CYTORI LIPOBANK™

Transplanted adipose tissue grafts must survive through diffusion until such time as new blood vessels can grow into the tissue to restore perfusion.[1,2] This simple biological fact limits the amount of tissue that can be transplanted within a single procedure inasmuch as adding tissue beyond the limits of diffusion results in necrosis of graft tissue that is too distant from a vascular bed. Consequently, surgeons wishing to treat large defects or to augment larger volumes frequently apply a staged approach.[3,4] This can be facilitated by storing excess tissue collected at the time of the initial treatment and delivering it at later times. This eliminates the need for repeated liposuction and, thereby, reduces cost and patient morbidity. A survey of 508 US-based plastic surgeons published in 2007 found that 6% freeze excess fat for this purpose.[5]

Protocols for long-term cryostorage of human sperm for artificial insemination and of bone marrow, peripheral blood stem cells, and umbilical cord blood intended for transplant into myeloablated recipients are well-established in the literature and have been applied for many years.[6,7] These protocols include the use of cryoprotective agents, control of the rate of cooling to approximately 1°C/minute, and storage at temperatures below the glass transition point of water (~130°C). When similar approaches are applied to the cryopreservation of adipose tissue they have been shown to dramatically increase post-thaw viability of both adipocytes and Adipose-Derived Stem Cells.[8-12] Thus, protocols that combine rate-controlled freezing, storage in liquid nitrogen, and the use of cryoprotectants such as glycerol, trehalose, and dimethyl sulfoxide result in recovery of up to 80% of the adipocytes present in freshly isolated tissue. Cytori scientists have now improved adipose tissue cryopreservation and thawing protocols as part of creation of an adipose tissue banking process that meets the standards necessary for clinical use.

Methods and Results:

In order to validate Cytori's proprietary Adipose Tissue Banking Process (LipoBank™), adipose tissue was cryopreserved and thawed in accordance with LipoBank™ Standard Operating Procedures (SOPs). When cryopreserving adipose tissue for use in autologous fat grafting, the physician needs to be assured of three key matters:

1) That they know with certainty the identity of the person from whom the stored tissue was taken.
2) That the tissue is healthy and viable.
3) That the volume of tissue present after thawing will be adequate for the intended use.

The Cytori LipoBank™ system has been developed and validated to deliver the highest levels of assurance on all three parameters.

The first issue, guaranteeing the identity of the person from whom the tissue was taken, is managed by a software-controlled system that ensures accurate labeling throughout the banking process–from the point-of-receipt by the bank all the way through to thawing at the point-of-use. The second and third issues–post-thaw tissue health, viability, and volume–are managed by use of the LipoBank™ SOPs that provide validated post-thaw recovery of viable, healthy adipose tissue.

It is important to recognize that no cryopreservation protocol is perfect, especially when storing tissue rather than a simple single cell suspension. For example, viability depends in part on uniform penetration of cryoprotectants and uniform cooling. These parameters can be difficult to achieve in aspirated adipose tissue as a result of the heterogeneous size of aspirated tissue fragments. Consequently, some level of tissue loss is inevitable. The goal, therefore, is to minimize this loss.

This requires application of assays that can accurately and reproducibly measure tissue viability and health. The obvious way of achieving this is the application of vital dye exclusion assays such as Trypan Blue, which can be compromised by contaminants such as microscopic lipid droplets present in lipoaspirate and digested adipose tissue. This can be managed by concomitant use of nucleic acid staining to discriminate between cells and droplets. However, in recognition of the limitations of this approach, Cytori scientists have applied a broader measure of tissue health. Specifically, the assay used by Cytori measures the health of the primary function of adipose tissue: storage and mobilization of energy. Stimulation of adipose tissue with adrenergic agonists activates a cascade of biochemical steps including activation of adenylate cyclase resulting in release of free fatty acids and glycerol into the blood stream **(Figure 1)**. Therefore, by measuring glycerol release in response to adrenergic stimulation, Cytori has been able to measure the function of this whole cascade and thereby quantify global adipose tissue health. Confirmation can be obtained by observing the effects of an inhibitor of adenylate cyclase on adrenergic-stimulated glycerol release.

Post-thaw Lipolysis

This assay was used with tissue cryopreserved using the LipoBank™ systems and methods versus using tissue simply placed into a conventional -20°C freezer **(Figure 2)**. The results show robust activation of glycerol release in response to the adrenergic agonist and inhibition of this release by an inhibitor of adenylate cyclase in tissue cryopreserved with the LipoBank™ systems. By contrast, tissue stored at -20°C showed no response to adrenergic stimulation. Further, the extent of stimulation (and inhibition) of glycerol release by the tissue cryopreserved with the LipoBank™ systems is the same as that seen in tissue prior to cryopreservation. Specifically, both fresh and thawed tissue showed a 40-50% increase over baseline and a 45-65% inhibition of this increased glycerol release in response to an adenylate cyclase inhibitor **(Figure 3)**.

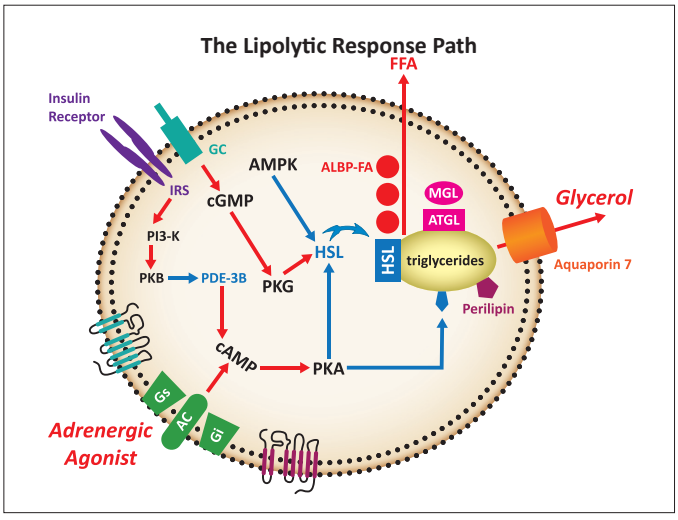

Figure 1. The biochemical cascade occurring following adrenergic stimulation of adipocyte lipolysis. This pathway includes adenylate cylase (AC), cyclic AMP, and Protein Kinase A (PKA), leading to activation of Hormone Sensitive Lipase (HSL) and the release of glycerol and free fatty acids (FFA) Inhibition acts at the level of AC.

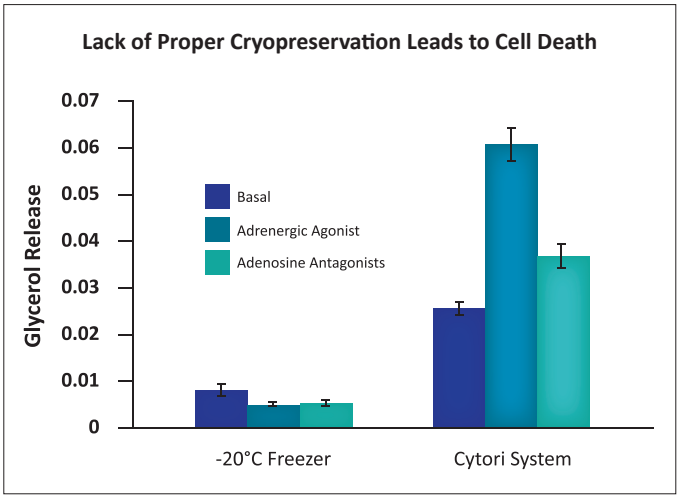

Figure 2. Post cryopreservation adipose tissue maintains inherent β adrenergic agonist-induced responsiveness when preserved using the Cytori LipoBank™ SOPs. This demonstrates that the normal cellular signaling cascades and functions are active in the cryopreserved adipose cells post-thawing.

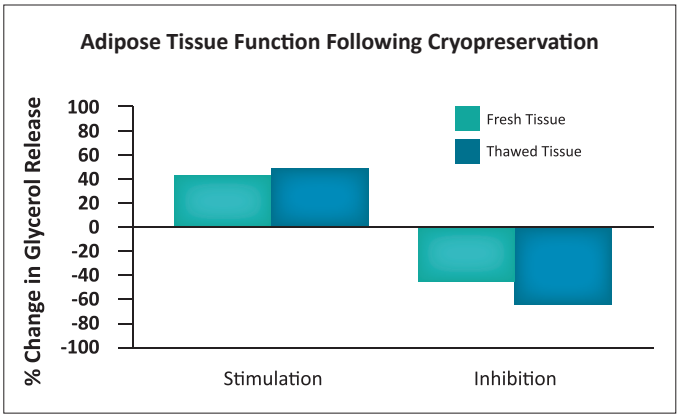

Figure 3. Cryopreservation using LipoBank™ systems preserves normal ability of adipose tissue to respond to adrenergic stimulation of lipolysis and inhibition of this stimulation by an inhibitor of adenylate cyclase.

Post-thaw Cell Recovery

While adipose tissue function is clearly the most global measure of tissue health, Cytori scientists also evaluated simple recovery of viable adipocytes using a two-dye fluorescent method in which one dye stained all cells (to discriminate between true cells and debris or lipid droplets), and the second stained only non-viable cells. In this study, adipose tissue was enzymatically digested following cryopreservation and thawing using Celase® to release individual adipocytes from the connective tissue matrix. The number of viable adipocytes was measured using a fluorescent vital dye to differentiate between live and dead cells. The results show 85–90% recovery of adipocytes with tissue cryopreserved using the LipoBank™ system. By contrast, tissue stored at -20°C showed recovery of only 20% of the adipocytes present in fresh tissue **(Figure 4)**.

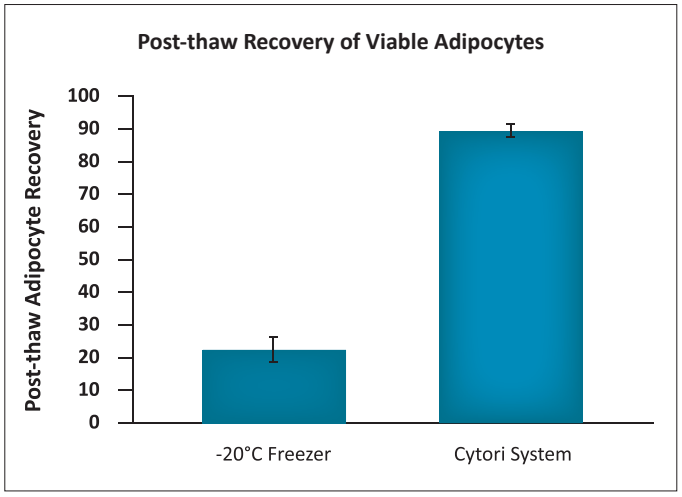

Figure 4. Post-Thaw Recovery of Viable Adipocytes. Storage of adipose tissue at -20°C without the addition of cryoprotectants and application of appropriate banking procedures leads loss of ~80% of viable adipocytes. Application of the LipoBank™ system results in recovery of 80–90% of viable adipocytes.

Post-thaw Tissue Volume Recovery

The LipoBank™ thawing process applies a Puregraft® washing step to remove cryoprotectant along with the small amount of free lipids and debris associated with this loss of adipocytes. On average, 86% of the original tissue volume stored was recovered after thawing **(Figure 5)**.

For example, tissue for staged procedures has been stored in aliquots that include 125 mL of adipose tissue along with the cryoprotectant and Lactated Ringers solution that acts as a carrier allowing the tissue to flow. Thawing and washing this tissue yielded, on average, 108 mL of adipose tissue, a yield of 86%.

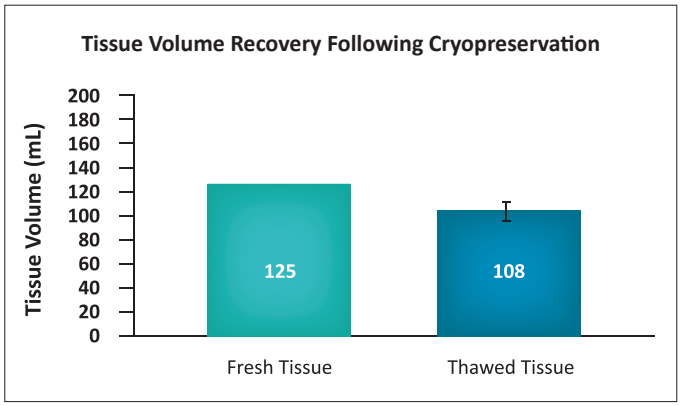

Figure 5. The graph above displays the tissue volume (mL) recovery following the cryopreservation. Average recovery of tissue was determined to be 86% of the adipose tissue volume prior to cryopreservation.

Conclusion:

The data shown here demonstrate that application of the Cytori LipoBank™ practices and procedures allowed recovery of approximately 86% of the volume and 85–90% of the adipocytes present in the tissue prior to cryopreservation. Importantly, thawed tissue retained normal tissue responsiveness to physiologic stimuli. In other words, the thawed tissue was healthy and viable.

Through a variety of different studies and over 40 different adipose tissue donors, Cytori has demonstrated that use of the Cytori's LipoBank™ SOPs to cryopreserve adipose tissue results in a product that retains normal biologic function with high viability and predictable volume.

Banking Process and Necessary Expertise:

The ability to recover viable tissue following cryopreservation is simply one aspect of adipose tissue banking. The processes that give rise to these excellent results must be executed within the context of a robust quality system that ensures that such results are achieved reliably and reproducibly. Further, in order to maximize safety and efficacy of cryopreserved adipose

tissue, it is essential to ensure that tissue is only implanted into the individual from whom it was collected. This requires that the facility providing tissue banking services ensures that the tissue sample is labeled in such a way as to preserve the link between the tissue and donor identity from the point of tissue collection through transport, processing, cryopreservation, cryostorage, retrieval from storage, and reimplantation. The problems associated with ensuring label integrity are magnified by the physical issues associated with ultra-low temperature storage and, even more so, by numbers. What may be relatively simple with just one patient becomes enormously more difficult when the number of patients with tissue in storage at any one time increases, let alone when tissue from more than one patient is being processed on the same day. However, these issues are not unique to adipose tissue and lessons learned in the wider field of tissue banking have been applied to Cytori LipoBank™, including the development of custom software that helps manage all these issues. The LipoBank™ system also includes a robust quality system that applies elements such as validated SOPs, staff training, process management software to track samples throughout the process, and controls for equipment, reagents and supplies, all of which are incorporated in the Cytori LipoBank™ system. A combination of technical advances in adipose tissue cryopreservation within the framework of a well-managed quality system have been applied in order to move the field of transplanting cryopreserved adipose tissue forward in a safe and effective fashion.

References

1. Nishimura T, Hashimoto H, Nakanishi I, Furukawa M. Microvascular angiogenesis and apoptosis in the survival of free fat grafts. *Laryngoscope*. 2000;110:1333-8.
2. Yamaguchi M, Matsumoto F, Bujo H *et al.* Revascularization determines volume retention and gene expression by fat grafts in mice. *Exp Biol Med* (Maywood). 2005;230:742-748.
3. Illouz YG, Sterodimas A. Autologous Fat Transplantation to the Breast: A Personal Technique with 25 Years of Experience. *Aesthetic Plast Surg.* 2009.
4. Donofrio LM. Structural autologous lipoaugmentation: a pan-facial technique. *Dermatol Surg.* 2000;26:1129-1134.
5. Kaufman MR, Bradley JP, Dickinson B *et al.* Autologous fat transfer national consensus survey: trends in techniques for harvest, preparation, and application, and perception of short- and long-term results. *Plast Reconstr Surg.* 2007;119:323-331.
6. Feldschuh J, Brassel J, Durso N, Levine A. Successful sperm storage for 28 years. *Fertil Steril.* 2005;84:1017.
7. Kobylka P, Ivanyi P, Breur-Vriesendorp BS. Preservation of immunological and colony-forming capacities of long- term (15 years) cryopreserved cord blood cells. *Transplantation.* 1998;65:1275-8.
8. Cui X, Pu LL. The search for a useful method for the optimal cryopreservation of adipose aspirates: part I. In vitro study. *Aesthet Surg J.* 2009;29:248-252.
9. Wolter TP, von Heimburg D, Stoffels I, Groeger A, Pallua N. Cryopreservation of mature human adipocytes: in vitro measurement of viability. *Ann Plast Surg.* 2005;55:408-413.
10. Pu LL, Cui X, Fink BF, Cibull ML, Gao D. Long-term preservation of adipose aspirates after conventional lipoplasty. *Aesthet Surg J.* 2004;24:536-541.
11. Pu LL, Cui X, Fink BF, Gao D, Vasconez HC. Adipose aspirates as a source for human processed lipoaspirate cells after optimal cryopreservation. *Plast Reconstr Surg.* 2006;117:1845-1850.
12. Cui XD, Gao DY, Fink BF, Vasconez HC, Pu LL. Cryopreservation of human adipose tissues. *Cryobiology.* 2007;55:269-278.

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