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# Differential effects of processing time and duration of collagenase digestion on human and murine fat grafts

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## Abstract

**Background—**Autologous fat graft retention is unpredictable and mechanisms of optimization are poorly understood. Attempts at improving retention utilize collagenase experimentally and clinically to isolate the stromal vascular fraction to "enhance" fat grafts. However, no standardized duration for collagenase digestion or time following fat graft harvest has been established. This study investigates the effect of 1.) time after fat graft harvest and 2.) collagenase digestion time on interstitial cell and adipocyte viability in murine fat and human lipoaspirate.

**Methods—**Murine fat and human lipoaspirate were incubated *ex vivo* after harvest at room temperature for 120 minutes. Additional groups were incubated with collagenase for increasing five minute intervals from 30-60 minutes. Samples from each group were stained with BODIPY to quantify intact adipocytes and LIVE/DEAD kit to quantify interstitial cell viability.

**Results—**With increased time post-harvest, the number of intact adipocytes in murine fat and human lipoaspirate remained unchanged. Human interstitial cells were resistant to the effect of increased time *ex vivo*, while murine interstitial cells decreased in viability. In both populations, increased collagenase digestion time significantly decreased the number of viable adipocytes (murine: p-value ≤ 0.001, human: p-value ≤ 0.001) and interstitial cells (murine: p-value ≤ 0.001, human: p-value ≤ 0.001).

**Conclusions—**Human and murine adipocytes and human interstitial cells appear resistant to deleterious effects of increasing time following harvest. However, murine interstitial cells including are sensitive to increased time and prolonged collagenase digestion. These studies highlight the complex cellular components of fat grafts and how they respond differentially to time and collagenase digestion.

### Keywords

fat graft; adipose tissue; collagenase; adipocyte

## INTRODUCTION

Clinical indications for autologous fat grafting have increased in both reconstructive and aesthetic plastic surgery in the past decade[1–8]. There has been a surge in both large-volume fat grafting utilized in breast and buttock reconstruction, as well as small-volume fat grafting in facial aesthetic and reconstructive applications[5,7,9].

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Intense interest in autologous fat derives from advantages including abundance of donor fat, ease of harvest, relative decreased cost compared to allogeneic and implant alternatives, and avoidance of foreign non-autologous implant material and related complications[8,10]. However, long-term volume retention of autologous fat grafts are highly unpredictable, with resorption rates ranging from 20-75%[10].

Fat grafts are heterogeneous and are composed of adipocytes and interstitial cells, including endothelial cells, macrophages, pericytes, fibroblasts, adipose-derived stem cells, and other blood-derived cells[11]. Methods for "enhancing" or supplementing the fat graft are being developed to improve graft retention rates[7,9,12,13]. Adipose stem cells (ASCs) have been studied extensively for this application and other therapeutic applications. A number of recent studies have suggested that supplementation, or "enhancing" fat grafts with adipose-derived stem cells (ASCs), increases the graft retention rates[7,9,10,12,13].

The most common technique for enhancing fat grafts in clinical practice and in pre-clinical murine models involves collagenase digestion of a portion of the fat graft to isolate the stromal vascular fraction (SVF) and supplementation of the fat graft with this ASC-rich solution. Collagenase dissociates the collagen-rich extracellular matrix (ECM) of the adipose tissue, which liberates the ASCs from the interstitial space. Although collagenase digestion is not approved for this indication in the United States, this technique is currently in clinical practice in a number of other countries, including Japan, Spain, and Korea[7,9,12,14]. In the laboratory setting, isolation of individual adipocytes using collagenase is commonly used in murine and human populations to study the adipogenesis cascade[15,16], adipokine secretion from adipocytes[17–19], adipocyte metabolism[19], and immune cell recruitment to adipose tissue/adipocytes[20,21].

Despite the prevalence of using collagenase digestion as a means to isolate the SVF and individual adipocytes, a standardized time for collagenase digestion of human and murine adipose tissue has not been determined. Collagenase digestion duration must be long enough to allow for adequate disaggregation of the adipose tissue and release of the interstitial cells, while also short enough to prevent deleterious cell disruption or cell death. Digestion times reported in the literature range from 30 minutes to 90 minutes, with no consensus supported by scientific evidence[22–24]. This study seeks to identify the optimal collagenase digestion duration that minimally disrupts viability of both adipocytes and interstitial cells.

A second important variable in both the clinical and laboratory settings is the time after adipose tissue removal from the organism and prior to transplantation (*ex vivo* time), which can be significant and is often overlooked as a factor affecting graft retention. For example, during large volume fat graft harvest, lipoaspirate may be *ex vivo* for two hours prior to reinjection. In a laboratory setting, time *ex vivo* following removal from a human or laboratory subject may also be significant prior to analysis. No studies to date have examined the effect of this time *ex vivo* on the viability of the adipocytes or interstitial cells in human and murine adipose tissue.

In the current study, we sought to determine: 1) how the amount of time after harvest and prior to injection transplantation (*ex vivo* time) affects adipocyte and interstitial cell health,

and 2) if the duration of collagenase digestion affects adipocyte and interstitial cell health. We hypothesize that increasing collagenase digestion will decrease adipocyte and interstitial cell viability in both human and murine adipose tissue. Further, we hypothesize that time *ex vivo* will not significantly affect the viability of adipocytes and interstitial cells in human and murine adipose tissue.

A better understanding of how time after harvest and collagenase digestion affects the adipocytes and interstitial cells within adipose tissue will inform procedures for fat handling and manipulation that will improve autologous fat graft retention in the clinical setting. Further, these studies may help to standardize potential confounding variables in the laboratory research setting – the effect of time *ex vivo* and collagenase digestion on murine adipose tissue.

## MATERIALS AND METHODS

### Murine adipose tissue harvest

All procedures were performed in accordance with the University of Virginia Institutional Animal Care and Use Committee. Eight to twelve week old BALB/c mice (Charles River, Washington, MA) were humanely euthanized with $CO_2$ asphyxiation with 3-4 mice used for each group. Immediately following asphyxiation, both inguinal fat pads were surgically excised and stored in phosphate buffered saline (PBS) until use. The time of removal (t=0 minutes) was noted to ensure accurate time points for subsequent studies.

### Human adipose tissue harvest

Subcutaneous adipose tissue was obtained according to an approved protocol by The University of Virginia's Institutional Review Board. All human adipose tissue was acquired from intraoperative suction lipectomy from nondiabetic patients undergoing elective surgical procedures at the Department of Plastic Surgery at The University of Virginia. All six patients were female, non-diabetics and ranged in age from 25-65 with body mass indexes ranging from 22.5-27.5. Adipose tissue was incubated in a sealed container at room temperature (25°C) and time of removal was noted to ensure accurate time points for subsequent studies.

### Ex vivo time studies

Murine and human adipose tissues were incubated *ex vivo* in PBS at room temperature (25°C) for time points up to 120 minutes. After incubation was complete, the tissue samples were divided evenly into two groups for each time point: one for BODIPY (Life Technologies, Grand Island, NY) staining and the other for LIVE/DEAD staining (Life Technologies, Grand Island, NY). Samples that were stained with BODIPY were submerged in 500 $\mu$L of 4% (v/v) paraformaldehyde (PFA) and incubated at 4°C overnight. Samples to be stained with the LIVE/DEAD kit were not incubated in 4% PFA prior to staining.

### Collagenase digestion of murine and human adipose tissue

Both murine and human tissue were digested at a concentration 1 g tissue/mL of collagenase-containing digestion buffer consisting of 0.1% (weight/volume) collagenase

Type I (Worthington Biochemical Corporation, Lakewood, NJ), 2.5% (weight/volume) bovine serum albumin (Jackson ImmunoResearch West Grove, PA), 20 mM HEPES (Life Technologies, Grand Island, NY), 200 nM adenosine (Sigma, St. Louis, MO), 1.2 mM $KH_2PO_4$ (Sigma, St. Louis, MO), 1.2 mM $MgSO_4 \cdot 7H_2O$ (Sigma, St. Louis, MO), 120 mM NaCl (Sigma, St. Louis, MO), 4.7 mM KCl (Sigma, St. Louis, MO), 1.3 mM $CaCl_2 \cdot 2H_2O$ (Fisher Scientific, Pittsburgh, PA)[23]. The murine tissues were divided into two control groups: untreated and mechanically dissociated only. Untreated murine tissue remained intact and was incubated in the same buffer above except without collagenase (collagenase-free buffer) for 60 minutes. Mechanically dissociated murine tissue was incised with scissors for one minute to simulate the effects of suction lipectomy in human tissue. Collagenase-treated murine tissue was also mechanically dissociated and subsequently enzymatically digested with collagenase-containing digestion buffer at increasing five minute intervals from 30-60 minutes. Tissue samples were incubated in a 37°C water bath and agitated every 5 minutes to ensure uniform digestion. After digestion was complete, the tissues were washed three times with PBS to remove remaining collagenase based on previous studies[25]. Tissue samples were divided into two groups for each time point: one for BODIPY staining and the other for LIVE/DEAD staining. Samples to be stained with BODIPY were submerged in 500 μL of 4% (v/v) paraformaldehyde (PFA) and incubated at 4°C overnight. Samples to be stained with the LIVE/DEAD kit were not incubated in 4% PFA prior to staining.

Untreated human tissue harvested from suction lipectomy was incubated without collagenase buffer for 60 minutes. The treated human tissue was enzymatically digested with collagenase-containing buffer according to the same protocol outlined above for murine tissue.

## BODIPY Staining

Samples were washed two times with PBS to remove residual 4% PFA. 100 μL of 10 μg/mL BODIPY 558/568 $C_{12}$ (Life Technologies D-3835)/Hanks Balanced Salt Solution (HBSS) was added to the samples and then incubated at 37°C for 20 minutes in the dark. Samples were washed three times with HBSS prior to mounting for imaging.

## LIVE/DEAD Staining

After incubation *ex* vivo or immediately following the wash steps after collagenase digestion, the LIVE/DEAD Cell Viability Kit (Life Technologies L7013) was used to stain tissue and assess cell viability. For murine tissue, Component A and Component B were diluted 1:500 in HBSS to make a working staining solution. For human tissue, Components A and B were diluted 1:250. 100 μL of the working staining solution was added to each of the samples and tissue was incubated at room temperature for 30 minutes protected from light. Following staining, samples were washed three times with HBSS prior to mounting for imaging.

## Whole mounting tissue

Samples were allowed to adhere to gelatin coated microscope slides for 5 minutes and sealed with coverslips in 50:50 PBS/glycerol solution.

### Confocal Imaging

Samples were imaged on a Nikon TE 2000-E2 microscope (Nikon Instruments, Melville, NY) equipped with a Melles Griot Argon Ion Laser System (Melles Griot, Carlsbad, CA) and a Nikon D-Eclipse C1 confocal attachment. To account for tissue depth, 40 $\mu$m Z-stacks were acquired with a 2 $\mu$m step size. For both LIVE/DEAD and BODIPY samples, three, 200x fields of view were obtained. The entire field of view (FOV) was filled by adipocytes when imaging the BODIPY stained samples. FOVs with large vascular structures when imaging the LIVE/DEAD stained samples were excluded.

### BODIPY Quantification

BODIPY stains the lipids in adipocytes and allows visualization of these cells. Images acquired of BODIPY stained samples were examined with confocal microscopy to assess architectural integrity of adipocyte, and images were analyzed using ImageJ Software (National Institutes of Health, Bethesda, MD). The number of intact adipocytes for each image was counted by a single, trained and blinded observer. Adipocytes were considered intact if no lysis was evident, no lipid droplets were present and if the shape of the adipocyte was consistent with untreated adipose tissue.

### LIVE/DEAD Quantification

Images acquired of LIVE/DEAD stained samples were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). Images acquired were split into the respective channels (red and green) and cells were counted manually by a single, trained and blinded observer. The quantity of red cells constituted the number of nonviable cells. The quantity of green cells constituted the total number of cells. Percent of viable cells were calculated using the following equation.

$$Percent\quad of\quad viable\quad cells=\frac{Total\quad cells-Dead\quad cells}{Total\quad Cells}\times 100$$

### Statistics

A One-way ANOVA with a Tukey's Post-hoc test was used to compare all measurements and test for significance as appropriate with significance asserted at p-value ≤ 0.05. For more details regarding statistical testing, please see figures and corresponding captions.

## RESULTS

### Duration ex vivo does not significantly affect adipocyte health in murine or human adipose tissue

Whole-mounted images of BODIPY stained murine and human tissue reveal no significant change in adipocyte viability as time *ex vivo* increases. The number of intact adipocytes did not change significantly for murine (Figure 1, bottom right) or human tissue (Figure 2, bottom right). Human adipocytes were larger in area than the murine adipocytes, consistent with previous findings[26].

### Duration ex vivo affects interstitial cell viability in both murine and human adipose tissue

Whole-mounted images of LIVE/DEAD stained murine adipose tissue reveal that the time *ex vivo* significantly affects the percentage of viable interstitial cells – as time is increased, the percentage of viable cells decreases. In murine fat grafts, after 20 minutes *ex vivo,* 80% of interstitial cells are viable and after 120 minutes 40% of interstitial cells are viable. Quantified numbers of murine cell viability are reported in bottom right panel of Figure 3. Images of LIVE/DEAD stained human tissue reveal the time *ex vivo* significantly affects the percentage of viable interstitial cells, albeit to a lesser degree in human tissue than murine tissue (Figures 3, 4). In human fat grafts, after 20 minutes *ex vivo,* 75% of interstitial cells are viable, and after 120 minutes 60% of interstitial cells are viable. Quantified numbers of human interstitial cells and the percentage that were viable are shown in the bottom right panel of Figure 4.

### Duration of collagenase digestion affects murine and human adipocyte viability

Collagenase digestion of murine adipocytes significantly decreases the number of intact adipocytes as the duration of collagenase digestion is increased (Figure 1). Mechanically dissociating the murine tissue (minced) resulted in a slight but not significant decrease in number of intact adipocytes. There is a significant decrease in intact adipocytes as collagenase digestion duration is increased in murine tissue. Similar to what was observed in murine adipose tissue, human adipose tissue exhibited a statistically significant decrease in the number of intact adipocytes as duration of collagenase digestion increased (Figure 2).

### Duration of collagenase digestion affects murine and human interstitial cell viability

Representative images of whole-mounted images of murine and human adipose tissue revealed that the amount of time that the tissue was exposed to collagenase digestion affected the viability of interstitial cells contained within the tissue (Figures 3, 4). Increasing the time required for collagenase digestion significantly decreased the percentage of viable interstitial cells in both murine and human adipose tissue.

## DISCUSSION

Autologous fat grafting has increasing indications in both reconstructive and aesthetic plastic surgery. The benefits of fat grafting have been well described, however the conditions to improve retention of fat grafting are still being elucidated[1,3–5,7–9,27]. The aim of this study was to evaluate the effects of time and collagenase on human lipoaspirate and experimental mouse adipose tissue to optimize conditions affecting fat graft retention in clinical and research laboratory settings. Human lipoaspirate and murine adipose tissue were systematically examined using multiple modalities over increasing time *ex vivo* and over increasing collagenase digestion intervals. Human adipocytes and murine adipocytes remained intact over increased time intervals up to two hours. Murine and human interstitial cells within adipose tissue exhibited decreased viability over increased time to varying degrees. Murine interstitial cells were seemingly more affected by time *ex vivo* than human interstitial cells. Increased collagenase digestion time significantly decreased viability of both human and murine adipocytes and interstitial cells.

Determining the effect of increasing temporal delays after harvest of the graft and prior to injection of the fat graft was a goal of this present study, which used multiple modalities to quantify viability of cell populations within adipose tissue. The finding that adipocytes (human and murine) are resilient enough to remain viable two hours *ex vivo* is consistent with findings by Eto et al.[28]. Specifically in a human fat graft, the adipocytes and the cells in the interstitial space, including endothelial cells and adipose derived stem cells, have been found previously to maintain viability one day after removal and when maintained in serum-free medium under hypoxic conditions[28]. We report that adipose tissue (human and murine) viability is not significantly impaired up to 2 hours when maintained in saline solution at room temperature, which we feel is more akin to a surgical setting or laboratory setting. Our findings agree with the findings of Eto et al. and build on their findings, potentially providing more insight into the survival of adipose tissue in a clinical or laboratory setting. Temporal delays after harvesting murine adipose tissue for pre-clinical experiments may be substantial, but the viability of murine adipocytes was not significantly attenuated over time in this study.

In our *ex vivo* studies, the interstitial cell component of the adipose tissue harvested was affected differently in both species studied. The human interstitial cells were resistant to the effect of increased time *ex vivo*, while the murine interstitial cells decreased in viability with increased time *ex vivo*. This may be in part explained by the technical differences in the treatment of the murine fat graft (manual dissociation required to simulate liposuction) as compared to the human fat graft (harvested via traditional liposuction). Further study into this is required to understand the differences in murine interstitial cells compared to human interstitial cells, as this is the first report of this observed difference interstitial cell viability between different species with respect to time.

Results revealed that increased duration of collagenase digestion significantly decreased adipocyte and interstitial cell viability in both human and murine populations. This is consistent with prior work by Piasecki and colleagues[29] who found that human fat grafts fragmented at higher rates with increased duration of collagenase digestion with times ranging from 0-180 minutes[29]. No other studies to date have examined the effect of collagenase digestion duration on murine adipocytes and murine interstitial cells. Although our studies were carried out in two different species (murine and human), the results for each species are valuable to the scientific and surgical communities, as pre-clinical murine models play a critical role in the therapy development pipeline.

Our data show that collagenase may be detrimental to interstitial cell viability and adipocyte integrity and that it is therefore very important to deactivate or wash out any residual collagenase prior to enhancing fat grafts with SVF or using adipose tissue for preclinical studies. Failure to do so may lead to unwanted fat necrosis, a false sense of correction of the deformity intraoperatively, and a low rate of retention of the fat graft in the postoperative period.

It is imperative to consider and tailor the time, temperature, concentration, and type of collagenase used for specific studies or experimental trials. Bacterial-derived collagenase remains the most common proteolytic enzyme used for tissue dissociation[30] and has

growing implications in the plastic surgery and reconstructive field for use in adipose tissue. Collagenase disrupts the collagen-rich extracellular matrix of adipose tissue allowing for easy harvest of interstitial cells and single adipocytes.

"Crude" collagenases (several types exist) are often used and are not pure collagenase, but rather a mixture of several enzymes in addition to collagenase capable of dissociating the ECM. For the present studies, type I collagenase was used. This is typically used for adipose dissociation but there is no standard in the literature, and other authors have utilized other collagenase types for dissociation. Also important in the collagenase digestion process is the temperature and collagenase concentration used, with varying temperatures and concentrations used in the literature. Although our studies used one type of collagenase (Type I), one temperature for digestion (37°C), and one concentration (1 mg collagenase/mL), they highlight the importance and the possible deleterious effects of collagenase on the adipocytes and interstitial cells in both human and murine tissue.

Currently, the use of collagenase to isolate SVF and enhance fat grafts is not an approved practice by the United States Food and Drug Administration (FDA) but is practiced in other countries[7,9]. Further, collagenase as a biologic has been recently approved by the FDA (XIAFLEX®, Auxilium Pharmaceuticals) to treat Dupuytren's contracture[31] and Peyronie's disease[32]. With proper characterization and future studies to examine the effect of collagenase in more detail, FDA approval to enhance fat grafts with collagenase-isolated SVF may be imminent.

A few specific limitations temper the conclusions drawn from this study. First, mice lack sufficient volumes of subcutaneous adipose tissue to perform liposuction *in vivo*, thus inguinal fat pads were manually dissociated as previously shown to adequately fragment the fat for the purposes of study[33]. This surgical technique is well established as an adequate proxy for liposuction; however, it may increase interstitial cell susceptibility to cell death. Additionally, the small sample sizes also limit the conclusions that can be drawn, however the evidence of parallel findings consistent across multiple modalities in this study add strength to the conclusions. All murine tissue was treated in the same manner and thus serves as an internal control. Secondly, we used architectural integrity after BODIPY staining as a marker for viable adipocytes (no lysis evident, no lipid droplets present, shape consistent with untreated adipose tissue). Because our chosen assays rely on architectural integrity, it is possible that only cells with severely compromised membranes are being detected as non-viable. There may be a subpopulation of cells with irreversible damage but do not have compromised membranes and thus we may be underestimating the percent of non-viable cells. Reliably determining a viable, intact adipocyte from a nonviable adipocyte has proven to be a challenge that has been identified by the literature, and it is necessary to employ a combination of viability assays or stains. Suga et al.[34] conclude that three common viability assays (XTT, MTT, trypan blue) cannot be used singly and must be employed in a combinatorial fashion for adipocyte specificity. Suga et al.[34] used Hoechst 33342 and propidium iodide to distinguish viable, intact adipocytes following collagenase digestion. We coupled our BODIPY staining with a LIVE/DEAD stain to similarly provide a global view of overall adipose tissue viability that is consistent with their use in other published studies[11,35,36]. The combination of these assays and the consistencies of the findings from

each assay adds to the strength of the conclusions that can be drawn. Our studies were conducted using liposuction samples from all female patients potentially limiting the generalizability of the findings to both sexes. However, this is consistent with published national trends showing that 89% of patients undergoing autologous fat grafting in 2013 were female[37]. Although this limits the generalizability of the findings in this study to the female gender, most patients undergoing autologous fat transfer nationally are female thus the findings are highly relevant. Further, there are several examples in the literature outlining the potential impact of donor gender on ASC behavior such expansion and differentiation capabilities. Aksu et al. showed that male-derived human ASCs differentiate down an osteogenic pathway more effectively[38], while Ogawa et al. showed that female murine ASCs differentiate down the adipogenic pathway more effectively[39]. To our knowledge, no studies have been conducted examining the viability of the interstitial cells or adipocytes between male and female donors. Our studies do not characterize the cell type composition of the interstitial cell compartment and do not examine the individual cell types. However, these future studies are feasible and would be interesting to couple these findings with an *in vivo* retention model of fat grafting (e.g. dorsal subcutaneous implantation) to correlated specific cell types responsible for fat graft survival.

Our approach provides insight into both the interstitial cells within the adipose tissue, as well as the adipocytes themselves. We are confident that these two staining protocols allow us to differentiate healthy and viable adipose tissue from adipose tissue that has been detrimentally affected. Altering the collagenase concentration, the temperature in which the digestion is performed, and the type of collagenase will very likely affect both clinical and research study outcomes. Thus at the present time, while we are not able to definitively identify an “optimal” digestion time as it is likely dependent on these aforementioned parameters; our results provide insight into the detrimental effects of increasing collagenase digestion duration.

## CONCLUSIONS

Our results suggest that adipose tissue can withstand an *ex vivo* time period of up to two hours with no significant effect on adipocyte viability. This information can inform guidelines for operating procedures and provide surgeons with a timeframe for fat grafting that ensures cell viability and may lead to better engraftment outcomes and volume retention. Autologous fat grafting is frequently performed in the setting of larger-volume suction lipoplasty, which would require the harvest of more adipose tissue and delay the surgical time to grafting. Our data suggest that surgeons may proceed without fear of loss of graft viability from this temporal delay of up to two hours.

While our study was not designed to identify an optimal digestion duration that would preserve adipocyte and interstitial cell viability while allowing for complete dissociation of adipose tissue for isolating of SVF, our findings show that prolonged collagenase digestion decreases the number of viable interstitial cells and adipocytes in murine and human tissue. Our study allows us to conclude that increased collagenase digestion duration may be detrimental to cell viability and not likely beneficial for autologous fat grafting.

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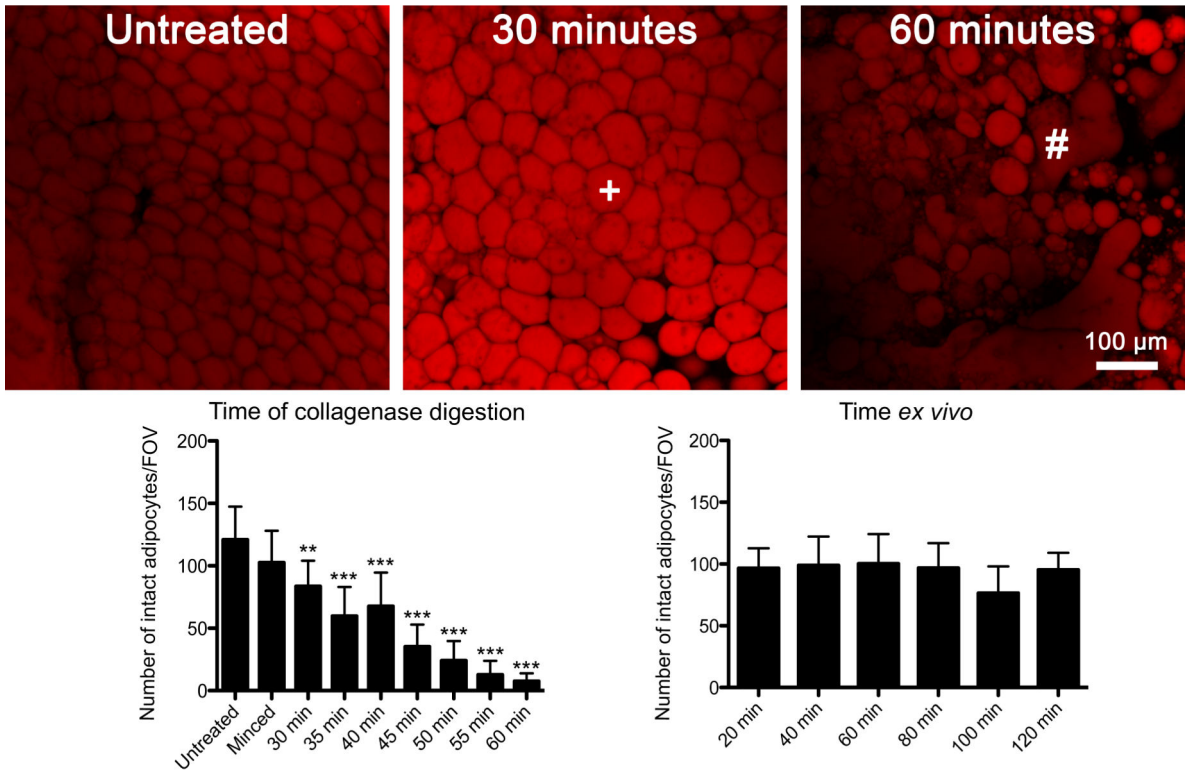


**Figure 1.**

Murine inguinal fat pads were digested with collagenase at increasing five-minute intervals, stained with BODIPY and imaged using confocal microscopy. The "Untreated" fat pad was neither minced nor digested with collagenase. Also shown are representative images for collagenase digestion durations of 30 and 60 minutes. Left graph: Four trials (n=4) using eight murine fat pads were quantified for intact adipocytes using three fields of view (FOVs) for each time point or control group. Confocal images and the quantification of intact adipocytes reveal that in murine inguinal fat pads, increasing the duration of collagenase digestion results in a decreased number of intact adipocytes when compared to untreated tissue (One-way ANOVA, Tukey's Post-hoc test, *: p-value ≤ 0.05 **:p- value ≤ 0.01 ***: p-value ≤ 0.001). Right graph: Three trials (n=3) using six murine inguinal fat pads were quantified for intact adipocytes using three fields of view (FOVs) for each time point. Confocal images and the quantification of intact adipocytes reveal that increasing time after fat harvest does not have a statistically significant effect on the overall number of intact adipocytes in murine inguinal fat pads when compared to 20 minute *ex vivo* tissue (One-way ANOVA, Tukey's Post-hoc test). A representative intact adipocyte is marked with "+," and a representative lysed adipocyte is marked with "#." Data shown are mean plus standard deviation.

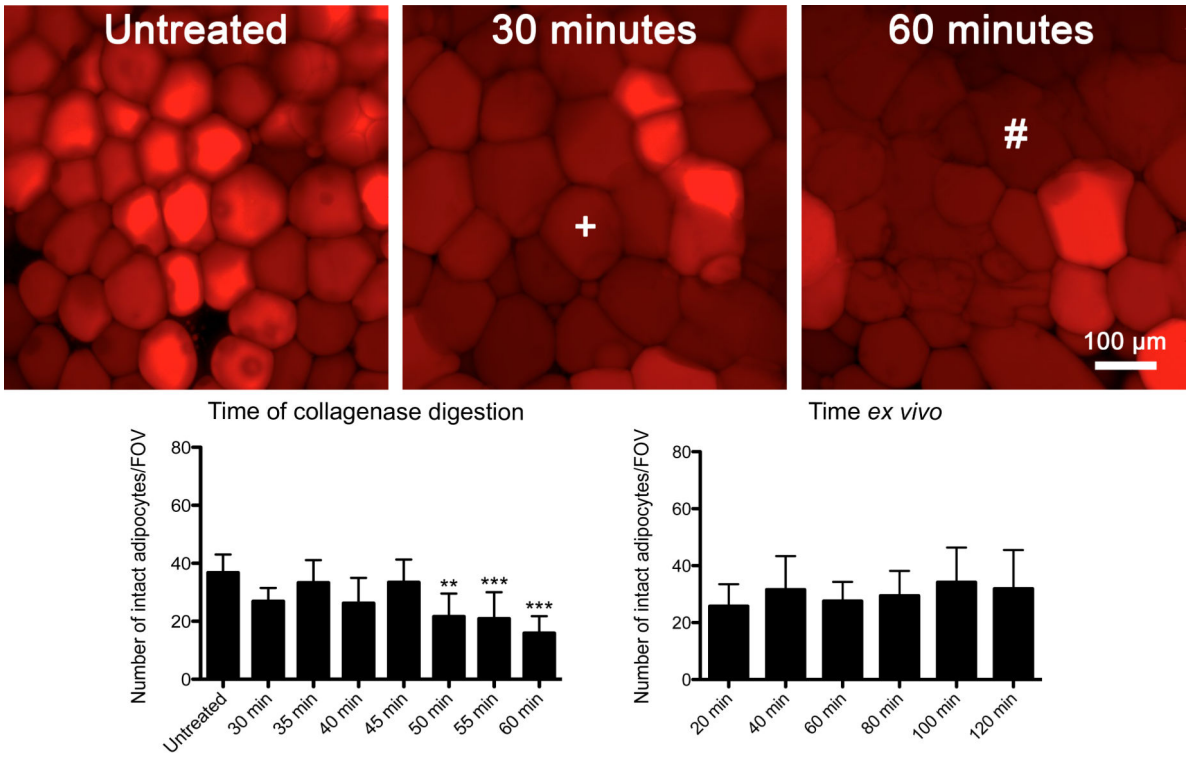


**Figure 2.**

Human lipoaspirate harvested using the Coleman technique was treated with collagenase at increasing five-minute intervals, stained with BODIPY and imaged using confocal microscopy. The "Untreated" fat pad was neither minced nor digested with collagenase. Also shown are representative images for collagenase digestion durations of 30 and 60 minutes. Left graph: Human lipoaspirate from three subjects (n=3) were quantified for intact adipocytes using three fields of view (FOVs) for each time point or control group. Both confocal images and the quantification of intact adipocytes reveal that in human lipoaspirate, increasing the duration of collagenase digestion results in a decreased number of intact adipocytes when compared to untreated tissue (One-way ANOVA, Tukey's Post-hoc test *: p-value ≤ 0.05 **:p-value ≤ 0.01 ***: p-value ≤ 0.001). Right graph: Human lipoaspirate from four subjects (n=4) were quantified for intact adipocytes using three fields of view (FOVs) for each time point. Confocal images and the quantification of intact adipocytes reveal that increasing the duration of time up to 120 minutes after fat harvest does not have a statistically significant effect on the overall number of intact adipocytes in human lipoaspirate when compared to 20 minute *ex vivo* tissue. A representative intact adipocyte is marked with "+," and a representative lysed adipocyte is marked with "#." Data shown are mean plus standard deviation.

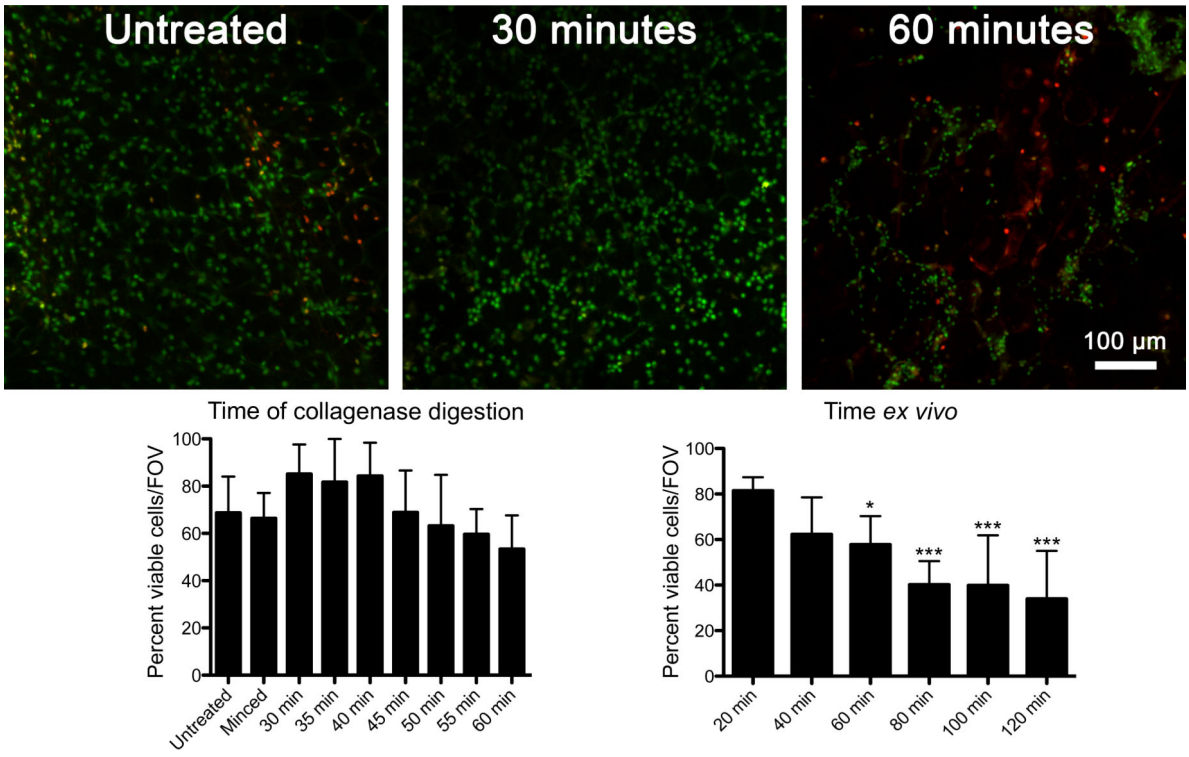


**Figure 3.**

Murine inguinal fat pads were treated with collagenase at increasing five-minute intervals, stained with LIVE/DEAD kit and imaged using confocal microscopy. Interstitial cells that are stained green are viable, and cells stained red or yellow are nonviable. The "Untreated" fat pad was neither minced nor digested with collagenase. Also shown are representative images for collagenase digestion durations of 30 and 60 minutes. Left graph: Four trials (n=4) using eight murine fat pads were quantified for the percent of viable interstitial cells using three fields of view (FOVs) for each time point. Confocal images and the quantification of interstitial cells reveal that in murine inguinal fat pads, increasing the duration of collagenase digestion results in a decrease in the percentage of viable interstitial cells. Right graph: Three trials (n=3) using six murine inguinal fat pads were quantified for the total number of interstitial cells using three fields of view (FOVs) for each time point. Confocal images and the quantification of interstitial cells reveal that in murine inguinal fat pads, increasing time after fat harvest results in a statistically significantly decreased percentage of viable interstitial cells when compared to 20 minute *ex vivo* tissue (One-way ANOVA, Tukey's Post-hoc test *: p-value ≤ 0.05 **:p- value ≤ 0.01 ***: p-value ≤ 0.001). Data shown are mean plus standard deviation.

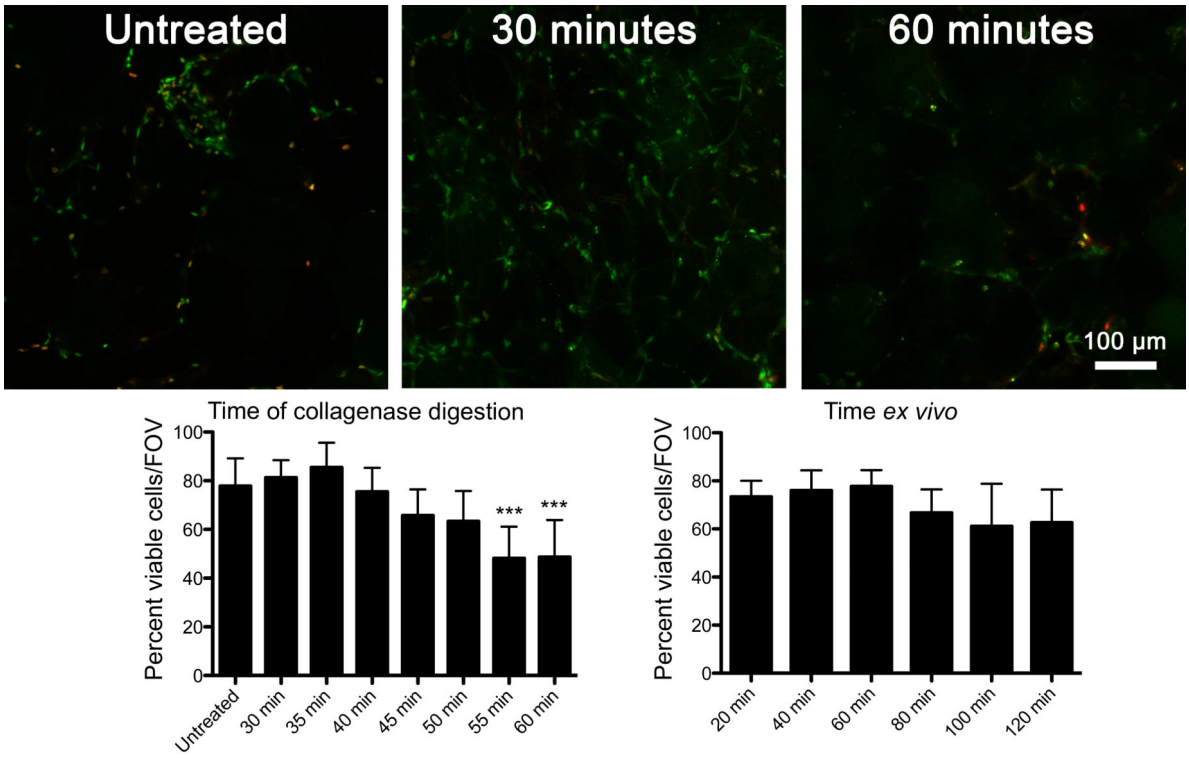


**Figure 4.**

Human lipoaspirate harvested using the Coleman technique were treated with collagenase at increasing five-minute intervals, stained with LIVE/DEAD kit and imaged using confocal microscopy. Interstitial cells that are stained green are viable, and cells stained red or yellow are nonviable. The "Untreated" fat pad was neither minced nor digested with collagenase. Also shown are representative images for collagenase digestion durations of 30 and 60 minutes. Left graph: Human lipoaspirate from three subjects (n=3) were quantified for the percent of viable interstitial cells using three fields of view (FOVs) for each time point. Confocal images and the quantification of interstitial cells reveal that in human lipoaspirate, increasing the duration of collagenase digestion results in a decreased percentage of viable interstitial cells when compared to untreated tissue (One-way ANOVA, Tukey's Post-hoc test *: p-value ≤ 0.05 **:p- value ≤ 0.01 ***: p-value ≤ 0.001). Right graph: Human lipoaspirate from four subjects (n=4) were quantified for the percent of viable interstitial cells using three fields of view (FOVs) for each time point. Confocal images and the quantification of interstitial cells reveal that in human lipoaspirate, increasing time after fat harvest does not result in a statistically significant difference in the percentage of viable cells when compared to 20 minute *ex vivo* tissue. Data shown are mean plus standard deviation.