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Research article

Patient use of autologous cryopreserved intact adipose tissue from lipoaspirate

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Abstract: Autologous fat grafting has now been extensively and successfully performed for more than two decades. Although most adipose grafts and adipose-derived MSC therapies are done with fresh tissue, cryopreservation of tissue allows much greater flexibility of use. Over the course of five years, 194 cryopreserved adipose samples were thawed and returned to the collecting physician for subsequent autologous applications. Samples were stored with a mean cryogenic storage time of 9.5 months with some samples being stored as long as 44 months. The volumes of tissue stored varied from 12 cc to as large as 960 cc. Upon thaw the volume of recovered whole adipose tissue averaged 67% of the original amount stored. The recovery ranged from a low of 21% (n = 1) to a high of 100% (n = 1). Recovery was not found to be a function of collection volume, amount of tissue thawed, or length of time in cryopreservation. No association was found between tissue recovery and patient age. Viability of thawed cells remained high with a mean value of 91%. While an average recovery of 67% of volume frozen indicates that the use of banked and thawed tissue requires a larger amount of sample be taken from the patient initially, this requirement is easily accomplished by an experienced clinician. As cryopreservation of adipose tissue becomes more commonplace physicians will find it helpful to know the amount and quality of tissue that will be available after thawing procedures.

Keywords: adipose tissue; cryopreservation; autologous; fat grafting; cryogenic storage; lipoaspirate; cosmetic surgery; regenerative medicine

Abbreviations: MSC: mesenchymal stromal cells; DMSO: dimethyl sulfoxide; PBS: phosphate buffered saline

1. Introduction

Autologous fat grafting has now been used extensively and successfully for more than two decades. First described late in the 19th century by Neuber [1], it was only minimally used throughout most of the 20th century. This lack of widespread adoption may have been due to poor performance as "blocks" of adipose tissue were usually transferred with little regard for the required vascularization [2]. Only with the advent of liposuction in the 1980s [3,4] and Coleman's work in the 1990s [5] did our current concept of adipose tissue transfer emerge. The increases seen after Coleman's systemization of the techniques in 1995 are such that the grafting technique is now commonplace. Twenty years of improved harvest techniques, increased knowledge of tissue handling, and a more experienced group of clinicians make adipose tissue grafting more efficacious than ever. In addition to clinical use of freshly harvested tissue, better cryopreservation protocols [6,7] and greater experience with post-thaw uses have made an equally strong case for cryopreserved adipose graft utility. Although most adipose grafts are still done with fresh tissue, cryopreservation of tissue allows much greater flexibility of use. A large variety of applications are now common in cosmetic uses [8,9] and stem cell therapies [10–12]. Many additional experimental applications show great promise from cardiac repair [13,14] to endocrine therapies [15,16]. More applications will be forthcoming as cryopreservation of adipose tissue becomes more commonplace.

One advantage of adipose tissue is that the physician can use whatever amount is needed for the treatment. Small scale cosmetic applications include treatment for facial rejuvenation [8,17], fine lines and wrinkles on face and hands [18,19], and scar repair [20,21]. Larger volumes are needed for applications such as breast reconstruction or enhancement [22,23], or other large area sculpting/remodeling. The amount of tissue needed for different applications can be exceedingly variable. While several hundred milliliters might be used for larger defects or for reconstruction, some uses will require far less. It, therefore, behooves the clinician to know how much fat would be a useful amount when it is time for the tissue to be applied. Many practitioners presently harvest adipose tissue as lipoaspirate and use it for reinjection during the same office visit [24]. This tissue, broken down only insofar as the canula and suction can accomplish, remains in this form for many reinjection applications. Since it is not further broken down mechanically or enzymatically, it can be described as "whole" or "intact" adipose tissue for purposes of grafting. As large blocks of adipose are known to graft poorly (if at all) [2], the lipoaspirate harvest comes though as large granules in the range of one to several millimeters. Recently, others have proposed the formation and use of adipose tissue pieces of other sizes. The concepts and use of "microfat" [25] and "nanofat" [26,27] are interesting and will doubtless be an important topic for many years. However, as none of the mechanical resizing nor enzymatic digestions have been done here, these concepts are outside the scope of this paper. As such, we show here that this whole and intact tissue from lipoaspirate is useful beyond the immediate reapplication on the same day.

With such widespread and common use of intact lipoaspirate, it has been clear for some time that cryopreservation of adipose tissue for future use is not only a viable option, in many ways it is advantageous. The adipose tissue collected from a single harvest can be cryopreserved into multiple aliquots of varied sizes [28]. This approach reduces not only cost to the patient but patient discomfort, morbidity and possible complications. As a general principle, cells that are younger are more healthy, hardy and generally more useful. As patients age it would be beneficial for physicians to have a bank of tissue from which they could draw. If these frozen tissues are adipose tissue aliquots from the

same patient (only younger) so much the better [29]. As adipose tissue is a rich source of mesenchymal stromal cells (MSCs) [30] and other cells future applications in cellular and regenerative medicine are amendable.

Cryopreservation of a large amount of tissue allows the medical professional to procure from a large store of tissue whenever additional adipose tissue is needed. This approach obviates the need for additional patient harvests with their associated cost and trauma. For those patients that may have less than optimal healing, this may be extremely useful. Diabetics, the elderly, rheumatoid arthritics, or others with chronic wounds and ulcerated conditions may benefit most from this approach. To aid the clinician in determining the amount of adipose tissue that should be harvested for cryopreservation, this study analyzes recovery, viability and sterility results supporting the utility of cryopreserved adipose tissue for future clinical use. We hypothesize that cryostorage, and subsequent thaw, of multiple aliquots of lipoaspirate provides the fat grafting surgeon with tissue volume, viability, and utility comparable to freshly harvested, and is a viable alternative to multiple tissue harvests.

2. Materials and methods

2.1. Patient selection

Adipose tissue samples were collected from properly consented patients undergoing elective lipoplasty. Adult patients ranging in age from 27 to 81 years were given the opportunity for private cryostorage of residual lipoaspirate. Tissue collected was solely for autologous use.

2.2. Statistics

All data was collected into Excel spreadsheets. Scatterplot graphs were generated by Excel and linear regression was calculated using Excel's best fit linear function. Comparison of viability was evaluated using Student's t-test.

2.3. Tissue collection and processing

Lipoaspirate was collected directly into sterile syringes (for smaller amounts) or sterile canisters (for larger volumes). If canisters were used, lipoaspirate tissue was subsequently packaged into 10 ml or 60 ml syringes to facilitate handling and shipping. While attempts were made to minimize the fluid packaged with the lipoaspirate tissue, an amount of liquid was co-packaged into each syringe. Samples were shipped via overnight or local courier in a room temperature insulated shipping box. Samples were sent to arrive at the processing laboratory within 24 hrs. Upon receipt, syringes were placed upright at room temperature for sedimentation. Any remaining liquid waste (blood and tumescent fluid) was removed from each syringe along with a small amount of adipose tissue and pooled into a single sterile tube. This pooled fraction was used for sterility and viability testing. If there was an insufficient volume of tumescent fluid, a small amount of adipose tissue was removed from the syringe and mixed vigorously with sterile PBS. The tissue washed PBS was then used in subsequent testing.

2.4. Viability testing

Pooled sample liquid was mixed with 0.2% trypan blue dye (Sigma-Aldrich, St. Louis, MO). Cells in this suspension were a mix of red blood cells and other cells released during the lipoaspirate harvest. Cells were counted and scored live/dead using a standard hemocytometer.

2.5. Sterility testing

Pooled sample liquid was either streaked with a 10 μl sterile loop onto growth plates or tested with the BacT/ALERT system (Biomerieux, Marcy l'Etoile, France). For plate testing, the sample liquid was streaked with a sterile loop onto MacConkey agar, Sabaroud-dextrose agar, and 5% sheep's blood tryptone soy agar plates (University of Arizona Bio5 Media Facility, Tucson, AZ), incubated and monitored for 7 days in a 37 ℃ dry incubator. Appearance of colonies at any point during the 7 days was scored as microbial growth present. For BacT/ALERT testing, 1 ml sample liquid was loaded into a sterile syringe and added through a rubber septum into the culture liquid as per manufacturer's protocol. Both aerobic and anaerobic media bottles were kept for seven days at 37 ℃. Any growth within seven days was scored as positive.

2.6. Cryostorage

Individual patient samples were handled separately. At no times were samples from different patients processed together. Adipose tissue was treated as previously described [28]. Tissue from an individual patient was centrifuged at 150 g for 5 minutes to reduce the liquid present in the sample. Separated from waste liquid, the tissue volume was measured by syringe gradations and loaded into sterile cryostorage bags (Origen Biomedical, Austin TX). Cooled cryoprotectant was added to achieve a final concentration of 5% DMSO, 1% dextran-40 (Protide Pharmaceuticals, Lake Zurich, IL), and 1% human serum albumen (Octapharma, Lachen, Switzerland). Tissue was sealed into a secondary sterile overwrap and frozen according to a preprogrammed cooling curve in a controlled rate freezer (Custom Biogenic Systems, Romeo MI) and stored in vapor phase LN2 dewar at −180 ℃.

2.7. Thawing of frozen adipose tissue

Samples were prepared for thawing when requested by the patient and physician. The amount of tissue and number of individual tissue aliquots was a decision made by the requesting physician. Individual patient samples were handled separately. Individual Cryostorage bags were thawed rapidly in a 37 ℃ water bath and washed twice with cold lactated Ringer's solution. Tissue was separated from wash liquid and the volume of tissue measured in the same manner as prior to cryostorage. Washed tissue was then transferred to 20 cc or 60 cc syringes in a 1:1 ratio with transport buffer consisting of lactated Ringer's solution with 1% human serum albumen. Tissue was kept refrigerated in a temperature controlled shipping container for overnight or local courier delivery to the administering clinician.

3. Result

3.1. Effect of intact adipose tissue cryopreservation on viability

Samples (n = 74) were assessed for viability upon arrival at the processing laboratory. Free MSCs released into the tumescent fluid and blood cells collected along with the adipose tissue were used for viability testing. The majority of samples were scored at approximately 100% viable. As a group, incoming samples had an average viability of greater than 99%. The majority of samples were tested approximately 24 hours after collection. This finding indicates that the time of shipping a sample to the central repository does not adversely affect the sample quality as measured by cell viability.

After thawing and washing of cryopreserved tissue, free cells suspended in the wash fraction were again used to assess viability. Trypan blue staining of residual blood and released cells in suspension showed that the large majority of samples had viabilities ranging from 80–100%. The mean value was 91% +/−8%. (Figure 1) Student's t-test indicates that this is significant change ($p < 0.05$). However, this observation is within normal results seen with other types of cells having been subjected to cryopreservation [31].

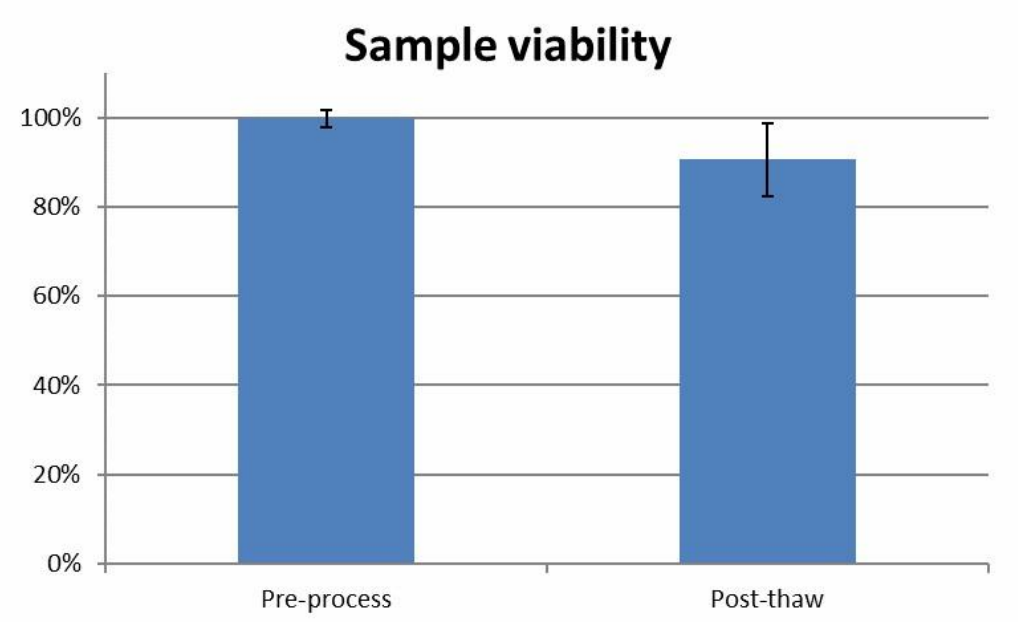

Figure 1. Sample viability. Residual blood from tumescent fluid and cells released from the adipose tissue were used for viability testing. There was measured reduction in viability after cryopreservation and subsequent thawing ($p < 0.05$). Samples had a mean viability of 91% +/− 8% post thaw.

3.2. Effect of freeze/thaw of whole adipose tissue on sterility

Collected adipose samples were tested for aerobic, anaerobic and fungal contamination upon arrival at the processing laboratory as well as after processing. Samples that were thawed and

returned to the collecting physician for use were also tested after the thaw process. In this study a contamination rate of 29% was seen in the samples incoming to the processing laboratory. As sterility testing was done immediately upon arrival these results were therefore likely due to contamination at collection. The nature of collecting lipoaspirate through the skin can lend itself to inclusion of various skin flora, thus contamination will occasionally be observed. After thawing procedures the contamination rate increased to 38% of the total samples.

3.3. Adipose tissue recovery after prolonged cryopreservation and thawing

A total of 181 samples were thawed after various lengths of time following cryopreservation. The samples had been stored for periods ranging from 13 days to more than 44 months. Average duration of cryostorage was approximately 9½ months. Upon thaw the amount of tissue recovered was compared to the amount of concentrated tissue originally cryopreserved for each sample. The majority of waste liquids (blood, tumescent fluid, oil) were removed prior to cryostorage. Likewise, the washing buffers after thaw were removed, therefore, not counted in the amount of tissue. The average recovery yield of cryopreserved tissue volume was 67% of the original stored amount. Tissue recovery ranged from a low of 21% to a high of 100%. The large majority of samples had yields of between 50% and 80%. Figure 2 shows the distribution of recovery yield has no relationship with length of time in cryostorage.

Patients in this study ranged from 27 to 81 years old. The majority of patients were 50 to 70 years of age. The yield of adipose tissue recovery after thawing appears to have only a very slight dependence on age of the patient. Linear regression analysis shows that for each additional decade of age, the reduction in recovery is less than one percentage point. The shallow negative slope of the linear best fit in Figure 3 shows the relationship of patient age to recovery yield.

The adipose samples collected in this study ranged from 12 cc to 960 cc (mean = 242 cc) of total sample collected. This volume included waste materials such as blood, oils and tumescent fluid that were packaged into the collection syringes along with the adipose tissue. These fractions were discarded prior to adipose cryopreservation. Samples smaller than 12 cc were excluded from this study but no upper limit was placed on the collection amount. The amount of lipoaspirate that was cryopreserved was a function largely dependent upon how much the physician harvested. No relationship was found regarding the recovery after thawing and the original amount of tissue collected. Figure 4 shows the recovery percentages varied randomly with regard to collection size.

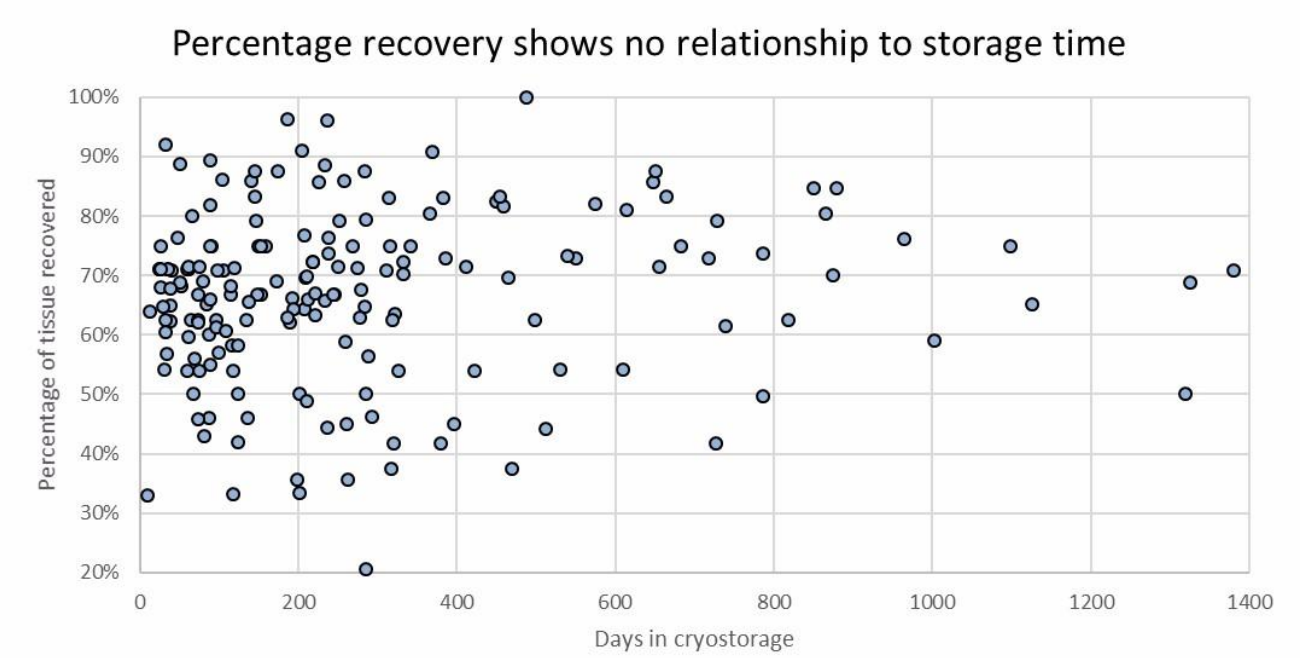

Figure 2. Percentage recovery shows no relationship to length of storage. Adipose tissue was cryopreserved for periods ranging from several weeks to several years. The yield of recovered tissue was not found to be dependent upon the length of time in cryostorage.

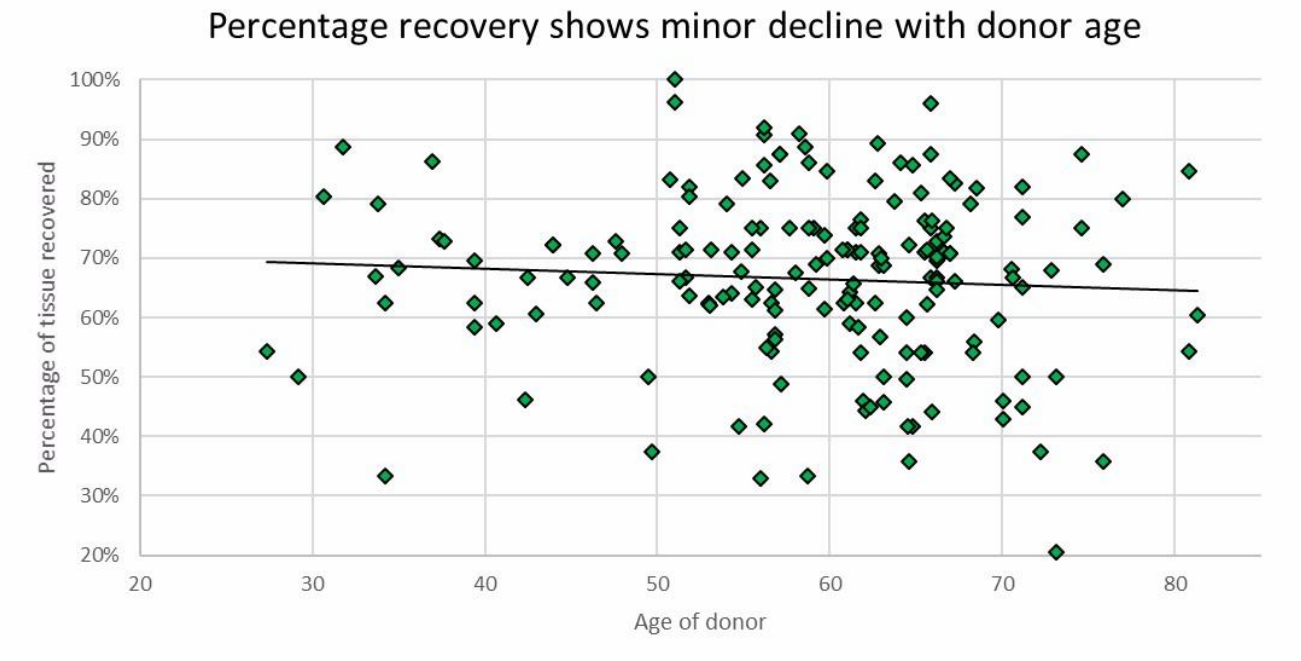

Figure 3. Percentage recovery shows minor decline with donor age. The age of adipose tissue donors ranged from 27 years to 81 years. There was no significant decrease in tissue yield after cryopreservation with respect to age.

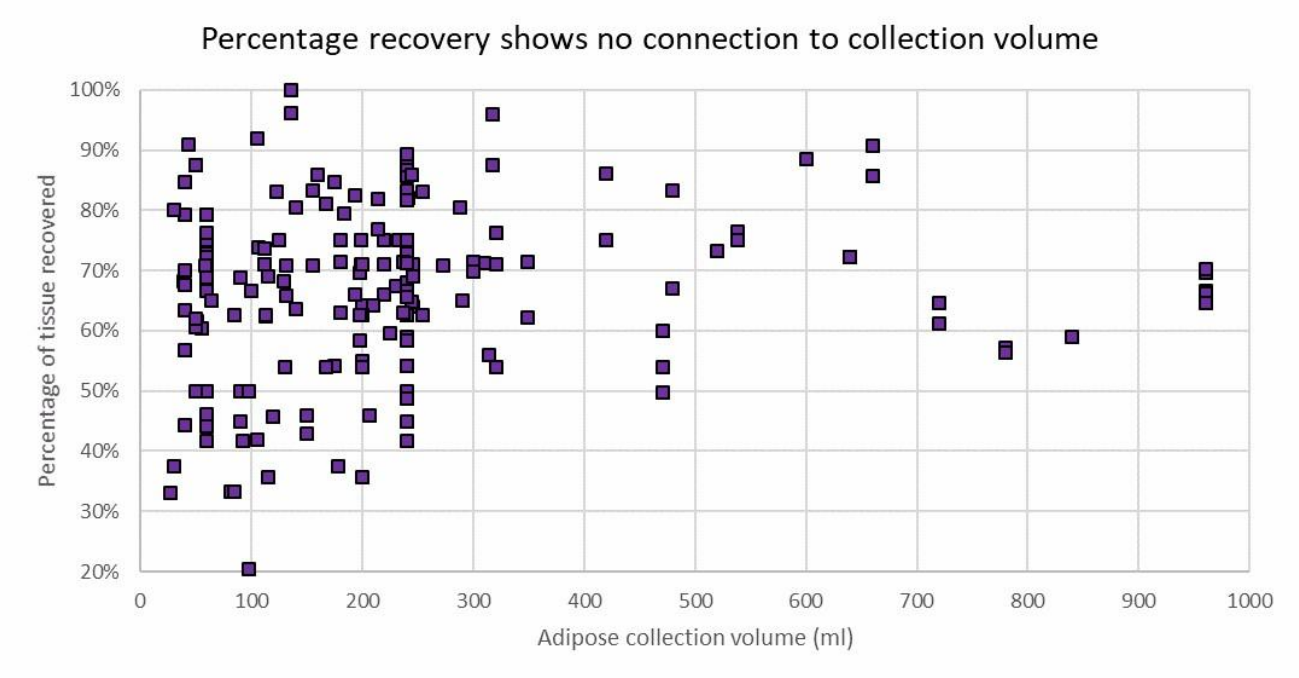

Figure 4. Percentage recovery shows no connection to collection volume. Adipose tissue was collected in volumes ranging from 12 cc to 960 cc. The size of the collection was not a factor in adipose tissue recovery.

4. Discussion and Conclusion

Adipose tissue has become useful for a great many conditions and has promise for a great many more. It is easy to harvest, easy to handle and, for many patients, rarely is it in shortage. It provides a rich source of adipose derived MSCs which are known to have large numbers of current and potential therapies. All else being equal, younger cells are better than older cells [29]. Therefore, it would seem beneficial for patients to have a bank of stored autologous adipose tissue taken while the patient is young. Additionally, only a limited amount of adipose tissue can be grafted at one time. The donor tissue needs time to vascularize and grow extra-cellular matrix to attach. After the grafted tissue has had time to adapt to the new area and the surroundings have healed properly, another graft adding more volume is possible. This strategy allows practitioners to stage several grafts into the same area. This can be particularly useful in reconstruction and enhancements of areas such as breast and buttocks. If the patient has a large cryopreserved collection, aliquots can be taken out periodically for small or large staged cosmetic needs, regenerative medicine, cellular therapies, and reconstructive applications which the patient may require in the future.

While the relatively high rate of microbial contamination is concerning, it is useful to remember that contamination was also a problem in the early days of umbilical cord blood collection. It took years for collecting physicians to attain better, microbial-free samples. Armitage et al. reported in 1999 on the first 1000 donations for the London Cord Blood Bank [32]. Monthly contamination rates were as high as 28% early on. They were able to reduce the monthly rate to 4% by the time they had 1000 samples. They eventually brought the rate down to approximately 1%. Similarly, a study by Clark et al. studied 13,344 cord blood samples from the Sydney Cord Blood

Bank between 1997 and 2009 [33]. Contamination rates of approximately 10% took 10 years to be reduced to a rate of less than 2%. Some may think this comparison less than useful. After all, we are not in the "early days" of adipose collection. Many countless thousands of adipose harvests have been performed in the last 20 years. However, it is important to note that most of those adipose samples were used immediately after collection and that no sterility testing was done. This means that there was no feedback to the collecting surgeon regarding the maintenance of sterility. Without knowledge of sterility testing results no collecting physician would see a reason for adjusting their harvest techniques to achieve cleaner collections. As physicians get feedback on the condition of samples collected, we presume that they have an interest in self-examination of techniques and practices to assure the best possible quality of material for future use. Indeed it would be useful for surgeons and professional organizations to consider establishment of guidelines to ensure the safest possible adipose harvests and grafts. Just as we have seen the improvements in sterility in umbilical cord collection over the years, it is not unreasonable to assume a similar pattern will emerge for adipose collection.

We have seen that the average amount of tissue yield after thawing is 67% (range 21% to 100%). The practical application is that after the thawed tissue is washed and recovered it allows the physician to easily predict the amount of adipose tissue available for use. A conservative approach allows a physician to estimate that one half to two thirds of the original banked amount will be available for fat transfer applications. Physicians can make this useful estimate for all additional tissue frozen regardless of aliquot size, overall harvest volume, or length of time in storage. Samples of adipose tissue act similarly to other types of properly cryopreserved cells in that cells can be stored indefinitely. The samples remained in the vapor phase of liquid nitrogen at a temperature of approximately −180 ℃ to −190 ℃. At this temperature essentially all biological activity is stopped. Under warmer temperatures we may expect to see degradation of the sample over time. However, properly stored under appropriate conditions, no reduction of recovery yield is seen as the time in cryostorage increases, even over several years.

An average recovery of 67% of the original volume of frozen adipose tissue indicates that the use of banked and thawed tissue requires a larger amount of sample be taken from the patient initially. This requirement is easily accomplished by an experienced clinician. Our samples in this study were stored in cryopreservation bags that allowed storage of aliquots of adipose tissue of 24 cc or 56 cc per bag. Some physicians had stated that a portion of the fresh lipoaspirate had been used that same day and the remainder had been sent to the processing lab for cryopreservation. Unfortunately, data regarding the number of these cases and the amount of tissue for each purpose is not available. Outside of this study our lab has stored adipose tissue in other types and sizes of vessels that resulted in aliquots of tissue ranging from 2 cc to more than 200 cc. A wide variety of cryostorage bag sizes are available from our supplier (Origen Biomedical, Austin TX), as well as other suppliers. This allows the adipose tissue to be stored in essentially any volume. It seems reasonable that large volumes of tissue are best cryopreserved in multiple aliquots. Multiple aliquots allow the clinician to thaw just the volume needed for the present application while holding the remainder in reserve. While it is possible to refreeze adipose tissue and still utilize it upon a second (or third) thaw, we have observed a reduction in both cell viability and overall volume yield. (data not shown). Once a clinician has an approximate idea of the amount of tissue required for a given procedure the amount of tissue aliquots (cryobags) to be thawed can be predicted. In this way the

clinician is assured of not running out of tissue but also of not thawing too many bags resulting in overage and waste.

As the adipose tissue is processed and cryopreserved a small amount of tissue is lost through the normal manipulations that are required. For example, tissue that cannot easily be removed from a syringe or an amount of tissue may become stuck in tubing is unrecoverable. The amount lost in the unloading of the collection syringes and transfer to cryostorage bags is minimal. However, some adipocytes burst and are destroyed during the various processes involved. Moving fragments of lipoaspirate through a small orifice, exposing cells to pressure differences and sheer stresses, changes in osmotic pressure with the cells during cryopreservation and thaw, washing and centrifugation all can have an effect on the tissue. Taken together these processes affect a change in the overall volume of the recovered sample. It was noticed that most often amounts of tissue were unrecoverable during the thaw and wash procedures and the subsequent transfer of tissue to packaging syringes for transport. This loss was minimized by ensuring the size of cryostorage bag was not overly large compared to volume of tissue stored. It was noticed early on that lowest yields were often from cryostorage bags that were filled to less than capacity with adipose tissue. Tissue can be stored in any amount and cryostorage bags come in many sizes. But more tissue was unrecoverable or lost when the bag was too large for the tissue stored. There was no significant correlation between the total amount of tissue stored and the percentage yield of individual samples.

There are different reasons for storage of adipose tissue; e.g., for the near-term use for cosmetic purposes. Many of our current tissue retrievals have been for reinjection into the face or hands. Other observed uses have been for larger scale remodeling/reconstruction such as breast or buttocks. Another reason for storage of tissue is that adipose tissue is abundant with mesenchymal stromal cells (MSCs). This stem cell rich tissue will no doubt be even more useful in the future as yet unknown, regenerative medicine applications are discovered. It is also known that the younger the stem cells are the more potent and more useful they are [29]. Therefore, it behooves patients to collect a sample of adipose tissue and store it at a time as early as is feasible and to keep it stored instead of for short term use for cosmetic applications.

Of the samples discussed here, the majority were used for plastic and/or reconstructive applications. Some cellular therapies will depend upon the extraction and expansion of MSCs. For this a very small amount of tissue can be of great use due to the expansion. However, in these cosmetic and reconstructive cases, the amount of tissue a physician has to work with is of great importance. As the tissue was processed for thaw (washings, transfers, etc.) only the actual amount of tissue that ended up in the physicians hands was measured as the final product. As cryopreservation of adipose tissue becomes more commonplace physicians will find it helpful to know what will be the likely amount of tissue that will be available after the thaw procedures. The final product ready to use was sufficiently washed and tissue yield was measured. As the tissue is packaged for use into syringes with osmotically balanced buffer, the physician can use the tissue immediately upon arrival by merely decanting the buffer.

The amount of adipose tissue that can be harvested from any given patient is dependent upon the amount of fat on the patient. However, more adipose tissue can nearly always be taken than usually is taken. Part of the reason for this is that many physicians are currently unaware of the value of harvesting adipose tissue for cryopreservation. It's taken 20 years since Coleman's systemization of liposuction technique [5] for the medical community to reach this current level of use of fat grafting. It's reasonable that many physicians are not yet aware of, or comfortable enough with

cryopreservation to utilize the practice fully. Therefore, cryopreservation is, as of yet, not something most harvesting doctors consider. As more information on its value and usefulness comes out, cryopreservation it will, undoubtedly become more popular.

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Conflict of interest

All authors declare no conflicts of interest in this paper.

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