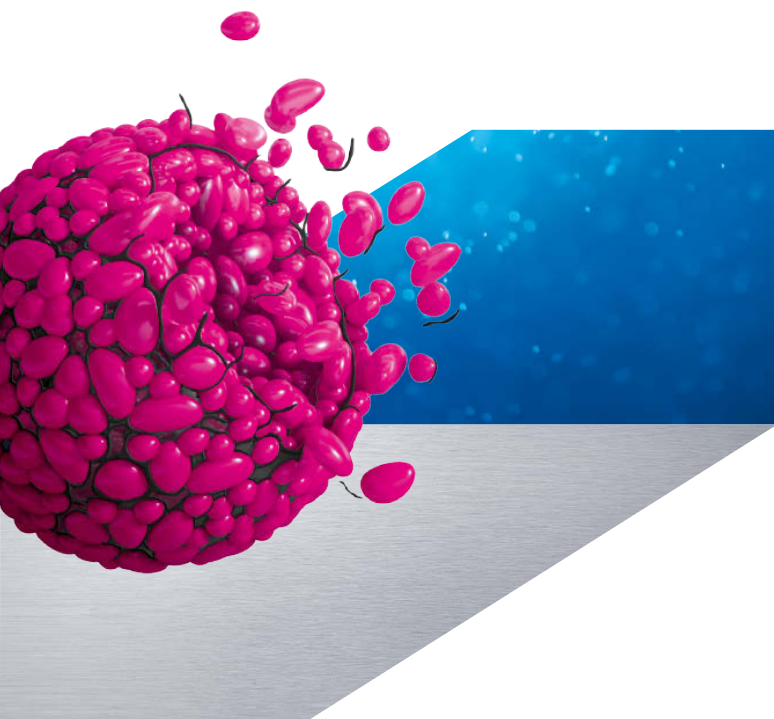# Application note

Comparative analysis of stromal cell isolation from adipose tissue using Roche Liberase MNP-S* and Worthington Collagenase I

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Introduction

The idea of cell therapy using the systemic or local injection of stem/progenitor cells in the area of injury to treat multiple chronic disorders received close attention in the last decade [1]. Bone marrow-derived mesenchymal stem cells (BM-MSC) and blood-derived endothelial progenitor cells were among the first cell types that were evaluated in regard to their therapeutic potentials [2, 3, 4].

Adipose stem/stromal cells (ASC) represent another type of mesenchymal cell and, in contrast to BM-MSC, are substantially more abundant in adults [5]. BM-MSC and ASC are among the clinically feasible and promising candidates for autologous cell therapy applications due to their pluripotency, immunomodulative properties, and secretory activity [6, 7]. Therefore, they are the subject of study in several cell therapy clinical trials worldwide. The most efficient way to generate a stromal vascular fraction (SVF) and then extract ASC from adipose tissue is through dissociation of a lipoaspirate by enzymatic digestion. Accumulated data, strongly supporting the therapeutic potential of ASC, motivated biotech companies to develop enzymatic blends and automated processing devices for adipose tissue dissociation applications. Currently, there are many different products on the market that are suitable for adipose tissue dissociation. Many of them are blends containing collagenase I (isolated from *C. histolyticum* cultures) and a neutral protease, such as dispase.

* Blended proteolytic enzyme for tissue dissociation.
  For further processing only.

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Introduction

Multiple preclinical studies have demonstrated that dissociation of adipose tissue with collagenase alone or in combination with neutral protease results in efficient extraction of viable and proliferative ASC, and that, post expansion, these cells are capable of multilineage differentiation into adipocytes, chondrocytes, osteocytes, and smooth muscle cells [8].

While most blends are acceptable for preclinical and basic science studies, the more strict requirements for clinical trials significantly narrow the options. Factors such as lot-to-lot enzyme activity inconsistency, insufficient purity from endotoxins, other protease activity, and animal-derived components raise safety and applicability concerns for clinical use. In order for the reagent to be accepted as an in-process reagent for the preparation of cells for trans-plantation, it must be GMP grade with standardized final blend purity and lot-to-lot consistency. Liberase MNP-S (Roche Diagnostics) is a novel, highly purified GMP-grade enzyme blend.

The goal of this study was to compare side-by-side human adipose tissue dissociation to single-cell suspension by Worthington Collagenase I and Liberase MNP-S, a Sterile-A, GMP-grade blend of collagenases I and II with a medium-content thermolysin component.

Materials and methods

Isolation and expansion of cells

The protocol for collection of human fat samples was conducted with patient informed consent and under the approval of the Indiana University Institutional Review Board (IRB). Tissue samples were collected in sterile containers during scheduled abdominal liposuction procedures. Samples were collected from four female donors, ages 28 – 67 years with mean body mass index of 25±1.4 SD. Samples were received within two hours of harvest. Lipoaspirates were washed with ice-cold phosphate buffered saline (PBS) and used directly or stored at 4°C overnight.

In the first part of the study, 10 ml of lipoaspirate was aliquoted into each of four 50 ml tubes. An equal volume of Liberase MNP-S (Cat. No. 06297790001, Roche Diagnostics) at 0.9 Wünsch units/ml was added to each tube. Tubes were incubated in an Enviro-Genie incubator with a rocking speed of approximately 40 cycles per minute at 37°C for 10, 20, 30, and 40 minutes.

In the second part of the study, 25 g of lipoaspirate, which was approximately 25 ml of tissue suspension, was aliquoted into each of six 50 ml tubes. An equal volume of Liberase MNP-S (Cat. No. 06297790001, Roche Diagnostics) at 0.9 Wünsch units/ml or of Collagenase I (Cat. No. LS004196, Worthington Biochemical) at 2 mg/ml was added to the tubes. In this study, three lots of each enzyme were tested (see Table 1). Tubes were incubated in an Enviro-Genie incubator with a rocking speed of approximately 40 cycles per minute at 37°C for 30 minutes.

Sample	Enzyme	Lot	Activity	Used at	Source
1	Collagenase I	X0M1295	280 U/mg	1 mg/ml	Worthington
2	Collagenase I	X2A13235	290 U/mg	1 mg/ml	Worthington
3	Collagenase I	X2B13287	275 U/mg	1 mg/ml	Worthington
4	Liberase MNP-S	14428121	5 WU/mg	0.45 WU/ml	Roche
5	Liberase MNP-S	14428122	5 WU/mg	0.45 WU/ml	Roche
6	Liberase MNP-S	14428123	5 WU/mg	0.45 WU/ml	Roche

Table 1: Lot numbers and parameters of enzymes used for digestion of adipose tissue.
U = CDU
WU = Wünsch unit
CDU units/mg ÷ 1000 = Wünsch units/mg

Following incubation, dissociation tubes were centrifuged at 300 x g for 8 minutes to separate the stromal cell fraction (pellet) from adipocytes. The cells were resuspended in EBM-2/5% FBS media (Lonza), filtered through 100 µm-pore membranes (BD Biosciences), and again centrifuged at 300 x g for 8 minutes. These pellets were resuspended in red-cell lysis buffer (eBioscience), incubated at 37°C for 10 minutes, diluted in double volume of EBM-2/5% FBS media, filtered through 40 µm-pore membranes (Becton Dickinson), and centrifuged at 300 x g for 8 minutes. Pellets were resuspended in EGM-2MV media (Lonza), counted manually using a hemocytometer, and plated on tissue culture plastic. For culturing: 100,000 of cells were resuspended in 2 ml of EGM-2MV media, plated into one well of a 6-well plate, and placed into an incubator with 5% CO_2 and 37°C. Medium on the cells was changed after 24 hours and then every three days. ASC were passaged when 60-80% confluent. Cell monolayers were treated with 0.05% Trypsin solution for 5 minutes at 37°C to the lift the cells. ASC were used at passage 1 for follow-up tests.

Analysis of SVF composition by flow cytometry

Freshly obtained samples of stromal vascular fraction (SVF) were reconstituted at 10^6 cell/ml, and 0.1 ml aliquots of SVF were dispensed into 5 ml tubes containing flow cytometry-validated fluorescently labeled antibodies. Cells were incubated for 20 minutes on ice with the following combinations of reagents:

1) IgG1-PE/IgG1-APC
2) CD45-PE/CD31-PE/CD34-APC
3) CD140b-PE/CD45-APC/CD31-APC
4) CD45-PE/CD31-APC

After incubation, cells were washed in PBS/2% FBS medium and fixed with 1% formaldehyde. Labeled SVF samples were analyzed on a Calibur APC FACS (Becton Dickinson).

Analysis of the adipogenic potential of the ASC

Adipose stem/stromal cells (ASC) at passage 1 were plated into 48-well plates at 60,000 cells/cm2 and exposed to control EBM-2/5% FBS media or Adipogenic Differentiation Medium (Cat. No. DM-2, ZenBio) for 10 days with media exchange at days 4 and 8. At the end of the incubation period, cell mono-layers were fixed with 10% formalin, incubated with 10 µg/ml of Nile Red dye for 30 minutes to reveal lipid droplets in the cells, and then followed by staining with DAPI to reveal nuclei. Quantitative analysis was performed by scanning the wells on a fluorescence-detecting plate reader with the wavelengths specific for Nile Red dye and DAPI.

Analysis of the vasculogenic potential of the ASC

The vasculogenic potential of expanded ASC was assessed using the *in vitro* model of cultivation of cord blood-derived endothelial cells (CBD-EC) on ASC monolayers as previously described [9]. Cell mixtures composed of ASC at passage 1 (6×10^4 cell/cm²) and CBD-EC (5×10^3/cm²) were plated in EBM-2/5% FBS and incubated for six days with media exchange at day 3. On day 6, cultures were fixed in methanol at -20°C for 5 minutes. The vascular networks formed by ASC/CBD-EC co-cultures were evaluated by probing the wells with the cells with biotinylated Ulex Europaeus Agglutinin I (Vector Labs) for 1 hour followed by incubation with Streptavidin Alexa 488 (Invitrogen) for 30 minutes. Fluorescently stained cultures were imaged using a Nikon Ti Eclipse microscope. Digital images were acquired using a 4x objective (5 pictures/cm² = 20% of the well surface). Images of vascular networks were processed with MetaMorph software using the "Angiogenesis Tube Assay" algorithm (Molecular Devices).

Statistical analysis of the data

Quantitative data are expressed as mean ± SEM. Comparisons between groups were performed with an unpaired t-test. Each experimental condition represents at least n = 4. Statistical analysis was performed using Prism 4 (GraphPad).

Our prior studies have shown that SVF and ASC yields may be substantially different between samples. This is probably dependent upon several factors, including donor age, BMI, region of tissue harvest, and method of its extraction. The significant variability in cell yield between samples makes it difficult to perform statistical analyses of the collective data and to graphically present it. To overcome this problem, in the graphs representing collective data, the cell counts for Collagenase I-treated samples are presented as 100%.

Results

Part I: Efficiency of SVF and ASC isolation based on tissue/enzyme incubation time

To define the optimal incubation time for efficient dissociation of lipoaspirate with Liberase MNP-S, two samples of fat were incubated with an equal volume of Liberase MNP-S at 0.9 Wunsch units/ml. Samples were taken at 10, 20, 30, and 40 minutes, counted, and plated. Analysis of SVF yield showed that there is a linear relationship between incubation time and cell yield (Figure 1A).

However, the efficiency of cell attachment (sample enrichment with ASC) only increased until 30 minutes with a sharp decline in attachment efficiency occurring at 40 minutes (Figure 1B), which eventually resulted in a non-significant difference in total ASC yield (per ml of starting material) in the samples incubated for 30 or 40 minutes (Figure 1C). This is the product of cell number multiplied by attachment efficiency. Based on these observations the second part of the study was performed with tissue exposure to enzymes for 30 minutes.
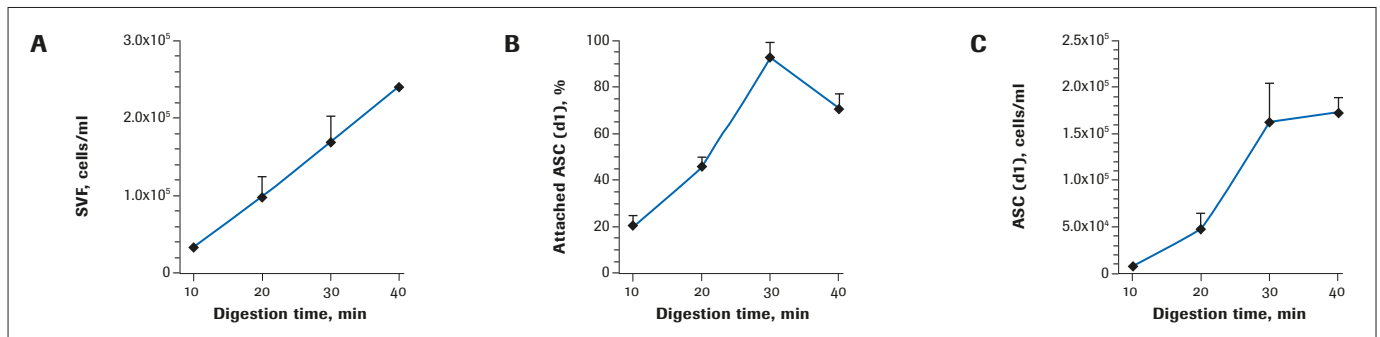

Figure 1: Dynamic of Efficiency of SVF and ASC isolation as a function of tissue/enzyme incubation time.

Part II: Side-by-side comparison of SVF yield, composition, and cell activity using Worthington Collagenase I and Roche Liberase MNP-S

Cell yield and attachment:
Analysis of cell yield after enzymatic dissociation of the lipoaspirate with three lots of Worthington Collagenase I and three lots of Roche Liberase MNP-S revealed that in both cases there was a low, seemingly insignificant, level of lot-to-lot variability. At the same time, when evaluated collectively, isolation with Liberase MNP-S gave consistently higher cell yields (Collagenase I: $100\pm5.81\%$; Liberase MNP-S: $156.8\pm13.6\%$, $p<0.001$) (Figure 2).

Analysis of efficiency of cell attachment, performed on day 1 post isolation/plating (Figure 3), demonstrated insignificant differences between the enzymes (Collagenase I: $67.3\pm4.9\%$; Liberase MNP-S: $77.4\pm6.3\%$). However, because the yield of SVF was higher in every Liberase MNP-S-treated sample, the total number of attached cells per gram of lipoaspirate was substantially higher for Liberase MNP-S than for Collagenase I (Collagenase I: $100\pm6.0\%$; Liberase MNP-S: $181.6\pm17.7\%$, $p<0.001$) (Figure 4).
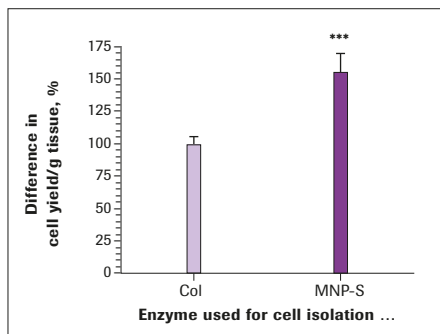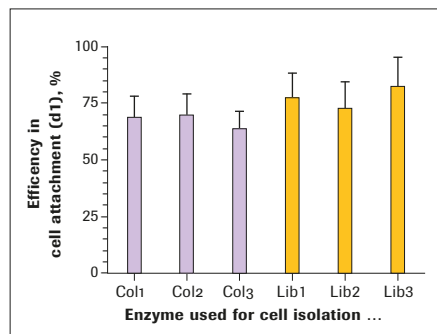

Figure 2: Yield of SVF.
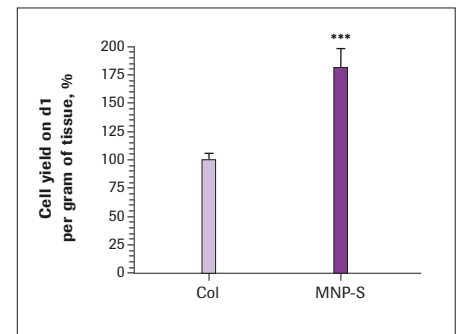
Figure 3: Efficiency of SVF attachment.

Figure 4: Yield of attached cells on day 1.

Characterization of SVF composition based on surface marker expression:

It is well known that SVF is a highly heterogeneous cell population, composed of adipose stromal/stem cells, endothelial cells, hematopoietic cell types, pericytes, and other cell types. The most interesting cells in SVF, in regard to clinical use, are ASC. Multiple surface markers are used to characterize cells within these populations. We used several methods to identify the ASC in SVF.

We defined ASC as CD34+/CD45-/CD31- cells or as CD140b+/CD45-/CD31- cells. Flow cytometry-based analysis revealed that the percentage of CD34+/CD45-/CD31- cells in SVF obtained with Collagenase I digestion was 33.0±2.4% and with Liberase MNP-S was 39.0±2.5% (Figure 5A).

The percentages of CD140b+/CD45-/CD31- were 23.9±3.1% and 26.7±2.8% for Collagenase I and Liberase MNP-S, respectively (Figure 5B). The percentage differences between enzymes are insignificant. However, because the total cell yield with Liberase MNP-S was higher, the number of ASC per gram of lipoaspirate was significantly higher. Specifically, independent from the way the ASC were defined in SVF (CD34+/CD45-/CD31- or CD140b+/CD45-/CD31- cells) there was a 90% increase in ASC yield with Liberase MNP-S (Figure 5C, D) (CD34+/CD45-/CD31-: Collagenase I: 100±7.1%; Liberase MNP-S: 189.3±19.1% (p<0.001); CD140b+/CD45-/CD31- cells: Collagenase I: 100±11.4%; Liberase MNP-S: 191.1±25.4% (p<0.01).
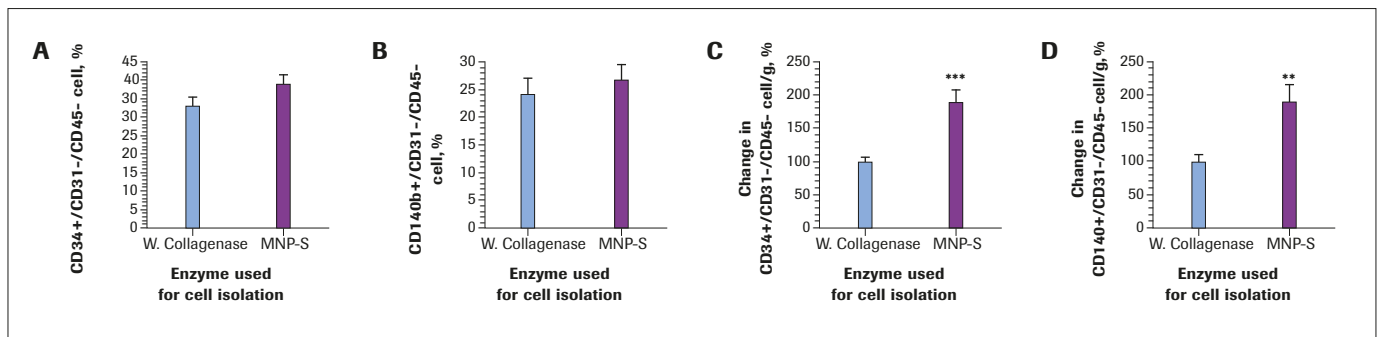

Figure 5: Percentages and total cell yield of ASC from SVF.

Further SVF analysis revealed that samples isolated with Liberase MNP-S had twice the number of endothelial cells (3.2±0.6%) (Figure 6A), defined as CD31+/CD45- cells, compared to the SVF isolated with Collagenase I (1.8±0.3%)

(p<0.05), which resulted in a three-fold increase in yield of EC per gram of SVF by Liberase MNP-S isolation (Collagenase I: 100 ±8.0%; Liberase MNP-S: 297.9±54.8%) (Figure 6B).
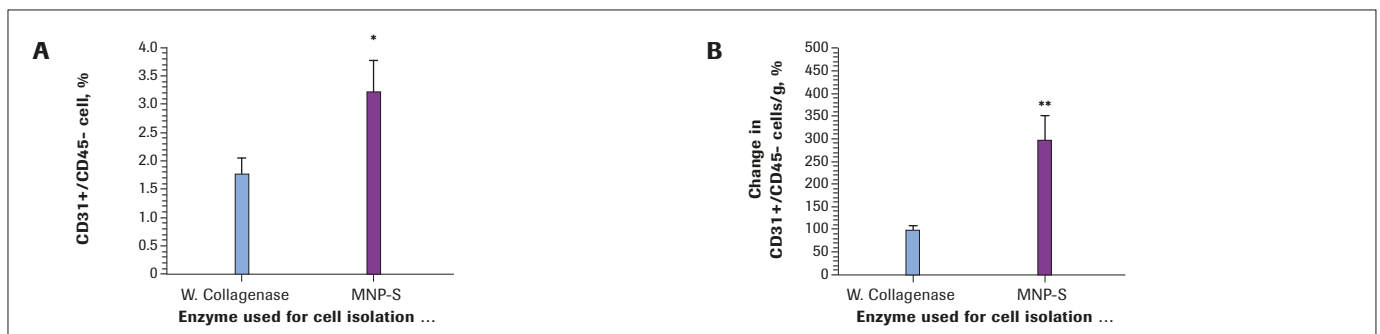

Figure 6: Percentages and total cell yield of EC from SVF.

Proliferative potential of SVF:

To evaluate the dynamics of ASC proliferation after isolation, SVF was harvested at day 1 and replated at 5,000 cells/cm^2 in EGM-2mv medium. Cells were subsequently harvested at day 4 and then on day 7 for sub-culturing. We found that the rate of cell proliferation was enzyme independent and resulted in 4.9 cumulative cell divisions between days 1 and 7 (data not shown).

Vasculogenic potential of ASC:

Co-cultivation of CBD-EC with ASC resulted in EC reorganization into vascular-like cord structures (Figure 7). Quantitative analysis of the density of the vascular networks, represented as density of the total tube length of the cords, has shown that vasculogenic potential of the cells was independent of type of enzyme used for cell isolation or its specific preparation (data not shown).

Adipogenic potential of ASC:

Exposure of ASC monolayers to Adipogenic Differentiation Medium for 10 days resulted in lipid accumulation in the cells (Figure 8). Quantitative analysis of the lipid accumulation performed by probing the monocultures with the lipophilic dye Nile Red revealed that all cells underwent the same degree of adipogenic differentiation independent of the enzyme used for cell isolation or its specific preparation (data not shown).
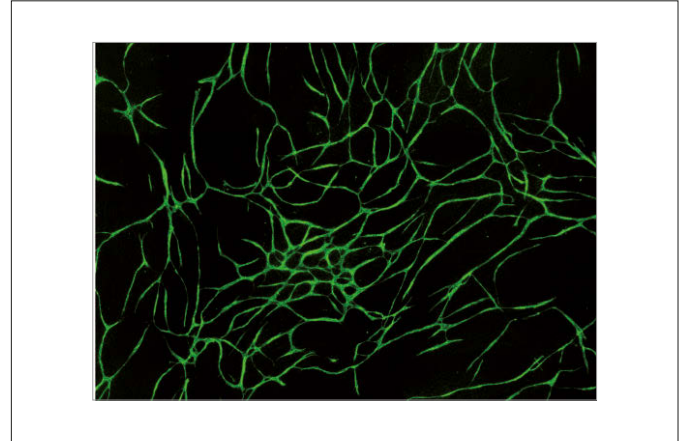

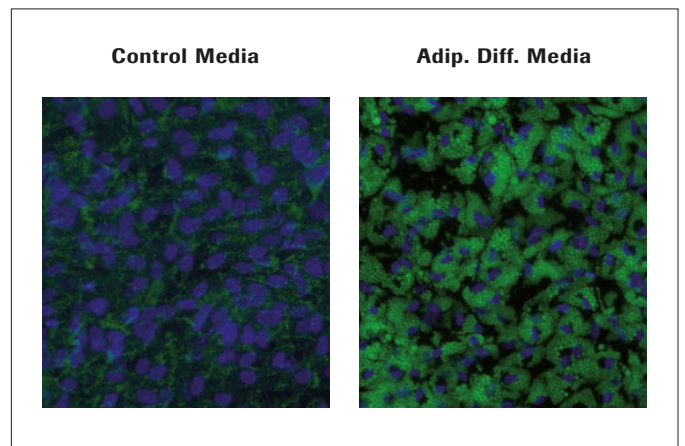**Figure 7:** Analysis of vasculogenic potency of ASC.*

Figure 8: Analysis of Adipogenic differentiation of ASC.*

* Source: Indiana University School of Medicine

Discussion and conclusion

This study compared the isolation of ASC from lipoaspirates with crude non-completely characterized Worthington Collagenase I and Roche Liberase MNP-S, a GMP-grade enzyme blend of collagenase I and II and thermolysin.

Researchers isolate SVF from adipose tissue via several protocols, using a wide range of enzyme concentrations and incubation times. This variability in digestion protocols is most likely due to the properties of the original material (dimensions of fat pieces) determined by liposuction technique employed, as well as the activity of the particular enzyme used for tissue dissociation. Additionally, we have no guarantee that in every case enzyme/tissue incubation time was optimized. Optimization of enzyme activity can be a time-consuming, laborious process if high lot-to-lot variability is present; in clinical settings this is expensive and unfeasible.

Non-optimized digestion protocols lead to inefficient tissue dissociation, or its over-digestion resulting in high dead/live ratios. Both scenarios will cause low yield of therapeutically active cells, and can jeopardize effectiveness of subsequent therapeutic applications. In the present study, we determined the efficiency of yield of viable cells as a function of incubation time of lipoaspirate with Liberase MNP-S. We found that 30 minutes of incubation gave us the best outcome — high cell yield with low dead/live cell ratio. In the rest of the study, the 30-minute incubation protocol was used. Because manufacturers of these enzymes use different tests to define proteolytic activity of the products, for side-by-side comparison of activity we used 1 mg/ml of Collagenase I (most accepted concentration determined by literature review) and 0.45 Wünsch units/ml of Liberase MNP-S (recommendation of Roche).

We saw a high lot-to-lot consistency for both tested enzymes to successfully isolate SVF. However, at the same time, we consistently saw a significantly higher cell yield for all tested lots of Liberase MNP-S. The efficiency of cell attachment was similar for both enzymes, but the higher cell yield with Liberase MNP-S resulted in much higher (up to 80%) yields of ASC — primary cells of interes —per gram of lipoaspirate. Evaluation of SVF composition revealed that percentages of ASC in the SVF, defined as CD34+ or CD140b+ but CD45-/CD31-cells, were the same for both enzymes, but Liberase MNP-S was more efficient in extracting endothelial cells from the tissue.

When isolating cells from tissue, one must always be concerned with whether the use of different enzymes might result in variations in the cell types, subsequent cell behavior, and *in vitro* and *in vivo* activity, such as mitogenic activity or differentiation potency. Analysis of cell activity after extraction with Collagenase I or Liberase MNP-S suggests that both methods of isolation gave rise to cells with indistinguishable physiological activities: cells have the same rate of proliferation, degree of adipogenesis, and potency to support endothelial cell reorganization into vascular-like structures, a process that relies on ASC paracrine activity and direct heterogeneous cell-cell interaction.

In conclusion, this study demonstrates that dissociation of lipoaspirate with Roche Liberase MNP-S to single-cell suspension is more efficient than dissociation with Worthington Collagenase I. Resultant cell population characteristics are virtually indistinguishable, but the efficiency of isolation with Liberase MNP-S in regard to getting higher yields of viable biologically active ASC as well as endothelial cells, suggests it is a better choice. This data, combined with the fact that Roche Liberase MNP-S is GMP-grade with high lot-to-lot consistency, makes the product well-suited for SVF isolation in clinical applications.

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