T75 flask (TC, 75 cm, Midwest Scientific, Cat. No. TP90076) with the aforementioned culture medium and incubated at 37°C in 5.5% CO2. The adherent cells were washed and trypsinized, following 18 hours of incubation. Cells were counted in a Neubauer chamber along with Trypan Blue staining for live/dead assessment. Live/dead staining was confirmed by Propidium Iodide staining as well (Picture 2, Picture 3). A sample of 100,000–500,000 cells was fixed for flow cytometry.

Apoptosis was assessed using the Annexin-V-FLUOS Staining Kit (Roche Diagnostics Corporation). Briefly, 20 µl of Annexin-V-FLUOS was prediluted in 1 ml of PBS. Following centrifugation, the cell pellet was resuspended in 100 µl of this diluted Annexin and incubated for 15 minutes at room temperature in the dark. A sample was analyzed under a fluorescent microscope equipped with a 490 nm filter (Picture 3).

Remaining cell samples were cryopreserved using 10% Dimethyl Sulfoxide (DMSO) protocol and stored in vapor phase of liquid nitrogen.
Results

Flow cytometry of processed lipoaspirate demonstrated the expression of MSC's specific markers CD73, CD90 and CD105 in the range of 28%–58%, along with high expression of PerCP-linked markers cocktail, which included CD14, CD20, CD34, CD45 and HLA-DR as markers for hematopoietic line (Figure 1).

1X Collagenase–60 min and 1X Liberase–60 min demonstrated highest and comparable initial harvest capacity (9 x 10^6 and 8.6 x 10^6, respectively) (Figure 2) and adherence rates (39.4% and 32.4%, respectively) (Figure 3). Viability of cells was affected by longer exposure to either collagenase or Liberase enzyme blend, compared to shorter exposure for 15 and 30 minutes to Liberase enzyme blend (Figure 4). These differences were significant between 1X Liberase–30 min and 1X Liberase–60 min (p<0.05).

Also, significantly increased apoptotic index was observed following 1X Collagenase–60 min treatment versus all Liberase groups: 8% in 1X Collagenase–60 min and 1.83%, 2.6% and 3.7% in 3X Liberase–15 min, 1X Liberase–30 min and 1X Liberase–60 min, respectively (Figure 5). Exposure to 1X Liberase–60 min resulted in higher apoptotic index among Liberase groups—40% higher than 1X Liberase–30 min and two-fold higher than 3X Liberase–15 min. Even though 3X Liberase–15 min group expressed the lowest apoptotic index, it demonstrated lower adherence rate than others (Figure 3) and lower viability than 1X Liberase–30 min group in lipoaspirate and following plating (Figure 4).
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Figure 2. Cell counts in processed lipoaspirate (SVF) and following adherence test. Abbreviation: L-15 – Protocol #1: 3X Liberase–15 min Coll-60 – Protocol #2: 1X Collagenase–60 min L-60 – Protocol #3: 1X Liberase–60 min L-30 – Protocol #4: 1X Liberase–30 min

1X Collagenase–60 min and 1X Liberase–60 min delivered highest and comparable number of cells in processed lipoaspirate (9 x 10^6 and 8.6 x 10^6, respectively) and following adherence test (2.5 x 10^6 and 2.1 x 10^6, respectively).

Figure 3. Percentage of adherent cells following different adipose tissue digestion protocols. Abbreviation: L-15 – Protocol #1: 3X Liberase–15 min Coll-60 – Protocol #2: 1X Collagenase–60 min L-60 – Protocol #3: 1X Liberase–60 min L-30 – Protocol #4: 1X Liberase–30 min

Exposure of tissue to either Liberase or Collagenase for 60 minutes following protocols #2 and #3, respectively, demonstrated higher, yet not significant (p>0.05) plastic adherence rate than by protocols with shorter enzyme exposure time.

Figure 4. Cell viability as assessed in processed lipoaspirate (SVF) and following adherence test. Abbreviation: L-15 – Protocol #1: 3X Liberase–15 min Coll-60 – Protocol #2: 1X Collagenase–60 min L-60 – Protocol #3: 1X Liberase–60 min L-30 – Protocol #4: 1X Liberase–30 min

Viability of cells has been affected by longer exposure of 60 minutes to either Collagenase or Liberase, compared to shorter exposure for 15 and 30 minutes to Liberase, whereas difference between L-30 and L-60 was significant (91% vs. 84%). *p<0.05

Figure 5. Apoptotic index of adherent cells following different adipose tissue digestion protocols. Abbreviation: L-15 – Protocol #1: 3X Liberase–15 min Coll-60 – Protocol #2: 1X Collagenase–60 min L-60 – Protocol #3: 1X Liberase–60 min L-30 – Protocol #4: 1X Liberase–30 min

Increased apoptotic index was observed following exposure to Collagenase and Liberase for 60 minutes, compared to shorter enzymatic exposure for 15 and 30 minutes.
Flow cytometry for specific mesenchymal stem cells markers identified a highly homogeneous population of cells with higher than 90% purity, following plating of washed SVF for 18 hours and assessment of adherent fraction (Picture 1, Figure 6). This level of homogeneity was identical throughout all study groups.

Figure 6. Expression of MSCs markers in adherent cells. MSC-specific markers CD90, CD105 and CD73 were assessed by flow cytometry after 18 hours of cell selection by plating and trypsinization of adherent cells. Expression in the range of 97%–99% was detected for all three markers, whereas live cells fraction comprises 84.9% of the sample.

Picture 1. Cells attachment as assessed following 18 hours of plating (A, B). Abbreviation: [A] 3X Liberase–15 min; [B] 1X Collagenase–60 min. Cells attached to the culture dish plastic 18 hours after initial plating. Unattached cells are seen out of focus floating in culture medium.
Collagenases, enzymes that break down the native collagen that holds animal tissues together, are made by a variety of microorganisms and by many different animal cells (6). The most potent collagenase is the “crude” collagenase secreted by the anaerobic bacteria *Clostridium histolyticum*. “Crude” collagenase refers to the fact that the material is actually a mixture of several different enzymes besides collagenase that act together to break down tissue (7, 8).

However, upon application of collagenase any new lot with higher specific activity could cause excessive cell death at an established concentration. Though, for research purposes it could be managed by adding additional protein substrate, it is not an option when standardization toward clinical quality is sought.

Effect of collagenase on released cell integrity has been reported in early studies, though are almost all on animal models (9, 10, 11, 12).

Thorough standardization of collagenase purity and lot-to-lot variability, along with adequate processing of cell preparations, appears therefore to be mandatory before initiation of human clinical trials. We demonstrated that the 1X Liberase–30 min group exhibited optimal parameters with highest viability rate, low apoptotic index and comparable to 1X Liberase–60 min adherence capacity. Tissue digestion by application of 1X Liberase for 30 minutes could be considered as a cost- and time-effective alternative to “crude” collagenase.

Application of Liberase MNP-S therefore would eliminate one of the variables for a standardized stem cells harvest protocol.

After the current study was summarized, we discovered that application of Calcium and Magnesium (Ca⁺⁺/Mg⁺⁺)-free medium at initial steps of digestion and washing does not compromise Liberase effectiveness, and furthermore, improves the consistency of SVF and eliminates micro-clotting and trapping of cells, hence facilitating the harvest process. In contrast, crude collagenase requires the presence of calcium in medium for effective digestion, making more difficult the release of cells from SVF for plating.

It should be mentioned that enzymatic digestion is only one of several steps comprising the entire stem cells harvest process. Our efforts focus on multiple factors that could affect this process. For instance, we believe that preliminary patient testing for viral infection is critical as it has been reported that presence of viruses could affect proliferation (13) and differentiation capacity of stem cells (14). Further, elimination of xenogeneic proteins, along with optimal culture conditions, is of paramount importance. We and others have elaborated replacement of fetal calf serum by human serum enriched by recombinant cytokines and/or growth factors (15).
References


Blended proteolytic enzyme for tissue dissociation.
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