

# Tissue Dissociation Enzymes for Adipose Stromal Vascular Fraction Cell Isolation: A Review

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## Abstract

With the increasing clinical translation of adipose stromal cell based technologies, it is important to thoroughly report on all aspects of these technologies. In this article, we review the commonly used enzymes for adipose tissue dissociation and stromal vascular fraction isolation. The enzymes reviewed include collagenase, trypsin, clostripain, and the neutral proteases dispase and thermolysin. Enzyme characteristics, the advantages of enzymatic isolation, and other relevant practical and regulatory issues are discussed.

**Keywords:** Enzymes; Cell isolation; Collagenase; Stromal vascular fraction

## Introduction

The use of stromal vascular fraction (SVF) cells has increased recently due to a variety of proposed therapeutic indications. These indications include breast augmentation/reconstruction, reduction of facial aging, stimulation of chronic wound healing, improvement of radiation damage and hypertrophic scars, as well as treatment for inflammatory and degenerative orthopedic conditions [1-3]. SVF is the population of cells which result from the enzymatic digestion or mechanical breakdown of lipoaspirate without culture or expansion. SVF is a heterogeneous mixture of various blood cells, preadipocytes, fibroblasts, smooth muscle cells, and both vascular endothelial progenitors and adipose-derived stem cells [4-6]. The composition of SVF varies depending on the method of isolation used.

Commonly SVF isolation is achieved using tissue dissociation enzymes (TDE) to breakdown adipose tissue. TDE mixtures are enzymes which digest the extracellular matrix proteins which hold tissue together. When isolating SVF, enzymatic digestion can be up to one thousand times more efficient than mechanical separation methods because the enzymatic breakdown of the extracellular matrix (ECM) frees many more cells from the fibrous stroma. While there are many enzymes used for tissue dissociation, like pronase, hyaluronidase and pancreatic elastase, this paper only pertains to enzymes which have been expressly described for the dissociation of adipose tissue and lipoaspirate to yield SVF and adipose-derived stem cells, specifically clostridial collagenase and neutral protease, trypsin, Clostripain and the neutral proteases Dispase and Thermolysin (Table 1).

## A Brief Overview of the Extracellular Matrix (ECM) Composition [7-9]

The extracellular matrix is the vast and complex network that holds all cells, tissues and organs together. The most abundant component of the ECM is collagens. Collagens act as the backbone of the extracellular matrix. Elastin is another major component. The amount of elastin present in a tissue is related to how much flexibility/stretch a tissue has. It is commonly found in abundance in arterial walls and ligaments, which stretch and contract frequently. Elastin has also been shown to play roles in cell adhesion, migration and cell signaling. While collagen and elastin are by far the most common, other molecules, while not as abundant, still play vital roles. Fibronectin functions to

guide assembly of the extracellular matrix by forming bridges between cell surface receptors such as integrins and the structural proteins of the extracellular matrix such as collagens and other proteoglycans. Laminins, another minor structural element, serve various functions including modulation of various cellular functions such as adhesion, differentiation, migration and survival.

## Clostridial Collagenases

The primary constituent of many TDE mixtures is collagenase derived from the bacteria *Clostridium histolyticum*. In many commercially available TDE mixtures, clostridial collagenases are included as a mixture of type I collagenase and type II collagenase. Collagenases are part of the matrix metalloproteinase (MMPs) family. MMPs are zinc-dependent endopeptidases involved in a variety of cellular processes including ECM degradation, cleavage of cell surface receptors, growth factor release from the ECM and more [10-12]. Collagenases digest collagens in the ECM. Collagens are the major components of the extracellular matrix and in mammals accounting for around 25% of their total body weight. Collagens are structural proteins with high tensile strength, which in addition to their structural roles influence cell differentiation, migration, and attachment. Collagens are present as a triple helix composed of three collagen polypeptides in their native form.

*C. histolyticum* produces a variety of collagenases in large quantities. The two main forms produced by *C. histolyticum* utilized in TDE mixtures are type I collagenase (ColG) and type II collagenase (ColH). Type I collagenase has been shown to preferentially digest the large, intact triple helices of native collagen fibers, while type II collagenase has been shown to preferentially digest the smaller collagen fragments generated by type I collagenase, although both enzymes will digest both

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Enzyme	Source	Optimum pH Range	ECM Substrates	Activator(s)	Common Inhibitors
Collagenase type I and type II	<i>Clostridium histolyticum</i>	6.3-8.5	Collagen I, II, III and IV	Ca <sup>2+</sup> , Zn <sup>2+</sup>	EDTA, EGTA Blood Plasma/Serum
Dispase	<i>Paenibacillus polymyxa</i>	5.9-7.0	Fibronectin, Collagen IV Collagen I (small amounts)	Ca <sup>2+</sup> , Mg <sup>2+</sup>	EDTA, EGTA
Thermolysin	<i>Geobacillus stearothermophilus</i> , <i>Bacillus thermoproteolyticus</i>	5.0-8.5	Non-specific Cell adhesion proteins	Ca <sup>2+</sup> , Zn <sup>2+</sup>	EDTA, EGTA
Trypsin	<i>Sus domesticus</i> (porcine) <i>Bos taurus</i> (bovine)	7.0-9.0	Non-specific Cell adhesion proteins	Ca <sup>2+</sup>	EDTA, EGTA Blood Plasma/Serum
Clostripain	<i>Clostridium histolyticum</i>	7.4-7.8	Non-specific Cell Adhesion protein	Ca <sup>2+</sup>	EDTA, EGTA

\*insufficient data pertaining specifically to CHNP

Table 1: Summary of tissue dissociation enzymes.

Enzyme	Unit	Definition	Conversions
Collagenase	Collagen Degrading Units (CDU) (Mandl Units)	One CDU catalyzes the hydrolysis of 1 μmole of L-leucine equivalents from collagen in 5 hours at 37°C, pH 7.4	1 Wunsch unit = ~1000 CDU 1 Wunsch unit = ~3.9 FALGPA units 1 Wunsch unit = ~10 HPU
	FALGPA unit	One FALGPA Unit catalyzes the hydrolysis of 1 μmole of N-(3-[2-furyl]-acryloyl)- L- leucylglycyl- L-prolyl-L-alanine (FALGPA) per minute at 25 °C, pH 7.5	
	Wünsch units (PZ Units)	One PZ unit catalyzes the hydrolysis of 1 μmole 4-phenylazobenzoyloxycarbonyl- L-prolyl- L-leucyl-glycyl-L-prolyl-D-arginine per minute at 25 °C, pH 7.1	
	Clostridiopeptidase A "HP Units" (HPU)	One HPU catalyzes the hydrolysis of 1 μmole N-carbobenzyloxy- glycyl-L- prolyl-lycylglycyl-L-prolyl-L-alanine per minute at 37°C	

Table 2: Summary of collagenase specific activity units [30-33].

substrates, but with lower affinity [13-16]. Bacterial collagenases are used instead of animal/human derived collagenases for tissue digestion because they are cheaper to produce, but more importantly, exhibit higher substrate specificity for native collagen and collagen fragments than vertebrate collagenases [17]. Table 2 summarizes units and unit conversions, where applicable, commonly used to describe collagenase activity [18-21].

## Neutral Proteases

The second major component found in many TDE mixtures is a neutral protease, usually *Clostridium histolyticum* neutral protease (CHNP) Dispase or Thermolysin. Neutral proteases exhibit mild proteolytic activity while preserving the integrity of the cell membrane. Dispase, sometimes referred to as Gentlyase, is purified from the bacteria *Paenibacillus polymyxa*, formerly known as *Bacillus polymyxa*. Dispase exhibits lower substrate specificity than collagenases. Dispase has been shown to be a rapid yet gentle agent for separating intact epidermis from dermis [22]. Dispase effects tissue separation by cleaving the basement membrane zone region. Dispase, activated by Ca<sup>2+</sup>, readily cleaves fibronectin, collagen IV, and a smaller amount of collagen I, while leaving collagen V and laminin intact [23-25].

Thermolysin is a thermostable zinc-metalloproteinase originally cultured from the bacteria *Bacillus thermoproteolyticus* [26]. Currently many companies producing thermolysin have switched over to thermolysin produced by the bacteria *Geobacillus stearothermophilus* (formerly classified as *Bacillus stearothermophilus*). Thermolysin tends to cleave surface proteins on the plasma membrane, however Thermolysin exhibits very broad specificity, with over 100 potential ligands reported. Unlike Dispase/Gentlyase, thermolysin is non-collagenolytic, but still provides gentle, non-specific protein cleavage beneficial in tissue dissociation. An important factor in the utility of

thermolysin is that it is highly thermostable, a trait attributed to the presence of four calcium cations present in the interior of the enzyme which prevent large conformational changes [27]. Table 3 summarizes units and unit conversions, where applicable, commonly used to describe neutral protease activity [28-31].

## Trypsin and Clostripain

Trypsin is a serine protease secreted by the pancreas which acts in the digestion of food proteins and other biological processes in the digestive system of many vertebrates. Most trypsin products sold for research use are derived from porcine or bovine sources [32,33]. Trypsin non-specifically cleaves peptides on the c-terminal side of Lysine and Arginine amino acid residues, unless a Proline residue is contained on the c-terminal side of the cleavage site [34-36]. Trypsin is also commonly used to dissociate adherent cells from culture vessels, a process known as trypsinization.

While trypsin has been described for a variety of uses relating to dissociation of various tissue types, it is rarely described for the dissociation of adipose tissue. Trypsin for adipose tissue digestion was described by Fadel et al. [37], and then again by Markarian et al. [38]. Markarian et al. on average recovered around 75,000 viable cells/cc of lipoaspirate processed using trypsin. Markarian et al. determined Trypsin to be a cheaper alternative to collagenases for enzymatic digestion in a laboratory setting because while it yielded fewer viable cells than collagenase-based methods, it did isolate more viable cells than many mechanical dissociation methods have been reported to yield from adipose tissue.

Clostripain (CP) is a cysteine protease that requires a thiol group on the target molecule for catalysis. Clostripain is most active under reducing conditions and cleaves on the carboxy terminal side of arginine. Clostripain exhibits trypsin like activity, but trypsin exhibits

Enzyme	Unit	Definition	Conversions
Dispase CHNP Thermolysin	Azocasein Units (AUC)	One AUC catalyzes an A440 equivalent to 1mg/mL of azocasein per mL per minute at 37°C	1 Dispase Unit = 181
	Neutral Protease Units (NPU)	An increase of 1 fluorescent unit per minute at 35°C BSAfromconjugatethecleava.	
	Protease units (PU)	One protease unit catalyzes the hydrolysis of casein to produce color equivalent to 1.0 μmole tyrosine per min at pH 7.5 at 37°C.	Protease Units (PU)
	Dispase Unit	One dispase unit catalyzes the hydrolysis of casein to produce 181 μmole tyrosine per min at pH 7.5 at 37°C.	
	DMC-U units	One DMC-U catalyzes the cleavage of 1 μmole of peptide bonds from dimethyl case in (DMC), at 25°C, pH 7.0	
	Caseinase Units	One caseinase unit will hydrolyze casein to produce color equivalent to 1.0 μmole (181 μg) of tyrosine per 5 hours at pH 7.5 at 37°C	

Table 3: Summary of common neutral protease specific activity units [42-45].

Enzyme	Unit	Definition	Conversions
Trypsin and Trypsin-like proteases	BAEE Units (S & T units)	One unit catalyzes a change in absorbance at 253 nm of 0.001 per minute at 25°C, pH 7.6. Substrate: N-benzoyl-L-arginine ethyl ester (BAEE)	1 TAME Unit = 57.5 BAEE units 1 TAME Unit = 19.2 USP units 1 U = 4.69 TAME Units 1 U = ~ 270 BAEE Units 1U = 90 USP Units 1 USP unit =3 BAEE units
	Neutral Protease Units (NPU)	An increase of 1 fluorescent unit per minute at 35°C BSAfromconjugatethecleava.	
	USP Units (NF Units)	1 USP-unit catalyzes a change in absorbance at 253 nm of 0.003 per minute at 25 °C, pH 7.6 Substrate: N-benzoyl-L-arginine ethyl ester (BAEE)	
	TAME Units	One TAME unit hydrolyzes 1 umole of p-toluene-sulfonyl-L-arginine methyl ester (TAME) per minute at 30°C, pH 8.2	
	DMC-U units	One DMC-U catalyzes the cleavage of 1 μmole of peptide bonds from dimethyl casein (DMC), at 25°C, pH 7.0	
	International Units (U)	One international unit catalyzes the hydrolysis of 1 μmole Na-benzoyl-L-arginine ethyl ester (BAEE) per minute at 25 °C, pH 8.0	

Table 4: Summary of trypsin and clostripain specific activity units [45,54-57].

broader specificity and around 100 times higher specific activity [39]. Due to its reduced activity compared to trypsin, Clostripain is typically ignored in most commercial TDE blends, but is noteworthy because it usually is present in small amounts with collagenase and CHNP as it is produced by *C. histolyticum* and therefore found in the bacterial extract [40]. Table 4 summarizes units and unit conversions, where applicable, commonly used to describe trypsin and trypsin-like activity [31,41-44].

## Buffer Selection

When isolating SVF cells with TDE mixtures, it is important to select a buffer which is both suitable for optimal enzymatic activity as well as preserving the viability of the cell population. The proteolytic enzymes reviewed in this article, Collagenase, Dispase, CHNP, Thermolysin, Clostripain and Trypsin all include calcium ions (Ca<sup>2+</sup>) as an activator of catalytic activity (Table 1). Therefore, it is important to have an adequate concentration of calcium in the buffer used. A final buffer concentration of 1.0-2.0 mM of Ca<sup>2+</sup> is usually sufficient. Collagenase and Thermolysin are also Zn<sup>2+</sup>-dependant proteases, however an adequate concentration of zinc ions is contained in the lyophilized form of the enzyme mixture to maintain optimal activity [45-47]. Therefore it is not necessary to use a buffer with added zinc ions. Commonly used buffers include Lactated Ringer's (LR) solution; Phosphate buffered Saline (PBS) and balanced salt solutions such as Hanks' Balanced Salt Solution (HBSS) with calcium. For summary of buffers see Table 5.

## Clinical Enzyme Inhibition

While there are a variety of molecular inhibitors that can be used

*in vitro* for TDE inhibition, many of these are not feasible for use in the clinical setting because they introduce added risk or lack human toxicity profiles altogether. In a previous study from our group we have shown that the residual levels of collagenase are negligible but, there is a simple process which can be done in tandem with the SVF isolation in order to significantly reduce the overall residual enzyme activity of the final product. This process involves the use of autologous blood plasma or serum which can be acquired with relative ease. Human blood plasma and serum are known to inhibit collagenase and trypsin activity. In our experience, plasma extracted from 50 mL of heparinized blood is sufficient to neutralize all collagenase activity in a 10 - 20 mL volume of SVF cell suspension. The use of autologous plasma or serum is ideal for clinical isolations because it does not introduce any added risk to the patient and is inexpensive to acquire.

## Enzymatic vs. Mechanical Isolation

When isolating SVF cells, there are two main pathways commonly used: enzymatic or mechanical. There are several mechanical methods of concentrating SVF cells from lipoaspirate without an enzymatic digestion process which have been proposed. These methods focus on washing, vibrating, shaking and centrifuging lipoaspirate in order to free up stromal cells from the adipose tissue [38,48-51]. When compared to enzymatic methods, enzymatic methods have shown to be much more effective in terms of SVF cell recovery. Enzymatic methods employ proteolytic enzymes to disrupt the extracellular matrix which holds adipose tissue together. Collagenase-based enzymatic methods have reported nucleated cell yields between 100,000 and 1,300,000 nucleated cells/cc of lipoaspirate processed [52-57]. This is much

Buffer	Composition	pH range
Lactated Ringer's Solution (LR)	130 mM Na <sup>+</sup>	6.0-7.5
	109 mM Cl <sup>-</sup>	
	28 mM lactate	
	1.5 mM Ca <sup>2+</sup>	
	4 mM K <sup>+</sup>	
Hank's Balanced Salt Solution (HBSS) with Calcium added	0.137 M NaCl	7.0-7.4
	5.4 mM KCl	
	0.25 mM Na <sub>2</sub> HPO <sub>4</sub>	
	0.1g glucose	
	0.44 mM KH <sub>2</sub> PO <sub>4</sub>	
	1.3 mM CaCl <sub>2</sub>	
	1.0 mM MgSO <sub>4</sub>	
	4.2 mM NaHCO <sub>3</sub>	
Dulbecco's Phosphate Buffered Saline (PBS or DPBS)	8 mM Na <sub>2</sub> HPO <sub>4</sub>	7.0-7.2
	135mM NaCl	
	1 mM CaCl <sub>2</sub>	
	3 mM KCl	
	0.5 mM MgCl <sub>2</sub>	
	1.5 mM KH <sub>2</sub> PO <sub>4</sub>	

**Table 5:** Common buffers used for tissue dissociation.

higher than mechanical methods which have reported yields in the range of 10,000 to 240,000 nucleated cells/cc of lipoaspirate [38,48-50].

Furthermore, the composition of the cell populations recovered through simple centrifugation and other mechanical methods has a greater frequency of peripheral blood mononuclear cells and a lower frequency of progenitor cells. Mechanical methods have reported ASC composition of SVF between 1% and 5% [38,48,49]. Comparatively, certain collagenase-based methods have been reported over 15% adipose-derived stem cells in the SVF cell population [53,58,59]. Without the chemical bond release afforded by enzymatic digestion, the resultant cell composition from mechanical methods is biased to be comprised of mainly white blood cells and certainly very few if any vascular endothelial or vascular progenitor cells which are contained within the sublayers of the blood vessels. It should be noted that the actual composition of the final SVF product is very dependent on the isolation methods used, and can vary widely between two different enzymatic methods as well as from patient to patient for a given method.

While enzymatic methods consistently yield higher nucleated cell counts, non-enzymatic methods are not without merit. Tissue dissociation enzyme mixtures for use in adipose tissue digestion tend to be fairly expensive, costing potentially hundreds or thousands of dollars depending on the amount of tissue being processed. In a laboratory setting, where number of progenitor cells is not as important, non-enzymatic methods can provide a cost-effective alternative, but while more expensive, the enzymatic isolation methods provide the better nucleated cell yield and produce a higher percentage of progenitor cells in the SVF than mechanical methods. The method employed by a lab or clinic ultimately depends upon their needs and financial capabilities.

## Does Enzymatic Digestion Alter Cellular Characteristics?

One topic which is debated across the literature is the effect that enzymatic digestion has on the resulting phenotype of the cellular population as well as the other cellular properties such as proliferation rate and differentiation capacity. In 2014, Busser et al. compared the

phenotypic characterization and cellular functions of resulting SVF cells isolated by two different methods: enzymatic digestion with collagenase and explant culture [60]. They noted similar phenotypes and functions of the ADSCs obtained with both methods in terms of surface marker characterization, trilineage differentiation, hematopoiesis supporting activities, population doubling-time and CFU-F formation. Similar findings were observed by Gittel et al. in 2013 when they compared characteristics of cells from equine adipose tissue isolated enzymatically versus by explant culture [61]. In 2014, Markarian et al. compared 9 different methods of ADSC isolation [38]. No significant differences were observed in terms of viability when comparing any of the mechanical methods to the traditional collagenase based method. In addition, they did not find any significant difference in population doubling time, but they did find significantly greater osteogenic differentiation when using trypsin in place of collagenase, suggesting that different enzymes may result in different cellular properties.

Overall, it would appear that the use of collagenase as the primary proteolytic enzyme with an incubation time of 2 hours or less does not alter the phenotypic or functional characteristics of ADSC populations of SVF when compared to cells isolated using non-enzymatic methods [50,51,62,63]. Articles not focused on mesenchymal stem cells from adipose tissue have shown that enzymatic digestion with collagenase can alter cellular properties. In 2008, Lopez et al. described their finding comparing mechanical and enzymatic isolation of chondrocytes [64]. They found reduced viability and altered differentiated phenotype in the enzymatic group. Another interesting consideration demonstrated by Abuzakuouk in 1996 is that collagenase and dispase can cleave some of the surface markers (CD4 and CD8) on peripheral blood mononuclear cells (PBMCs) [65], possibly leading to skewed results from surface marker analysis. While there is documented evidence that enzymatic digestion is capable of altering cellular characteristics, the data specifically pertaining to adipose-derived stem cells and SVF cells is fairly limited and suggests that ADSC populations remain unaltered when an incubation time of 2 hours or less is used.

## Incubation Time

Most SVF cell isolation protocols follow the same basic steps which involve washing the lipoaspirate to remove excess blood and tumescent solution, enzymatic digestion to dissociate the tissue, followed by centrifugation and additional washing to collect the stromal vascular fraction cells, but there is significant variation in how these methods are executed across different isolation methods. In terms of enzymatic digestion, methods tend to differ on the enzymes used (ie a mixture or pure/crude collagenase), the concentration of enzymes used, and amount of time the tissue is digested.

A 2008 study by Pilgaard et al. [66] compared the output of SVF cells isolated using variable enzyme blends and variable incubation times. They compared nucleated cell yield, viability, CFU-F formation and frequency of specific cellular lineages (CD34, CD90 and CD45). Enzyme blends compared included the Blendzyme 1-4, Liberase H1 (Roche Diagnostics GmbH, Mannheim, Germany) and a crude collagenase mixture (CCM). The Blendzyme 1, 2, 3 and 4 mixtures were measured at a constant collagenase activity of 0.28 Wunsch U/mL but varied in the activity of neutral protease which included 30, 60, 120 and 240 caseinase U/mL respectively. Blendzyme 1 contains the neutral protease Dispase, while Blendzyme 2-4 contain the neutral protease thermolysin. Liberase H1 and the crude collagenase mixture contained only 0.28 Wunsch U/mL and 0.26 Wunsch U/mL of collagenase activity, respectively and no neutral protease. Lipoaspirate samples were incubated for 1, 2 or 3 hours. No difference in the percentage of

viable cells was observed with longer incubation times, but the relative yield of CD34+/CD90- cells did increase with longer incubation time. No differences were observed between enzyme mixtures in terms of nucleated cell yield, but there was a significant increase seen overall as digestion time increased. While the average viabilities seen across all groups in this study were high (87.6% - 92.8%), there was significant variation in the ranges of viability observed in all groups, highlighting the vast differences that can be observed on a patient to patient basis. Overall, taking all factors measured into account, they determined that a 2 hour incubation time would provide optimal yields and CFU-F numbers. This study gave no clear suggestion on which enzyme mixture was preferred, but found that the Blendzyme 1, Blendzyme 2 and Liberase H1 outperformed other groups.

### Mixed Enzyme versus Mono-enzymatic Protocols

Another facet of enzymatic isolation is whether or not to use a mixture of enzymes or just one enzyme, usually collagenase. Highly purified GMP grade collagenases yield good results during isolation and tend to be highly regular from a manufacturing standpoint, but synergistic effects have been documented when using collagenase in combination with other proteolytic enzymes. Neutral proteases were shown to be a valuable component in tissue dissociation enzyme mixtures as demonstrated by McCarthy et al., who showed that the combination of neutral proteases and collagenase type I and type II yielded greater tissue dissociation than any of the three enzymes individually [67]. Thermolysin, CHNP, and Dispase share similar profiles for specificity and share significant amounts of structural homology across species and strains. These three are much more commonly found in TDE blends than trypsin and clostripain. In addition, in 2015 Dendo et al. demonstrated synergistic effects of neutral proteases and clostripain in the isolation of rat pancreatic islet cells [68]. Another study by Williams et al. in 1995 suggests that a mixture of trypsin and highly purified collagenase also provides a synergistic advantage in adipose tissue digestion [69]. While the literature suggests that a mixture of proteolytic enzymes will yield better dissociation and subsequently higher cellular yield, there is not a common consensus as to the optimal combinations and concentrations which should be used.

### Crude Collagenase versus Purified Collagenase

Crude collagenase mixtures are often overlooked when choosing enzymes for tissue dissociation. Crude collagenase mixtures are isolated collagenases which have not been purified to levels similar to other highly purified enzyme mixtures. As a result, they contain various other clostridial proteases such as neutral protease and clostripain. While based on previous research, this would not seem like an issue, as mixtures are preferred, but there tends to be significant lot to lot and vial to vial variation as well as enzyme impurity, pigment contamination, imbalances of key active components and high endotoxin levels which all result in lower digestion efficiency [70-73]. A 2011 study by Wang et al. highlighted the utility of crude collagenase in the lab setting [74]. Wang et al. compared the Sigma V crude collagenase and SERVA NB1 purified collagenase for human islet isolation. The crude collagenase, once sterile filtered, was comparable in terms of efficacy and activity, as well as demonstrated significantly lower levels of endotoxin. They concluded that crude collagenase can be a cost saving option for laboratory research. Purified enzymes are still preferred for use in the clinical setting though because of reduced variability and contamination and better compositional characterization.

### Clinical Safety

From a clinical standpoint, tissue dissociation enzymes introduce

minor risks to patients. Proposed *in vivo* risks due to proteolytic enzymes include allergic reaction and unwanted tissue degradation. Preclinical and clinical studies have shown relatively low risk from small amounts proteolytic enzymes as a result of systemic exposure or localized injection.

According to a 2013 study by Chang et al., there were no concerns of toxicity relating to residual collagenase activity in ASC related procedures [75]. They demonstrated the lack of toxicity *in vivo* using a mouse model. Mice were injected with a fat graft containing SVF cells isolated using collagenase. Mice were monitored over 4 weeks, and then dissected for histopathological analysis. There were no significant changes observed between treatment and control group in this study. In addition, Chang et al. demonstrated that levels of residual collagenase activity are negligible if at least 3 washes of the SVF cells are conducted following tissue digestion. In 2009, the Arthritis Advisory Committee issued a briefing document on Collagenase *Clostridium histolyticum* [76]. They gave an extensive review of clostridial collagenase as it pertains to use in the drug Xiaflex for Dupuytren's contracture. They reviewed both clinical and preclinical evidence, which supported the clinical safety of collagenase. Three animal based studies that were reviewed showed that after local injection with clostridial collagenase there was no systemic toxicity and only a small amount of systemic exposure which resulted only if collagenase was administered into highly vascular areas [77-80]. Another animal based study of collagenase studied the effects of systemic circulation of collagenase by administering collagenase intravenously to rats [76]. In this study, it was noted that when collagenase was detected in systemic circulation that there was no accumulation following repeated dosing and it was cleared rapidly from the system. When administered intravenously at a high dose of 0.29 mg/animal, which is equivalent to almost 22 times the clinical dose of Xiaflex, type I collagenase was not detectable after 30 min and type II was not detectable after 2 hours. Overall, collagenase was determined to have a very low level of toxicity [81-83].

The levels of residual collagenase activity reported in SVF [59,84] are significantly lower than other collagenase based products which have been approved by the FDA, specifically Xiaflex and Collagenase Santyl. Santyl is a collagenase based ointment (250 U/g) which is topically applied for enzymatic wound debridement [85,86]. Xiaflex is a highly concentrated injection of collagenase (~3600 U/dose) used to treat Dupuytren's contracture, a contracture of the hand due to palmar fibromatosis [87-89], and Peyronie's disease, a similar contracture located in the penis [90,91]. Xiaflex is administered via subcutaneous injection at the site of contracture. Both of these products, Xiaflex and Santyl, underwent a high level of safety testing and were deemed safe by the FDA. The levels of residual collagenase activity reported for SVF isolation are thousands of times lower than those in Santyl and Xiaflex. While only some comparison can be made between Santyl, Xiaflex and the use of collagenase in SVF cell isolation because they are administered in different manners, it does offer some insight into the levels of collagenase activity that the FDA has deemed safe for human use.

### Regulatory Issues

In the United States, the main regulatory issue associated with the isolation of SVF cells from adipose tissue is minimal manipulation. In December of 2014, the FDA released a series of draft guidances for industry dealing with the minimal manipulation and homologous use of HCT/PS from adipose tissue [92,93]. In these draft guidances, the FDA very clearly states that the isolation of SVF cells from adipose tissues, with or without the use of proteolytic enzymes, results in a final

product which is considered to be “more than minimally manipulated” because the original structure of the adipose tissue has been significantly altered. Additionally, the addition of proteolytic enzymes is considered more than minimal manipulation because they are not water, crystalloids, or a sterilizing, preserving, or storage agent. This has very serious clinical implications for ADSC and SVF-based therapies should these draft guidances become finalized. Being considered “more than minimally manipulated” means that SVF cells are considered to be a drug and not a minimally manipulated autologous cellular therapy, and therefore subject to an additional set of regulatory guidelines, section 351 of the Public Health Service (PHS) Act. The finalization of these draft guidance will make the approval process for SVF-based therapies significantly longer and more expensive for clinicians and private companies alike. Prior to this guidance, a combination of unclear regulatory status and lack of action on the part of the FDA has allowed SVF-based therapies to side step almost all regulation. Currently, most clinical experimentation with SVF-based therapies is typically done only under the regulatory guise of an institutional review board (IRB), but with finalization of this guidance, will require a much more formal and extensive Investigational New Drug (IND) or Investigational Device Exemption (IDE) from the FDA in order to proceed clinically. The IND/IDE processes are both long and expensive, but ultimately will result in a higher level of patient safety, uniform and improved reporting of clinical data across trials, and greater transparency in the clinical setting.

## Conclusion

There are many different enzymes which can be used in the isolation of SVF cells from adipose tissue. Various factors need to be considered when designing a proper protocol for SVF cell isolation. While this review does not cover the specific isolation methods, it does provide information for the proper use and preparation of tissue dissociation enzymes in order to help with protocol design. Additionally, there are various regulatory and practical considerations which need to be considered.

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