Cryopreservation and banking of adipose tissue obtained by liposuction for a later clinical use in the context of lipofilling: a systematic review

Elien LAMBERTYN

Promotor: Prof. Dr. H. Beele
Co-promotor: Dr. F. Stillaert

Dissertation presented in the 2nd Master year
in the programme of
MASTER OF MEDICINE IN DE GENEESKUNDE
“C’est comme la magie noire, parfois ça marche et parfois non.”
- Ellenbogen (1, 2) -
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VOORWOORD

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8.5 TABLE OF COMPARATIVE STUDIES OF FRESH AND FROZEN FAT

8.6 TABLE OF COMPARATIVE STUDIES OF FAT FROZEN WITH OR WITHOUT CRYOPROTECTANT AND DIFFERENCE IN CRYOPROTECTANTS

8.7 ANALYSIS OF VIABILITY DECLINE: COMPARISON OF VIABILITY OF FRESH AND FROZEN ADIPOCYTES
1. Abstract

Nederlands

Achtergrond: Autologe vettransfer met lipofilling is een techniek die een toenemende populariteit kent in de wereld van de plastische chirurgie. Absorptie van het vet na lipofilling (tot 70%) zorgt er echter voor dat herhaalde procedures nodig zijn om tot het beoogde resultaat te komen. Eén liposuctie gevolgd door het invriezen van de bekomen vetcellen zou herhaaldelijke liposucties onnodig maken.

Methoden: Er werd een systematische studie uitgevoerd van de bestaande literatuur m.b.t. het invriezen van vet. Klinische studies m.b.t. de procedure van invriezen van vet werden grondig bestudeerd.

Resultaten: Traag afkoelen van het vet, gecombineerd met snel ontdooien blijkt de basis van de procedure om vet in te vriezen. Het gebruik van een cryoprotectans zou de viabiliteit van het ingevroren vet verhogen. Over de optimale bewaar temperatuur en de maximale duur van het bewaren van vet in ingevroren toestand is geen consensus bereikt.

Conclusie: Er is geen consensus in de literatuur. Klinische studies zijn nodig om de procedure van het invriezen van vetcellen te optimaliseren.

English

Background: Autologous fat transplant is one of the most popular techniques used in plastic surgery. Unfortunately, because of fat resorption, results of lipofilling are variable. Using cryopreserved fat, repeated liposuction procedures and their secondary effects can be avoided.

Methods: Literature search of the existing literature related to cryopreservation of fat grafts.

Results: The major points in cryopreservation of fat are slow cooling and fast thawing. Use of a cryoprotectant should increase viability after freezing. The optimal storage temperature and the maximum duration of freezing have not been elucidated.

Conclusions: There is no consensus in literature. More clinical studies are needed to design an evidence-based-medicine procedure of cryopreservation of fat cells.
2. Nederlandse samenvatting

Liposuctie, gevolgd door lipofilling, wint de laatste decennia wereldwijd aan populariteit in de wereld van de plastische chirurgie. Vet, bekomen uit liposuctie, biedt grote mogelijkheden op het vlak van klinisch gebruik. Vet is in het menselijk lichaam overvloedig aanwezig, is lichaamseigen en goedkoop. Indicaties voor lipofilling zijn heel uiteenlopend, gaande van correctie van volumedefecten en wekedelenletsels tot esthetische correctie van littekens, rimpels, atrofie, acnelittekens etc.

De positieve eigenschappen van lipofilling worden echter overschaduwd door een onvoorspelbare absorptie van het vet na lipofilling. Daardoor zijn herhaalde liposuctie en -fillingprocedures nodig om het beoogde resultaat te bereiken. De mogelijkheid om het vetweefsel, bekomen uit liposuctie, in te vriezen, zou herhaalde liposucties en hun bijwerkingen vermijden.

De doelstellingen van deze masterthesis zijn dan ook het bestuderen in hoeverre vetcellen kunnen ingevroren worden en verder in de literatuur op zoek te gaan naar de beste procedure om vetcellen in te vriezen.

Als conclusie kan gesteld worden dat het traag invriezen met een snelheid van 1°C per minuut en het snel ontdooien de basis van de invriesprocedure vormen. Het gebruik van een cryoprotectans wordt aangeraden. Bij gebruik van een cryoprotectans gaat de voorkeur uit naar 7, 6 % trehalose in combinatie met 3, 3 % DMSO.

Gebruik van een voedingsmedium tijdens het invriezen moet worden vermeden. Voorkeur gaat uit naar fysiologische zoutoplossing (0, 90 % NaCl) om de cellen in te bewaren.

Over de optimale bewaarstemperatuur bestaat geen consensus. Ook over de mogelijke maximale duur van bewaren in ingevroren toestand bestaat geen duidelijkheid. Voor het onderzoek naar viabiliteit van vetcellen gaat de voorkeur uit naar celkleuring en histologisch onderzoek in combinatie met MTT/XTT en G3PDH, respectievelijk voor celfunctionaliteit en celmorfologie.

De procedure om vet in te vriezen bevat nog heel veel onopgehelderde deelaspecten. Literatuur toont veel positieve case reports, maar er zijn tot nu toe weinig objectieve data gepubliceerd om deze successen te verklaren. Er is dus een duidelijk positieve publicatiebias aanwezig in dit vakgebied.

Bovendien zijn de gepubliceerde studies heel moeilijk met elkaar te vergelijken door de uiteenlopende procedures en viabiliteitsmetingen. Daarenboven is het mechanisme van de onderliggende resorptie, basis van de nood aan herhaalde liposuctieprocedures, onvoldoende opgehelderd.

Bijgevolg zal meer onderzoek nodig zijn om tot een procedure te komen die gebaseerd is op wetenschappelijke evidentie.
Merk op dat in deze review geen aandacht werd besteed aan ASCs en preadipocyten die ook aanwezig zijn in het vet. Deze worden mee ingevroren en geïnjecteerd. Waarschijnlijk zijn ASCs en preadipocyten gedeeltelijk verantwoordelijk voor de outcome van de vettransplantatie.

### 3. Introduction

In 1601, the Archduke Albert, a Dutch surgeon, brought bags filled with human fat to the battlefield. He used the fat as a remedy for wounds and diseases of the soldiers. This remarkable story was the first report of the use of human fat in medicine and the beginning of the history of fat transplantation (3, 4).

In 1889, Van der Meulen described the first autotransplantation of human fat. He used the free omentum to fill the space between liver and diaphragm (4).

The first use of a free fat graft in humans leads us to the German surgeon Gustav Adolf Neuber in 1893. In his ‘Verhandlungen der Deutschen Gesellschaft für Chirurgie’ Neuber describes the use of upper arm fat in the face of a 20-year-old man. Neuber claimed that ‘grafts larger than an almond would not give good results’. But his findings haven’t conquered the world because his report was written in German, which proved to be a stumbling block to non-German speaking colleagues (4-7).

Later, in 1895, Dr Czerny, a German physician documented the first breast augmentation (4).

In 1912, Eugene Holländer published a photographic documentation of naturally appearing changes after infiltration of fat in two patients suffering from lipoatrophy of the face (8).

In 1926, Charles Conrad Miller infiltrated fatty tissue through cannulas. He believed that application of fat through a hollow metal cannula resulted in a better long-term correction and a more natural-appearing result than when fat is grafted with an open incision. His technique has never become popular, although he reported good results (8, 9).

Thirty years later, Lyndon Peer developed his ‘Survival theory’. He claimed that neo-vascularization at the edge of the graft caused fat cells to survive. Peer also stated that only 50 % of the adipose cell survived in free fat grafts (4, 7).

Peer’s theory of neo-vascularization contradicted the theory of cell replacement of phagocytes. This theory was developed by Neuhoft and Hirschfeld in 1923. They observed that fat grafts were dominated by degenerative phenomena by phagocytes two or three months after grafting. After this period some regeneration occurred. Five months later regeneration was complete. Neuhoft and Hirschfeld concluded that after the transplanted fat had died, it was replaced by connective tissue or metaplastic fat (8).
The liposuction technique, introduced by Dr. Fisher in 1974, followed by the tumescent technique, introduced by Dr. Klein in 1985, accelerated the development of the lipofilling technique. The tumescent technique allowed patients to have a liposuction performed by local anesthetics using small cannulas (4, 9).

Important to mention is the introduction of Coleman’s lipostructure® in 1987. Coleman developed a new technique to decrease traumatic handling of fat by liposuction. His lipostructure® consisted of three steps: manual lipoaspiration with low pressure, centrifugation for 3 minutes on 3400 rpm followed by reinjection in 3D. This technique is still the gold standard for liposuction and lipofilling, but some changes have been made due to the technical implementation (7, 9, 10).

The problems observed when using the Coleman technique were written down by Ferraro et al. (2011). The first problem observed, was a decrease in the number of fat cells because of damage caused by the aspiration procedure and the centrifugation. The second issue was the requirement to infiltrate cells in direct contact with well-vascularized tissues (55).

According to Pu et al. (2008), the Coleman’s technique is promising. It yields a greater number of viable adipocytes and sustains a more optimal level of cellular function compared with conventional tumescent local anesthesia liposuction. So the Coleman technique should be considered as the standard and preferred method for harvesting and processing. But it should also be taken into account that the Coleman technique can be operator dependent and time-consuming if performed by less experienced surgeons. It’s also a fact that the Coleman method can only obtain a small amount of fat graft. So it may not be the preferred technique to obtain large amounts of fat graft in order to graft the breasts or buttocks (11).

Since the 1980s, autologous fat transplant has been one of the most popular procedures performed by plastic surgeons (12). In 2009, fat grafting represented 5, 9 percent of the nonsurgical procedures within aesthetic surgery (13).

But optimization of the procedure is needed. The results of lipofilling are variable. The long-term results of fat grafting are often disappointing, because of the unpredictable partial absorption, reaching up to 70% of the volume of the fat graft. A number of studies report a resorption of 30 - 70% within a year (14).

The most acceptable explanation for this resorption is based on Peer’s cell survival theory, which states that the number of viable adipocytes at the time of transplantation may correlate with the ultimate fat graft survival volume (15, 16). This is because fat cells survive through osmosis prior to the growth of new nourishing blood vessels (17). The first day after lipofilling, the adipocytes are ischemic. Macrophages, histiocytes and polymorphic neutrophils are attracted to remove the debris. They produce TGFβ (transforming growth factor), which promote fibroblasts to produce collagen. So
they leave fibrotic and cystic changes behind. The living fat tissue is revascularized within 48 hours (1, 18).

Another theory (the theory of cell replacement of phagocytes of Neuhof and Hirschfeld) claims that histiocytes, attracted of the host, are charged with lipid droplets of the injected adipocytes. Those cells remove the debris and take the characteristics of adipocytes. So the graft is totally removed and replaced by scar tissue, such as fibrocytes (1, 19).

There is no consensus between physicians about survival of fat cells in grafts. Do fat cells really survive or is postsurgical fibrosis the true mechanism of action?

Michael Kaminer claims that the most likely answer is that fat grafts are successful due to a combination of adipocytes survival and host-response fibrosis (19, 20).

Because of absorption, liposuction surgery must be repeated and cell harvesting procedures are needed. This increases patient morbidity and discomfort. It results not only in a less than optimal appearance but also extra costs are incurred (18, 21). More extensive liposuction and subsequent storage of adipose tissue, using cryopreservation techniques could avoid repeated liposuction and the associated complications and costs (22).

The aim of cryopreservation is to enable stocks of cells to be stored. It is invaluable when dealing with cells with a limited life span. Cryopreservation has a lot of advantages: low risk of microbial contamination, low risk of cross contamination with other cell lines, low risk of genetic drift and morphological changes and low costs (23). Freezing fat should prevent degradative changes due to enzymatic activity, free radicals, oxidation and bacterial growth. Generally pathogenic bacteria will not grow when they are frozen (17). The use of frozen fat also has benefits for the patient. Frozen fat causes less swelling after the lipofilling. Frozen fat causes less postoperative inflammation (3).

This report is the result of a review of the existing literature on cryopreservation of fat grafts. This study is designed to be followed by an experimental phase. A protocol of the experiment will be based on the results of a review of the literature. The purpose is to be able to store fat longer than the recommended reinjection time which can vary from 2 to 18 months. The stored fat could be used for lipofilling of places where resorption took place. The contribution of the student in this study is minimal. No interpretations are made. The structure of this paper is based on the different steps in the procedure of fat transplantation (figure 1).

Figure 1: Different steps of the procedure of fat transplantation.
4. Methodology

The first exploratory literature search dates from October 2012. A systematic review of literature was performed till February 2014. Every three months the literature was studied again to detect recent publications.

At first, MeSH (Medical Subject Headings) terms were selected. These are cryopreservation, freezing, adipocytes, transplantation (autologous), adipose tissue, lipectomy and cryoprotective agents. Other terms, not included as MeSH term were used: fat cells, fat graft, grafting, liposuction, lipofilling, adipose aspirates, lipoplasty, cryoprotectant… Use of these terms resulted in new search results.

To seek for more literature, “snowballing” was used. New publications were found by consulting reference lists and the Science Citation Index.

The search for publications on fat storage was performed using a combination of search terms in different databases. The search technique, used in PubMed, was also used in other databases: Web of Science, Cochrane, TRIP-database and clinicaltrial.gov.

The abstract of the retrieved articles was read. Literature in English and French was read. One article, written in Chinese (Lei H, Li F. [Study on viability measurement of fat for grafting] (24)), was read by Siyuan He (何思源). She translated it to me in English. Articles were excluded using the following exclusion criteria: retrospective studies, case reports, fat grafting for non-plastic surgery application and inaccessibility to full text. The remaining papers were read in their entirety and their bibliographies were studied for additional publications. In search of further publications to broaden the view on the procedure of fat grafting, selected search terms were used.

Publication years were centered between 2000 and 2012, except for 2 publications of Dr. Coleman from 1998 and 1995 and a publication from 1963 about the effect of freezing in the cell (9, 10, 25).

Taking into account the aim to find the optimal procedure for cryopreservation of fat, seventeen clinical studies were selected. Studies not including freezing of fat were eliminated. The findings of the 17 selected papers were summarized in an excel table. The following data were collected: author, publication year, journal, impact factor, human/animal/in vitro study, harvest, anesthetic, donor site, number of participants, patient/mice conditions, amount of lipoaspirate, transport to laboratory, washing, centrifugation, statistical analyses, cooling and thawing method, freezing time and temperature, transplantation site, methods and results.

EndNote Web was used to insert references and to create a reference list. References were shown in Vancouver style.
5. Results

5.1 ORIGIN AND FUNCTION OF FAT
Fat develops in utero in the fourth gestational month in humans. Adipocytes originate from a multipotent mesodermal cell. A mature adipocyte has a roundish form and a diameter of 10 - 120 μm (7).

Adipose tissue is a connective tissue existing of white, brown and medullar adipose tissue. Medullar adipose tissue can be found in adults in the central cavity of the bone. White and brown adipose tissues are the main types of fat tissue that can be found in humans. White fat tissue is completely different from brown fat tissue. Brown adipose tissue can be found in newborns and generates body heat. It contains numerous small droplets of fat and has a lot of mitochondria, which results in a brown color of the cell (26).

The white adipose tissue consists of a stromal-vascular fraction and lipid inclusions containing mature adipose cells called adipocytes. A white fat cell contains one single lipid droplet. Its main function is storage of energy. White fat tissue produces leptin and has receptors for insulin, growth hormone, noradrenalin and glucocorticoids. The connective tissue matrix is made up of collagen and elastin, stromal cells and neurovascular structures (27). The white adipose tissue is the most important fat tissue for surgical purposes (28).

In people with a normal body mass index, white adipose tissue constitutes 15 - 20% of the mass in males and 25 - 30% in females. According to age, gender, nutritional and hormonal stimuli fat has a different distribution (7).

The functions of fat are passive energy storage, endocrine and paracrine production of hormones and signaling molecules, called adipokines. Leptin is the most important adipokine. It is mainly synthesized by adipocytes and this makes it an original marker for identification of mature fat cells. With regard to fat grafting, leptin is a stimulant of neo-angiogenesis. The hormone stimulates endothelial cells and therefore microvascularization. Another hormone is NGF (nerve growth factor). This factor is synthesized by white adipose tissue. Its expression in adipocytes is associated with wound healing and plasticity and it exerts metabotrophic effects on glucose, lipid and energy homeostasis (7).

5.2 FAT HARVEST
The different steps of liposuction, cryopreservation and lipofilling should be carried out without causing damage to the adipocytes, especially during harvesting, refining and reinjection (1). Manipulation of the fat results in reduced cell viability (29).
In 2000, Sommer et al. claimed that fat graft survival did not depend on harvesting and reinjection methods, but mainly depended on the anatomic site, the mobility and vascularization of the recipient tissue or on the underlying causes and diseases (30).

5.2.1 SURGICAL CHARACTERISTICS OF ADIPOSE TISSUE
Several surgical approaches are used to restore or replace a certain amount of adipose tissue. Examples are the use of autologous tissue flaps, free fat grafting and the implantation of prosthetic material. Injectable soft tissue fillers are in high demand because of the shorter recovery time. The result of the injection of soft tissue fillers is immediate and both safer and more cost-effective than surgical implantation. The popularity of autologous fat transplantation is due to the ease of the technique, the unlikelihood of scar formation and the low morbidity and cost (table 1) (18).

An ideal soft tissue filler should be physically and chemically stable, long lasting and immobile in the body, non-immunogenic, noninfectious, non-pyogenic and non-allergic. It should not require pretesting and is cheap and easy to store (7).

Adipose tissue is abundant, readily available, inexpensive, host compatible and can be harvested easily and repeatedly if needed (21). So autologous fat is considered to be the ideal soft tissue filler (31).

Table 1 (7, 32): Strength and weaknesses of fat used for soft tissue augmentation.

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FAT</strong></td>
<td>No techniques to expand the adipose tissue</td>
</tr>
<tr>
<td>Abundant</td>
<td>Subcutaneous use only</td>
</tr>
<tr>
<td>Autologous</td>
<td>Variable longevity</td>
</tr>
<tr>
<td>Biocompatible</td>
<td>Variable resorption</td>
</tr>
<tr>
<td>Cheap</td>
<td></td>
</tr>
<tr>
<td>Easy to harvest</td>
<td></td>
</tr>
<tr>
<td>Full integration</td>
<td></td>
</tr>
<tr>
<td>Natural appearing</td>
<td></td>
</tr>
<tr>
<td>Non-immunogenic</td>
<td></td>
</tr>
<tr>
<td>Not palpable</td>
<td></td>
</tr>
<tr>
<td>Polyvalent usable</td>
<td></td>
</tr>
<tr>
<td>Possibility to repeat</td>
<td></td>
</tr>
<tr>
<td>Stable</td>
<td></td>
</tr>
</tbody>
</table>

5.2.2 INDICATION
Autologous fat injection is suitable for soft tissue defect correction or volume augmentation. Autologous fat injection is useful in the aesthetic improvement of facial abnormalities, such as Romberg’s disease, depressed scars, wrinkles, eyelid depressions, pitting acne and posttraumatic defects (17), as well as subcutaneous adipose atrophy of senility, buttock or breast augmentation, improvement of function and appearance of irradiated tissues, correction of asymmetry in Poland’s syndrome etc. (18, 27). But take in consideration that many surgeons think that the technique of breast augmentation impedes breast cancer diagnosis because the possible formation of micro-calcifications
(33). In general, compared to reconstructive surgery, esthetic surgery has better results because of better quality and better blood supply to the recipient site (7).

5.2.3 PATIENT CHARACTERISTICS
Do age and BMI (Body Mass Index) have an impact on the numbers and the viability of ASCs and adipocytes?

Investigation shows a positive correlation of BMI with age, mature fat cell size and total number of ASCs/ fat cell per body. They also mention a negative correlation of BMI with the number of adipocytes, stromal cells/ gram of adipocyte tissue and differentiation capacity. Another study shows the difference between age and activity of adipocytes. Younger people may have higher lipolytic activity, higher pre-adipocyte proliferation and differentiation, and a lower level of apoptosis (27).

Restrictions in patient population for clinical studies are minimal. Only people with major systemic metabolic diseases or lipid disorders are excluded (34).

These are the exclusion criteria reported by Condé-Green et all. (2010): prior abdominal surgeries, prior chemotherapy or radiotherapy, chronic use of corticoid, connective tissue diseases, hematological and other systemic metabolic disorders, diabetes mellitus, previous obesity and pathological lipodystrophies (35). Padoin et all. (2008) use the same criteria (36).

5.2.4 PREPARATION OF THE PATIENT
The patient’s lifestyle, expectations, prior aesthetic procedures and medical history are questioned. The patient should be informed about the details of the procedure, the outcome and the postoperative care. The patient should be prepared for the postoperative swelling and bruising.

Surgeons must evaluate the patient’s appearance. Photographs should be taken and physical examination should be carried out (9).

The usual preoperative instructions for liposuction include use of preoperative antibiotics and the avoidance of anticoagulants (33).

5.2.5 DONOR SITE
The optimal place for harvesting is an important subject for investigation.

Wilson et all. (2011) consider the abdomen to be the optimal donor site, because there is little vascularization which means that there is less blood in the lipospirate. Another reason why they prefer the abdomen as the optimal donor site is the presence of ASCs in the superficial abdominal fat. These are more resistant to apoptosis in contrast to ASCs of the medial thigh and knee, where apoptosis is the highest (27).
The study of Padoin et al. (2008) reveals that the lower abdomen and the inner thigh may have higher processed lipoaspirate cell concentrations, compared to upper abdomen, trochanteric region, knee and flank (figure 2) (36).

Pu (2008) states that inner thigh and lower abdomen do not only have a higher concentration of processed lipoaspirate cells than found in other donor sites, but they are also easily accessible for fat graft harvesting by the surgeon if the patient is in a supine position. He would certainly choose the lower abdomen or the inner thigh in his practice (37).

![Figure 2 (36): Areas for collection of adipose tissue. 1. Upper abdomen 2. Lower abdomen 3. Trochanteric region 4. Inner thigh 5. Knee 6. Flank.](image)

On the other hand, Hudson and Lambert (1990) consider the gluteal-femoral region as the optimal donor region because of larger adipocytes and more lipogenic activity due to elevated activity of lipoprotein lipase (7, 16).

Mac Kinney (2000) quotes the pubic area as superior because of the granular aspect of the fat and the presence of smaller lobules (1).

Markey et al. (2000) prefer to recruit fat from the outer buttck/flank. This area seems relatively more resistant to alterations in body weight due to dietary change than other regions. This dietary resistance is probably beneficial for the augmented tissue (38).

However, after studying 319 lipofilling cases, Chajchir (2004) detected no difference in fat grafts survival between donor areas (16). Rohrich et al. (2004) and Mojallal and Foyatier (2004) didn’t find any significant difference in cell viability in fat removed from abdomen, thigh, flank or medial knee (13, 39, 40).

Li et al. (2013) detected no difference between donor sites either. In their study no significant difference in graft weight, volume or histological parameters was found in a single patient and among
a group of six patients. These findings should encourage surgeons to choose the donor site which is easy and safe to access taking into account the preference and wishes of the patient (41).

In 2006 Coleman concluded that current studies did not indicate increased viability from any donor site. So a harvesting site is chosen in function of convenience of access and in order to enhance the patient’s contour. Abdomen and medial thighs are the most commonly chosen areas (8, 9).

Mojallal and Foyatier (2004) show no difference in quantity of fat between the donor sites. They studied 100 patients with the abdomen (59 patients), the hip (27 patients), the medial thigh (8 patients) as the donor site and 6 patients with multiple donor sites. No superior donor site was found (1).

The difference in viability of cells between the posterior side of extremities or body and the anterior part of the body has not been investigated yet. Investigators expect no difference (42).

Depending on the amount of fat needed, numerous sites could be used, but Khater and Atanassova (2012) advice to use fat harvested from one place. This because fat differs in cell size and activity between different harvest localizations (7).

A clear preference for a particular donor site has not been established, although the choice of donor site seems to be important with respect to the outcome. Most studies have shown no difference in viability between fat grafts harvested from different donor sites (1, 13, 39-41). But because of increased ASCs, higher amount of processed lipoaspirate cells, easy accessibility and safety, lower abdomen and medial thighs are most commonly chosen as the preferred donor site, compared to other regions (8, 9, 27, 36, 37).

Although the ideal donor site has not been found yet, the lower abdomen and medial thighs should be preferred, taking into account the wishes of the patient and the preference and experience of the surgeon.

5.2.6 ANESTHESIA TECHNIQUE

Is there a preference for local or general anesthesia when harvesting fat? If local anesthesia can be used, which local anesthetic should be preferred? Local anesthesia can be performed using lidocaine, epinephrine or amide local anesthetics.

Sommer (2000)’s review of literature shows similar survival rates for aspirated fat, whether or not local anesthesia has been applied. There is no clinical evidence in any study of his review that epinephrine reduces the blood supply, which could harm the graft. On the contrary, he claims that vasoconstriction before extraction will help tissue to maintain viability due to reduced bleeding. In his study he cites Moore et al. (1995). Moore et al. (1995) show that lidocaine potently inhibits glucose transport, lipolysis of adipocytes and their growth in culture. But after washing the cells, they fully regain function.
Finally, Sommer (2000) concludes that amide local anesthetics enhance wound healing by reducing leukocytes migration, reducing metabolic activity of leukocytes and reducing toxic substances (30).

The use of lidocaine should give less glucose transport, lipolytic activity and growth of adipocytes when compared with a control group. But those effects should disappear when the adipocytes are washed (7, 42).

Keck et al. (2009) examined pre-adipocytes. They found that cell viability of pre-adipocytes was reduced with lidocaine (13).

Shiffman (2010) concludes that the use of both epinephrine and lidocaine has no significant effect on cell attachment in culture, cell morphology, proliferation or adipocyte metabolic activity in comparison with the use of lidocaine (3).

A study of Mojallal and Foyatier (2004) shows no significant difference between the use of saline solution and the use of epinephrine in terms of weight and volume of the fat graft. They conclude that epinephrine does not influence the viability of adipocytes whereas lidocaine does reduce viability when it isn’t washed out (1).

Coleman (1998) most commonly uses local anesthesia (with an infiltrator 0, 5 % lidocaine with 1:200 000 epinephrine), but epidural and general anesthesia may be preferred for removal of larger volumes or when multiple sites are used to harvest (9).

Wilson (2011) usually performs liposuction under general anesthetics with in situ infiltration of local anesthesia with or without adrenaline. Smaller volume fat graft can be performed under local anesthetics such as lidocaine and adrenaline, occasionally with addition of bicarbonate (27).

Mojallal and Foyatier (2004) and Moscatello (2005) show that cell viability is the largest when performed under general anesthesia. But also the risks of general anesthetics must be considered. Use of vasoconstrictors and/or large volume of physiologic serum at the donor or recipient site could reduce the viability (1, 32).

Local anesthesia can be used. Lidocaine has no effect on fat cells when it is washed out after the procedure. When general anesthesia is used, it has no effect on the fat graft. So it can be concluded that, depending on the volume of fat harvest needed, epidural, local and general anesthetics or combinations can be used.
5.2.7 **THE TUMESCENT TECHNIQUE**

The tumescent technique, so called due to the tightness of the donor area that results from large amounts of subcutaneously infused fluid, is the use of fluid (containing local infiltration solution) up to four times the volume of expected aspirate at the donor area.

It has been hypothesized that resulting distension of tissues and compression of blood vessels result in better homeostasis (27).

Coleman (1998) claims that the superwet or tumescent technique can disrupt the parcels of fatty tissue and decrease survival (9). Also Moscatello et al. (2005) state that use of large volumes of physiologic serum at the donor or recipient site reduce the viability of adipocytes (32).

**In general tumescent technique is not used in the liposuction procedure.**

5.2.8 **HARVEST TECHNIQUE**

Techniques for harvest of fat grafts affect not only the viability of adipocytes and ASCs, but also their level of adhesiveness to key adhesion proteins. Mature adipocytes are, compared to immature progenitors, more susceptible to damage of tissue harvesting techniques (15).

The fat grafts need to be composed of fat of the deep layer of the skin, because of its genetic and metabolic difference with upper layer fat. Deep fat has a larger density of alpha 2 receptors. Alpha 2 receptors inhibit lipolysis and adipose tissue blood flow (1).

5.2.8.1 Liposuction versus excision, manual versus pump-assisted

Harvesting by liposuction or by excision doesn’t make a difference in fat cell damage, according to Sommer et al. (2000). Also no correlation has been found between survival of fat harvest with syringe or with machine aspiration. Fatty tissue breakage and vaporization can only occur when maximum negative pressure of machine aspiration is used (30).

Atanassova (2012) also states that manual lipoaspiration is noninvasive and as atraumatic as surgical excision (7).

Histological examination by Shiffman et al. (2001) to compare the vacuum pressure shows mild cell damage at the edges of the core of the fat when harvested at - 700 mm Hg and when harvested with a lesser vacuum, specimens show 98 – 100 % intact cells in the periphery of the core (43).

The preferred option for fat harvesting according to Tremolada et al. (2010) is a gentle aspiration with a fine cannula and the reduction of aspirated fat particles (15). MacRae et al. (2004) also prefer a gentle manual 10 - cc syringe aspiration (15, 42).
Crawford et al. (2010) confirm that an atraumatic harvest of fat grafts continues to yield high viable cell counts and that the adipose cell density is the greatest at the lowest level of centrifuged fat. Additionally, the difference between hand-aspiration and power-assisted liposuction samples is significant. Hand-aspiration provides a more efficient and user-friendly system which is convenient to operate (44).

Smith et al. (2006) found higher viability with syringe aspiration at low vacuum pressure, in comparison with the standard liposuction (with Byron liposuction pump, no further specification mentioned). There is no difference in viability between excised fat or syringe-aspirated fat, and no difference between liposuction and syringe aspiration (29).

Pu et al. (2012) also state that the syringe aspiration is superior to other techniques. But it is time-consuming and it may be difficult to obtain large quantity of fat graft (39).

A study of Leong et al. (2005) demonstrates comparable metabolic activity and adipogenic responses between cells derived from pump-assisted liposuction and cells from hand-held syringe liposuction (14).

Figure 3 (39): Manual syringe aspiration.

**No significant differences were found between the different harvest techniques. Because of the ease of the technique, manual syringe liposuction is the best option (figure 3).**

5.2.8.2 Large versus small cannula

The use of a large bore and low pressure will give the best result, says Atanassova (2012) (7).

Gonzalez et al. (2007) contradict this statement. They conclude that the viability of fat grafts is significantly better when fat graft is harvested with a 2 mm diameter cannula with a blunt tip and several side holes connected to a 10 cc syringe as compared to a 3 mm diameter blunt tipped cannula connected to a 60 cc syringe. They recommend the use of smaller syringes (10 cc) to maintain a minimal negative pressure during harvesting (39). The introduction of smaller blunt cannula for adipose tissue harvesting should allow a much gentler harvesting technique as well as a better
recovery because of skin entrance holes less than 2 mm diameter, which do not require any stitches (15).

The authors of ‘The impact of liposuction cannula size on adipocytes viability’ conclude that the use of larger aspiration cannulas will lead to the improvement of graft retention and quality. The use of a 5 mm cannula should lead to better results than a 3 mm cannula. They assume that the result is due to the decrease in shear stress force. The larger the fat globules obtained, the more protected the adipocytes are in the inner core (45). Comparing a 3 mm and 5 mm cannula, Tambasco (2013) finds it easy to understand how in the first one the shear stress on the adipocytes is greater. The adipocyte is conducted at a higher speed and at a shorter distance of the walls. This is based on the non-Newtonian fluids, where shear stress at a point is directly proportional to the velocity gradient and inversely proportional to the distance from the walls of the duct, being the whole multiplied for the dynamic specific viscosity of that fluid (46).

Gir et all. (2012), Erdim et all. (2009) and Wilson et all. (2011) conclude that a large cannula diameter (> 4mm) should correlate with improved cell viability (13, 16, 27).

No differences in fat cell integrity between harvesting with an 18-gauge needle, a 2, 5 mm cannula or a 3 mm cannula and between injecting through an 18-gauge needle or the open end of a 10 cc syringe have been observed by Shiffman and Mirrafati (2001) (43).

**Most authors conclude that large cannulas should be preferred.**

5.3 FAT TRANSPORT

No specific studies have been found in which transport from the operating room to the laboratory is investigated.

Studying the clinical studies of cryopreservation, five protocols mention that the adipocyte aspirates are collected in a bottle and immediately transferred to the laboratory at room temperature (34, 47-50). Other transport techniques are on ice (12), transported in insulated containers and processed within two hours of harvest (32) or placed in 10 ml Falcon tube (42). In other clinical studies no transport method is mentioned, so fat is transported without any precaution.

5.4 FAT PROCESSING

The most commonly used methods to prepare fat are washing, centrifugation, decantation, metal sieve concentration, cotton gauze concentration and sedimentation. Fat processing is necessary because the lipoaspirate does not only contain adipocytes, but also fibers of collagen, blood and debris. Those elements can cause inflammation in the body of the recipient which can be detrimental for the fat graft (1). Blood must be extracted because blood also accelerates the degradation of the transplanted fat
(30). Even more, the injection of debris gives an erroneous impression of the volume of correction, because the debris will be absorbed after a few hours (1).

How debris should be removed, is still a controversial issue.

Sommer et al. (2000) conclude that most authors favor a closed technique. Advantages are sterility, no risk of contamination, no exposure to air and no dry out of adipocytes. The open technique gives the opportunity to separate bigger fibers from the tissue so that the graft is easier to inject (30). In any case the contact with air should be avoided. No studies have been found to determine differences in contact with filthy air or clean air in the operation room.

Atanassova (2012) denies filtration for its traumatic mechanical impact on fat cells and the long exposition to air. Decantation (no washing or centrifugation) is also not preferred because of its duration (7).

Ramon et al. (2005) studied a method using a cotton towel as a platform for concentrating adipocytes. They compared this method with the method of centrifugation. No significant differences were found in graft weight or volume between the two methods, but histological study revealed significantly less fibrosis in the animals with cotton towel processed the fat graft (39, 51).

Minn et al. (2010) harvested fat tissue from the remnants of transverse rectus abdominis musculocutaneous flaps. Fat was prepared using three different methods: centrifugation, metal sieve concentration and cotton gauze concentration. Centrifugation was performed at 1800 g for 3 min at 25 °C. When using the method of metal sieve concentration, the fat was shaken on a metal sieve for 3 minutes. Finally, when using the method of cotton gauze concentration, the fat was gently shaken for 3 minutes. They found the metal sieve group to have a significantly lower viability (XTT assay) and more inflammation than the other groups. The findings also suggested that the centrifugation technique had no advantages over the open gauze concentration in terms of fat viability. No significant difference was observed in fat graft survival and histological analysis (52).

In the analysis of Rose et al. (2006) cell count of intact adipocytes was significantly greater in samples processed by sedimentation compared with centrifugation or washing. Sedimentation should result in more viable cells and greater adipocyte size than washing or centrifugation (53).

Zhu et al. (2013) compared three different fat grafting preparation methods: gravity separation, Coleman centrifugation and simultaneous washing with filtration using a commercial available system (Puregrafts; Cytori therapeutics). Washing with filtration produced fat grafts with the highest tissue viability and with a lower presence of contaminants than fat grafts prepared with other methods. Note that Dr. Zhu and colleges are shareholders and employees of Cytori Therapeutics (54).
Condé-Green et al. (2010) published a study about the influence of decantation, washing and centrifugation (3000 rpm 3 minutes). Decantation maintained the integrity and number of adipocytes, while washing significantly reduced the number of intact cells. Centrifugation destroyed the great majority of fat cells. But decantation showed a great quantity of contaminating blood cells. Centrifugation cleared the fat of most blood remnants. When interpreting the results, washing may turn out to be the best processing technique for adipose tissue graft take, as it preserves the integrity and viability of the most important components of adipose tissue (35).

There are different ideas in literature related to washing. While Nguyen (1990) and Lewis (1991) proposed the washing of the fat grafts, Chajchir (1990) stated that washing removed the fibrin content which is necessary for fat graft adherence (16). Mojallal and Foyatier (2004) also recommend no washing. They prefer centrifugation instead (1). Rohrich (2004) showed no difference in cell viability between washing or centrifugation (16, 40).

The most commonly used technique for isolation of fat is the Coleman’s method, recommending centrifugation at 3000 rpm (= 1200 g) for 3 minutes. Several authors suggested that forces greater than 3000 rpm caused cellular damage. Centrifugation has a clear advantage in longevity and aesthetics, according to Coleman (9).

![Centrifugation Image]

After centrifugation three layers are observed: layer one includes lipids, which can be poured off using absorbent material. Layer 2 consists of fatty tissue. And layer 3 contains blood, tissue fluid, local anesthetic… and is ejected from the base of syringe. The middle layer is routinely used for adipose tissue graft (13, 27). A small fourth layer can be observed containing cell debris, pellets and red blood cells (figure 4) (14, 35).

Figure 4 (14): Image showing the centrifugal separation of the different layers of the product of liposuction.

Ferraro et all. (2011) observed a decrease in the number of fat cells because of damage caused by the centrifugation, but he also claimed that centrifugation is an essential process. It is needed to isolate fat cells and to separate them from substances such as blood cells, lipids, proteases and lipases (55).

Interestingly, Rohrich et all. (2004) concluded that centrifuged and non-centrifuged fat specimens were equally viable. Centrifugation was performed during one minute at low pressure (40).

Gir. et all. (2012) noticed no difference in viability between centrifugation techniques and non-centrifugation techniques. Gentle centrifugation resulted in the highest viability. Centrifugation for a longer time led to the isolation of the most proliferative cell types (13).
Botti et al. (2011) compared fat injected in the face. On one side of the face fat was injected that was simply filtered using a common strainer and then it was washed with a 0, 9% saline solution. The other side of the face was treated with centrifuged fat (3000 rpm 3 minutes). Subjective methods, such as a questionnaire and objective methods such as an evaluation of preoperative and postoperative photographs by a three-member jury, revealed no significant differences between the two sides of the face. A follow-up was done twelve months later (56).

The study performed by Lee et al. (2013) focused on the effect of pressure and shear on autologous fat graft viability. For negative pressure they found no difference in weight or histology with high versus low suction pressures. Adipocytes were also resistant to the forces of positive pressure. But adipocytes were remarkably sensitive to the effects of shear stress. Based on their findings, Lee et al. (2013) recommend the use of high suction pressure (57, 58).

Negative pressure is pressure applied by suction. Positive pressure is applied to fat during injection and during centrifugation. Shear stress is present as fat flows through a cannula or tube (57).

Ferraro et al. (2011) investigated different centrifugation forces. They concluded that a centrifugal force of 1300 rpm for 5 minutes resulted in a better density of adipose tissue, with good viability and increased ability to preserve a significant number of progenitor cells in comparison to centrifugation at 3000 rpm for 3 minutes (55).

In the study of Xie et al. (2010) freshly harvested adipose aspirates underwent centrifugation with four different centrifugal forces (1000 - 4000 rpm). When compared to the group not undergoing centrifugation, a significant decrease in viability of fat grafts was shown. They showed linear reduction of viability with increase of centrifugation forces (59).

Hoareau et al. (2013) subjected adipose tissue to different centrifugation techniques: strong centrifugation (3 min at 3000 rpm = 900, 1800 g), decantation and low centrifugation (1 min at 3000 rpm = 100, 400 g). The volume of interstitial liquid and the released oil were measured to choose the optimal method. Cytokine interleukin-6 and chemokine monocyte chemotactic protein-1 were assayed 24 h after fat injection in mice. After one month a macroscopic and histological analysis with oil gap area measurement was performed. Hoareau et al. (2013) concluded that strong centrifugation was deleterious for adipose tissue, because it led to threefold more adipocyte death compared to low centrifugation. After strong centrifugation more supernatant volume of oil was seen. This oil release was claimed to be caused by adipocyte death (60).

Hoareau et al. (2013) also stated that the more the tissue was centrifuged at a higher speed, the more it was possible to extract interstitial liquid. But the volume of liquid extraction did reach a maximum. So it was obvious that the volume of interstitial liquid was higher when adipose tissue was not washed
or centrifuged. This interstitial liquid contained lidocaine, adrenaline and inflammation molecules that were toxic for adipose tissue (60).

Higher rates of blood IL-6 and MCP-1, indicating blood inflammation, were observed in mice injected with strong-centrifuged and non-centrifuged adipose tissue, compared to those injected with soft-centrifuged fat. This was observed 24 hours after injection. One week after grafting, levels of IL-6 and MCP-1 were the same as in the control mice (60).

Hoareau et al. (2013) concluded that it was necessary to wash and centrifuge adipose tissue before re-injection in order to remove infiltration liquid and toxic molecules. Soft centrifugation, preceded by washing, seems to be the most appropriate protocol for fat grafting (60).

Boschert et al. (2002) centrifuged specimens at 10, 30, 50 and 100 g for a total of 8 minutes. When reaching 100 g, the quantity of oil increased. They believed that higher gravity forces led to a larger quantity of oil being separated from the adipocytes because more cells were destroyed in this process. They also examined centrifugation time by comparing centrifugation samples that were centrifuged for 2, 4, 6 and 8 minutes. Centrifugation beyond 2 minutes did not further stratify the cells by viability. And the highest number of viable cells was found at the bottom layer, because Boschert et al. (2002) showed that the bottom layer had a greater number of intact cells (61).

Chinese investigators (2005) showed no difference in cell viability between fat centrifuged at 1000 to 4000 rpm. On the other hand the amount of intact adipocytes after centrifugation at 5000 rpm was significantly (P < 0.05) less than after centrifugation between 1000 and 4000 rpm (24).

Shiffman and Mirrafati (2001) concluded that centrifugation at 3600 rpm for 10 seconds or for 1 minute did not result in significant cell damage but in compacting the cells (43).

**What’s the ideal processing technique?** Evidence-based literature didn’t offer an answer. But it is obvious that centrifugation is the most commonly used technique. Centrifugation time does not affect the viability of fat cells, but there is a lot of discussion about the forces that should be used. However, most authors have reached consensus about the correlation between force and fat cell viability; when increasing the force, fat cell viability decreases. The ideal force should be 3000 rpm ( = 1200 g), according to Coleman.
5.5 FAT STORAGE

The question remains if fat can be frozen, how it should be done and how long fat can be preserved.

5.5.1 COOLING AND THAWING

The greatest challenge during cryopreservation is the lethality of the cooling and thawing processes (62). A significant portion of the adipocytes die on the first day of cryopreservation, but those cells that survive are viable for a long time under the conditions of cryopreservation (48). The lethality of the cooling and thawing process is due to solutions effect, extracellular ice formation, dehydration and intracellular ice formation.

Solution effects are the result of high concentration of solutes because ice crystals grow in freezing water. When tissue is cooled, water migrates out of the cell and ice is formed in the extracellular space. This cellular dehydration damages the cell directly and too much extracellular ice can crush the cell membrane. Instead of large amounts of extracellular ice formation, small amounts of intracellular ice formation are always fatal to cells (63). The intracellular water of a cell that is dropped in a freezing medium has a higher vapor pressure than the external medium. If the cell remains cooled and if no water moves out of the cell, the ratio of the internal to external vapor pressure increases progressively. But cell membranes are permeable to water, so the vapor pressure differential causes water to leave the cell. The content of the cell concentrates. Survival of fat cells is improved by using slow cooling of 1°C/min. When using slow cooling, enough water will leave the cell to eliminate the vapor pressure differential and keep the protoplasm at its freezing point. The rate of cooling is affected by the permeability of the cell to water and its surface area-volume ratio (25).

Cryopreservation causes damage due to intercellular ice formation and osmotic stress. This is called the two factor hypothesis. Ice formation can be prevented by controlled slow freezing and rapidly thawing in a 37°C water bath (12, 34, 48).

In eight of the seventeen clinical studies slow cooling was used. The adipose tissue in a cryovial was put in a methanol bath. The temperature of the bath was set at 1-2°C/minute from 22°C to -30°C. Once the adipose tissue reached the ending temperature, it was held at -30°C for 10 minutes. Then fat were transferred into liquid nitrogen (-196°C). Below -85°C was considered to be optimal for cryopreservation.

Survival of fat cells is also improved using fast thawing. Fast thawing consisted of adaptation of the adipose tissue to room temperature for 2 minutes to let the liquid nitrogen vapor out of the cryovial. Then each cryovial was dropped into a stirred 40°C water bath until the adipose tissue was thawed. Seven of the seventeen selected clinical studies used fast thawing.
Table 2. Comparison of techniques used for cooling and thawing. In the first column the reference of the relative article is written down (this is also applicable to following tables).

<table>
<thead>
<tr>
<th>Cooling</th>
<th>Thawing</th>
</tr>
</thead>
<tbody>
<tr>
<td>(12) slow cooling (1°C/min) to - 20 °C in freezer</td>
<td>fast thawing: 2 minutes at room temperature, 5 minutes in 37°C water bath</td>
</tr>
<tr>
<td>(64) slow cooling (1°C/min) from 22°C to - 30°C, secondly transferred in - 196°C</td>
<td>fast thawing: 2 minutes at room temperature, 5 minutes in 40°C water bath (35°C/minute)</td>
</tr>
<tr>
<td>(48) cooling by placing in a deep freezer</td>
<td>fast thawing: 2 minutes at room temperature, 5 minutes in 37°C water bath</td>
</tr>
<tr>
<td>(16) cooling by placing in a deep freezer</td>
<td>fast thawing: 2 minutes at room temperature, 5 minutes in 37°C water bath</td>
</tr>
<tr>
<td>(34) slow cooling (1°C/min) from 22°C to - 30°C, 10 minutes at - 30°C, transferred in - 196°C (below - 85°C = optimal)</td>
<td>fast thawing: 2 minutes at room temperature, 5 minutes in 37°C water bath</td>
</tr>
<tr>
<td>(49) slow cooling (1°C/min) from 22°C to - 30°C, 10 minutes at - 30°C, transferred in - 196°C (below - 85°C = optimal)</td>
<td>fast thawing: 2 minutes at room temperature, 5 minutes in 37°C water bath</td>
</tr>
<tr>
<td>(18) in freezer: - 35°C</td>
<td>fast thawing: 2 minutes at room temperature, 5 minutes in 37°C water bath</td>
</tr>
<tr>
<td>(33) frozen at - 40°C in an upright freezer</td>
<td>fast thawing: 2 minutes at room temperature, 5 minutes in 37°C water bath</td>
</tr>
<tr>
<td>(50) slow cooling (1°C/min) from 22°C to - 30°C, 10 minutes at - 30°C, transferred in - 196°C (below - 85°C = optimal)</td>
<td>fast thawing: 2 minutes at room temperature, 5 minutes in 37°C water bath</td>
</tr>
<tr>
<td>(32) directly frozen at - 20°C or 1°C/ min to - 80°C and transferred to liquid nitrogen vapor phase storage</td>
<td>fast thawing: 2 minutes at room temperature, 5 minutes in 37°C water bath</td>
</tr>
<tr>
<td>(65) slow cooling (1°C/min) to - 20°C/ - 80°C</td>
<td>fast thawing: 2 minutes at room temperature, 5 minutes in 37°C water bath</td>
</tr>
<tr>
<td>(47) slow cooling (1°C/min) from 22°C to - 30°C, 10 minutes at - 30°C, transferred in - 196°C (below - 85°C = optimal)</td>
<td>fast thawing: 2 minutes at room temperature, 5 minutes in 37°C water bath</td>
</tr>
<tr>
<td>(17) cooled to - 18°C</td>
<td>fast thawing: 2 minutes at room temperature, 5 minutes in 37°C water bath</td>
</tr>
</tbody>
</table>

From the thirteen clinical studies who mentioned cooling and thawing, eight authors used slow cooling (1°C/min). Seven authors used fast thawing in their process, instead of five authors who preferred to thaw the fat slowly at room temperature. So according to the reported clinical studies and the previous literature slow cooling and fast thawing should be preferred (table 2).

5.5.2 COOLING TIME AND TEMPERATURE

An optimal method must be likely to preserve fat grafts for months or even years (21).

In 2000 Lidagoster et all. made a histological comparison between fat that was immediately implanted in mice, fat stored at 1 °C for 1 or 2 weeks and fat stored at -16°C for 1 or 2 weeks and later implanted in mice. The larger the storage time the more severe changes became. They also preferred to use a freezer (-16°C) instead of a refrigerator (1°C) (66).

MacRae et al. (2004) confirmed that cells frozen at -196°C were less viable that cells frozen at -20°C. But the difference was not significant (42). Pu et all. (2009) remarked that the longer the storage time and the higher the temperature of storage, the less viable the adipocytes became (21).

Son et all. (2010) reported that a significant portion of adipocytes were lost after one day of cryopreservation. Viability and metabolic activity of adipocytes were much higher for cryopreservation at -70°C than at -15°C (48).
Table 3 (23): Comparison of ultra-low temperature storage methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electric freezer (-135°C)</td>
<td>Ease of maintenance</td>
<td>Requires liquid nitrogen back-up</td>
</tr>
<tr>
<td></td>
<td>Steady temperature</td>
<td>Mechanically complex</td>
</tr>
<tr>
<td></td>
<td>Low running costs</td>
<td>Storage temperatures high relative to liquid nitrogen</td>
</tr>
<tr>
<td>Liquid phase nitrogen (-196°C)</td>
<td>Steady ultra-low temperature</td>
<td>Requires regular supply</td>
</tr>
<tr>
<td></td>
<td>Simplicity and mechanically reliable</td>
<td>High running costs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Risk of cross-contamination</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low volume</td>
</tr>
<tr>
<td>Vapor phase nitrogen (-135°C – (-196°C))</td>
<td>No risk of cross-contamination</td>
<td>Requires regular supply</td>
</tr>
<tr>
<td></td>
<td>Low temperatures achieved</td>
<td>High running costs</td>
</tr>
<tr>
<td></td>
<td>Simplicity and reliability</td>
<td>Temperature fluctuations between -135 and -196°C</td>
</tr>
</tbody>
</table>

Table 4 Comparison of storage temperature.

<table>
<thead>
<tr>
<th>Number of participants</th>
<th>Comparison of...</th>
<th>Freezing time</th>
<th>Methods</th>
<th>Conclusion of authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>(12)</td>
<td></td>
<td>2-4 weeks</td>
<td>MTT (viability), histological examination</td>
<td>NONSIGNIFICANT DIFFERENCE IN MTT AND HISTOLOGY</td>
</tr>
<tr>
<td>(48)</td>
<td></td>
<td>1,3,7,14, 28,56 days</td>
<td>Staining with fluorescein diacetate and propidium iodide by fluorescence microscopy, G3PDH activity (cell stability), XTT reduction assay (metabolic activity)</td>
<td>NONSIGNIFICANT DIFFERENCE BETWEEN -15°C AND -70°C BY STAINING, XTT / SIGNIFICANT DIFFERENCE IN G3PDH AT DAY 3 AND 7 BETWEEN -15°C AND -70°C (-70°C: HIGHER ACTIVITY) / SIGNIFICANT DIFFERENCE IN LIPOASPIRATE XTT AT DAY 7 BETWEEN -15°C AND -70°C (-70°C: HIGHER ACTIVITY)</td>
</tr>
<tr>
<td>(16)</td>
<td></td>
<td>2 weeks</td>
<td>Supravital dye staining</td>
<td>FREEZING AT -20°C, -80°C: SIGNIFICANT LOWER VIABLE ADIPOCYTES &lt;= FRESH / 4°C: NONSIGNIFICANT DIFFERENCE &lt;= FRESH</td>
</tr>
<tr>
<td>(18)</td>
<td></td>
<td>6 months</td>
<td>MTT (viability), histological examination</td>
<td>NONSIGNIFICANT DIFFERENCE IN VIABILITY AND HISTOLOGY</td>
</tr>
<tr>
<td>(32)</td>
<td></td>
<td>48 to 244 days (average 120 days)</td>
<td>Fluorescein diacetate and propidium iodide staining (viability measurement), Ceiling culture (viability determination)</td>
<td>SIGNIFICANT DIFFERENCE =&gt; -20°C = NON VIABLE CELLS</td>
</tr>
<tr>
<td>(65)</td>
<td></td>
<td>2,7,14,30 days</td>
<td>Trypan blue staining, MTT, G3PDH</td>
<td>SIGNIFICANT DIFFERENCE IN XTT (P = 0,047) AND GPDH TEST / NONSIGNIFICANT DIFFERENCE IN MTT</td>
</tr>
<tr>
<td>(66)</td>
<td></td>
<td>1 or 2 weeks</td>
<td>Histological examination</td>
<td>SIGNIFICANT DIFFERENCE</td>
</tr>
</tbody>
</table>
Table 5 Comparison of storage time.

<table>
<thead>
<tr>
<th>Number of participants</th>
<th>Comparison of...</th>
<th>Cooling</th>
<th>Freezing temperature</th>
<th>Thawing</th>
<th>Methods</th>
<th>Conclusion of authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>(48) 16 persons</td>
<td>freezing time of 1, 3, 7, 14, 28, 56 days</td>
<td>cooling by placing in a deep freezer</td>
<td>freezing at -15°C and -70°C</td>
<td>thawing at room temperature</td>
<td>Staining with fluorescein diacetate and propidium iodide by fluorescence microscopy, G3PDH, XTT</td>
<td>NONSIGNIFICANT DIFFERENCE</td>
</tr>
<tr>
<td>(65) 24 persons</td>
<td>freezing time of 2,7,14,30 days</td>
<td>slow cooling to -20°C/ -80°C</td>
<td>freezing at -20°C and -80°C</td>
<td>thawing in a water bath at 37°C</td>
<td>Trypan blue staining, MTT, G3PDH</td>
<td>NONSIGNIFICANT DIFFERENCE IN XTT AND GPDH TEST AT -20°C or -80°C BETWEEN 2,7,12 AND 30 DAYS/ SIGNIFICANT BETWEEN DAY 0 AND 2, AND 2 AND 30.</td>
</tr>
</tbody>
</table>

The different storage temperatures of cryopreservation have been compared but the results are not consistent. Cryopreservation in liquid nitrogen is the most commonly used method because temperature below - 85°C is considered to stop metabolism in fat cells (table 4).

Storage time should not influence adipocytes viability. But a lot more clinical studies are needed to make evidence-based conclusions based on literature (table 5). In four of the selected clinical studies storage time was twenty minutes. This time was considered to be equivalent to long-term-cryopreservation. In five studies two weeks was used as storage time, but no motivation was mentioned for this amount of time.

5.5.3 USE OF FREEZING MEDIA
Adding media (DMEM + 10% FBS) decreases the viability of cryopreserved fat cells significantly. This could possibly be explained by the increased metabolic activity encouraged by these media. They induce cells to leave dormancy (42).

5.5.4 USE OF CRYOPROTECTANT (CPA)
Because of the success of preserving autologous skin cell with cryoprotective agents, the use of cryoprotective agents should be investigated in fat cell preservation.

Lee L.Q. Pu (2005) comments, based on his experience, that effective cryopreservation of fat grafts should first involve adding cryoprotective agents. Then the mixture undergoes slow, controlled freezing to -30°C and is ultimately stored in liquid nitrogen at -196°C. A complete stop of metabolic
activity is achieved at -130°C, he says (48). Before implantation, the frozen fat graft undergoes fast thawing to 37°C and the cryoprotective agent must, depending on the type, be removed. Just simply freezing without adding cryoprotective agents is not adequate and would not be a reliable method for long-term preservation (65, 67).

Pu et al. (2009) remarked that the longer the storage time and the higher the temperature of storage, the less viable the adipocytes became. Adding a cryoprotective agent led to a preservation of up to 54% of the baseline activity (21).

Pu et al. (2010) underline the importance of a cryoprotective agent. The cryoprotectant acts like antifreeze: a cryoprotectant decreases the freezing temperature and increases the viscosity. It reduces cell injury due to intercellular ice formation, extracellular ice formation, dehydration and solution effects.

- DMSO (dimethyl sulfoxide) is a membrane-permeating cryoprotective agent. The optimal concentration when used alone is 10%. DMSO is toxic for humans. Its toxicity is most prominent at high temperatures. So because of its toxicity at body temperature, it must be removed after thawing (62).

- Trehalose, a disaccharide of glucose, is a non-permeable non-tissue toxic cryoprotective agent. Trehalose prevents ice formation (the vitrification theory), dehydrates cells and reduces water content before freezing (the water displacement theory). It stabilizes the membrane and proteins and forms a glassy matrix. DMSO and trehalose possibly have synergetic effects. The optimal combination is 7, 6% (0, 2 M) trehalose and 3, 3% (0, 5 M) DMSO (21, 34, 62, 63).

- More recent detected cryoprotective agents are HES (hydroxyethyl starch) 10% and glycerol. HES is a non-membrane-permeating cryoprotective agent. So it is less toxic to cells. In general membrane-permeating cryoprotectants seem to be beneficial. Glycerol is a membrane-permeating agent, but it needs repeated washing to remove it after thawing (12).

- Extrapolation of the freezing tolerance of wood frogs, urea and glucose are used as cryoprotectants. They limit the formation of ice and reduce osmotic shrinkage of cells (63).

Interestingly, Pu et al. (2010) compared fresh fat and fat graft frozen with a cryoprotective agent (DMSO) with slow cooling and fast reheating. Viability was slightly lower in the frozen fat graft. He noticed a significant lower level of enzyme activity when fat was cryopreserved. But there was no evidence of degeneration or necrosis (64).
A significantly higher adipocyte cell count was observed by Cui et all. (2007) in the group with use of 0, 5 M DMSO and 0, 2 M trehalose compared with groups using CPA in other concentrations (34). Cui et all. (2007) also observed a higher viability of fat cells when cryopreserved with CPA. When cryopreserved without CPA, observations were made of significant crimping of cells, viability loss, weight and volume loss of the fat graft (34).

A comparison of fresh adipose aspirates, optimal cryopreservation (with CPA: 0, 5 M DMSO and 0, 2 M trehalose) and simple cryopreservation was made by Pu et all. (2006). The fat was injected in the scalp of mice. Volume, weight and histology (H & E) were compared. The results of the cryopreserved group were less satisfactory than those of the fresh group. Optimal cryopreservation showed more maintained volume, weight and fatty tissue structure than the simple cryopreservation (50).

Atik et all. (2006) showed no difference in viability between immediately transplanted fat cells and cells frozen in liquid nitrogen at -35°C for 6 months. On the other hand, fat cells that were dry-frozen (lyophilized) or immersed in glycerol (cryoprotectant) at -35°C had an increase of cell loss and an increase of the vacuolar fibrotic area. The viability of dry-frozen fat showed no difference with dead control fat cells, but the enzymatic activity was higher than in the control group (18).

At last, cryopreservation was easier for thin samples and small clumps of individual cells, because these could be cooled more quickly and so required a lesser dose of cryoprotectant (63).

Pu (2009) concluded: “The major steps of modern techniques of cryopreservation are the following: a) add cryoprotective agents before cooling, b) cool the cells slowly to a low temperature (1°C/minute), c) quickly thaw the cells by incubation in a 37°C water bath for 3 - 5 minutes and last but not least d) remove the cryoprotective agents after thawing (21).”
Table 6 Comparative studies of fat frozen with or without cryoprotectant and differences between cryoprotectants.

<table>
<thead>
<tr>
<th>Compared groups</th>
<th>Methods</th>
<th>Conclusion of authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>(12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. In normal saline 2. In 10% HES</td>
<td>MTT (viability), histological examination</td>
<td>NONSIGNIFICANT DIFFERENCE WHEN ADDING HES AS CPA</td>
</tr>
<tr>
<td>(64)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Mixed with CPA (DMSO 0, 5M or 3, 3% and trehalose 0, 2M or 7, 6%): 1ml fat graft + 1 ml CPA in 3 cc vial, incubated for 10 min at room temperature =&gt; methanol bath 2. No CPA: 1 ml fat + 1 ml normal saline at room T</td>
<td>Trypan blue viability, G3PDH activity (cell stability), routine histology</td>
<td>NONSIGNIFICANT DIFFERENCE IN ADIPOCYTE COUNT AND HISTOLOGY BETWEEN CRYOPRESERVATION WITH AND WITHOUT CPA/ SIGNIFICANT DIFFERENCE (P &lt; 0,001) IN G3PDH ACTIVITY</td>
</tr>
<tr>
<td>(34)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Fresh control 2. Cryopreservation without CPA 3. cryopreservation with CPA: 0, 5 M DMSO + 0, 2 M trehalose with 3 X centrifugation 100 g to remove CPA (15 mice each group)</td>
<td>Weight, volume, histological examination, cell count</td>
<td>NONSIGNIFICANT DIFFERENCE IN ADIPOCYTE COUNT BETWEEN FAT FROZEN WITH AND WITHOUT CRYOPROTECTANT / GROUP WITH 0. 5 DMSO AND 0. 2 TREHALOSE SIGNIFICANT HIGHER CELL COUNT THAN OTHER CRYOPROTECTANT GROUP</td>
</tr>
<tr>
<td>(18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Immediately transplanted to donor 2. Dry-frozen 3. Immersed in glycerol 4. frozen in liquid nitrogen (10 mice each group)</td>
<td>MTT (viability), histological examination</td>
<td>SIGNIFICANT DIFFERENCE IN VIABILITY BETWEEN FROZEN WITH OR WITHOUT GLYCEROL</td>
</tr>
<tr>
<td>(50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Fresh control group 2. Simple cryopreservation group 3. Optimal cryopreservation group: with CPA (DMSO 0, 5 M and trehalose 0, 2 M)</td>
<td>Gross appearance of fat graft over their posterior scalps, volume, weight, histological examination</td>
<td>SIGNIFICANT INCREASE IN VOLUME,WEIGHT AND HISTOLOGY WHEN CRYOPRESERVED WITH CPA</td>
</tr>
<tr>
<td>(65)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. DMEM 2. DMEM + 10% HES 3. DMEM + 10% glycerol 4. DMEM + 10% glycerol + 5% dextran + 5% PVP (polyvinylpyridione)</td>
<td>Trypan blue staining, MTT, G3PDH</td>
<td>NONSIGNIFICANT DIFFERENCE IN MTT, XTT</td>
</tr>
<tr>
<td>(42)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. 2 ml of DMEM and 10% FBS: mixed and incubated at 37°C 2. No medium 3. -20°C freezer, no medium 4. “Snap” frozen by immersion in -196°C, without medium 5. Fresh adipocytes</td>
<td>MTT (viability)</td>
<td>SIGNIFICANT DIFFERENCE IN ADIPOCYTE VIABILITY: SIGNIFICANTLY LESS IN GROUP INCUBATED WITH MEDIA</td>
</tr>
</tbody>
</table>

Cryopreservation decreases adipocytes viability, but by adding a cryoprotectant, viability increases (table 6). When using a cryoprotectant, a preference is made for 0, 5 M (3, 3 %) DMSO and 0, 2 M (7, 6 %) trehalose. In general membrane-permeating protectants seem to be beneficial. But because of the toxicity of DMSO, there is a need to develop DMSO-free methods.
5.6 FAT REINJECTION

5.6.1 REINJECTION TECHNIQUE

The adult fat cell survives the trauma of tissue harvesting and transplantation after revascularization within 4 - 8 days after implantation. Pre-adipocytes are more likely to survive because of their resistance to trauma. This is due to the fact that progenitor cells have minimal metabolic requirements and can survive longer with minimal nutritional and oxygen requirements compared with mature adipocytes (15).

Washing with sterile saline before injecting the fat removes the oily component that could trigger undesired inflammatory events (15).

Reinjection of fat is preferred in multiple small-volume sessions over one single injection (3). This is because revascularization starts at the periphery and ischemic time is longer in the centre (1). The diameter of fat grafts should be less or equal to 3 mm for effective neovascularization (3). This because tissue nutrients only diffuse 1, 5 mm into the transplant (33).

Lee et all. (2013) recommend slow injections between 0, 5 and 1 cc/second for optimal fat graft survival (57, 58).

The use of a small injection blunt needle minimizes trauma at the receiving tissue site and reduces local anesthesia. Thinner strands of transplanted adipose tissue also result in a reduced post implantation swelling. Less-blunt cannulas give the surgeons more control for placement in the immediately subdermal plane, in fibrous tissue and in scars (9).

The advantage of blunt needles is the reduced risk of vascular tearing during introduction into the tissue. On the contrary, the use of blunt needles often requires more anesthesia because of the greater difficulty in passing the blunt needle through the subdermal tissue (38).

Multiple small tract injections performed with smaller needles show improved results. This could be the result of localized oxidative stress, which triggers cell migration and proliferation, including endothelial cells. Reinjection of the purified fat tissue through a three-dimensional implantation technique creates multiple tunnels in the recipient tissue that could be vascularized more easily (15).

Overcorrecting the volume defect must be avoided. Overcorrection may increase the incidence of fat necrosis, calcification or infection (39).

In clinical studies in mice, fat is preferably transplanted in the skull, because no endogenous fat is present at that site and there is a low blood supply and thus a lower level of fat retention (68). In humans, fat should be transplanted in an area without trauma or any pathology. Especially scars should be avoided. So in general the optimal condition for reinjection is an anatomically and
physiologically undamaged tissue structure with good blood supply. Many authors consider the muscle as optimal for graft intake because of its blood supply. But injection in the muscle may have a much higher risk for hematoma formation and fat cell lyses (1).

Khater and Atanassova (2012) demonstrated most favorable results with statistical significance (p < 0.05) in the head and neck region. The authors attributed the latter to the higher blood supply to this region (7).

The effect of the lipofilling can be observed after 2 till 4 weeks, when the edema, and if relevant, hematoma have disappeared. So any refilling can be performed within several weeks following the initial procedure (15).

**Reinjection of fat should involve washing with sterile saline to remove oily components. Reinjection is performed in multiple small-volume sessions with small, blunt needles to increase vascularization of the fat graft which decreases the reabsorption rate.**

### 5.6.2 USE OF ANABOLICS

Because adipocytes are subjected to a period of ischemia, investigators proposed the use of growth factors or hormonal factors to raise the survival.

Yuksel (2000) showed a significant increase in survival when those factors were added before injection, especially with insulin, IGF-1 and bFGF. Those factors should have effect on pre-adipocytes (1).

The addition of a medium enriched with nutritional elements such as amino acids, vitamins, glucose, growth factors, insulin and thyroxin increases the survival of adipocytes with 10% in 4 months. The mixture permits the cells to overcome the period of ischemia before revascularization takes place (1).

Pu et al. (2010) observed that better results in viability could be reached when fat graft was pretreated with cell culture medium or growth factors before implantation, compared with no medium. This will lead to increasing survival because cells may resume their optimal intracellular activity much faster. Other suggestions are purified viable adipocyte graft or cell-assisted transfer with adipose-derived stem cells (64).

The survival rate of fat grafts augments when transplanted with the stromal vascular fraction, which is known as cell-assisted lipotransfer (68).

Recent positive interaction between transplanted adipose tissue and hyaluronic acid fillers may open the possibility to improve long-lasting results (15). Hyaluronic acid is thought to stabilize the filling volume and result in a solid fibro-vascular network (27).
Coleman concludes: “When the fat graft is performed in good conditions, there is no need to add any growth factor (1).”

Clinical studies show a positive effect of the use of anabolics on the survival of fat grafts. More clinical studies are needed to confirm this data. Using an anabolic agent could decrease fat reabsorption and thus the need for repeated liposuction procedures. Investigations should be able to identify which anabolics can be used and how it should be integrated in the process of lipofilling. This could boost the possibilities of fat grafts.

5.6.3 AFTERTREATMENT

Literature recommends treatment with antibiotics and non-steroidal anti-inflammatory drugs for one week. Donofrio (2000) also adds vitamin therapy E (1). A biopsy for fatty acid analysis of the graft is the most informative method to report the outcome. Magnetic resonance imaging and the 3D tomodensitometry are too expensive and therefore not suitable for routine clinical practice. Follow-up often includes standardized photo documentation (7).

There can be a considerable swelling in the recipient tissue at 2 to 4 weeks after operation and it can last up to 16 weeks. Elevation, cold therapy and external pressure with tape can help prevent the swelling (9).

5.6.4 COMPLICATIONS

There are some complications associated with fat grafting. Common side effects are swelling, redness, loss of volume, tingling and bruising. Hematoma, cellulitis, fibrosis, oil cysts and calcifications are less common (figure 5, figure 6). On rare occasions, fat grafting can be followed by fat emboli of the retinal or cerebral arteries, hemi paresis, aphasia and loss of vision (9, 69). This especially occurs when fat grafting is performed in the upper third of the face. Another side effect is hypertrophy. This unwanted result is seen when abdominal fat is grafted to the face. When abdominal fat increases, the grafted fat increases (69).

Usually complications in the recipient place are due to too much or too less fat transferred to the recipient area, which results in irregularities (9). Liposuction deformities at the donor area especially occur in thin patients or patients who have already had liposuction (8).

Figure 5 (7): Postoperative complications in donor site. Figure 6 (7): Postoperative complications in recipient site.
5.6.5 OUTCOME: FRESH VERSUS FROZEN FAT

Is there a difference between fresh and frozen fat? Does frozen fat differ in histology or function compared to fresh fat? Is there a difference in outcome when frozen fat is injected instead of fresh fat?

Retention of fat grafts is likely to be affected by its degree of vascularization. Grewal et al. (2009) investigated the effect of freezing on the expression of the vascular endothelial growth factor (VEGF). They concluded that freezing had a dramatic effect on its function and morphology. VEGF was quantified using a commercial ELISA assay. To conclude, after 1 week of freezing most grafts lost their ability to secrete VEGF. In media with 50% DMEM, 40% FBS, 10% DMSO some VEGF secretion seemed to be preserved after 1 week of freezing. Moreover, after 2 weeks of freezing no VEGF-secretion could be detected. Only in the medium with 30% sucrose in PBS a significant VEGF-secretion was preserved after 2 weeks of storage (70).

In 2000 Lidagoster et al. made a histological comparison of immediately implanted fat, fat stored at 1 °C for 1 or 2 weeks and fat cryopreserved at -16°C for 1 or 2 weeks. A decrease in viable adipocytes and an increase in signs of inflammation and necrosis were demonstrated when animals received a transplantation with stored fat instead of immediate implanted fat (66).

Comparison of fresh adipose aspirates, optimal cryopreservation (with CPA: 0,5 M DMSO and 0,2 M trehalose) and simple cryopreservation was made by Pu et al. (2007). The fat was injected in the scalp of mice. Volume, weight and histology (H & E) were compared. All results were less satisfactory in the cryopreserved group than in the fresh group (67).

Interestingly, Pu et al. (2010) compared fresh fat and fat graft frozen with a cryoprotective agent (DMSO) with slow cooling and fast reheating. Viability was slightly lower in the frozen fat graft. He noticed a significant lower level of enzyme activity when fat was cryopreserved. But there was no evidence of degeneration or necrosis (64).

Wolter et al. (2005) believe that the effect of reinjection of fat, frozen at -20°C, mostly relies on injecting dead material. Initially, the cell debris still gives an increased volume, but removal of the dead material explains the volume loss (65).

Moscatello et al. (2005) concluded that fat frozen at -20°C was not viable. Viability was determined by staining and culture. No culture could be established from samples of frozen fat at -20°C. Viable adipocytes could be retrieved from fat mixed with cryoprotectant, frozen at 1°C/min and stored in liquid nitrogen vapor phase (32).

Shoshani et al. (2001) showed no statistically (p > 0,05) significant difference in fat graft weight, volume and histological evaluation between fat frozen at -18 °C for two weeks and fat reinjected 30 minutes after harvesting. Fat cells were sucked under negative pressure at 1 atm. After 2 sessions of
centrifugation (1500 rpm) for 5 minutes, the fat cells were separated from fluid-cell debris. One milliliter of fat was injected in 6-week old nude male athymic mice (limit ability to reject) in the mouse scalp, using a 14 G needle. The mice were divided into a study and a control group. In the control group, the time interval between harvesting and reinjection was less than 30 minutes at room temperature. The study group was frozen at -18 °C for two weeks. Graft investigation 15 weeks post-injection and after thawing at room temperature showed no difference in weight and volume (17).

Kim et al. (2007) compared fresh adipocytes and adipocytes frozen in liquid nitrogen (-196°C) for 6 days. At this cold temperature all biological activities, including the biochemical reactions that would cause cell death, were effectively stopped (63). He found no difference in morphologies and volume between both groups. Viabilities of freeze-thawed adipocytes were identical to fresh adipocytes, but the yield of freeze-thawed adipocytes was 10 % lower after cryopreservation. The results of the measurement of adipogenic expression by Oil red O staining, leptin secretion and RT-PCR revealed no significant difference. The study is not generalizable because of the small number of mice. And as they know that cell viability mostly depends on the freezing and the thawing process, there is a need for experiments with longer preservation (22).

Atik et al. (2006) showed no difference in viability between immediately transplanted fat cells and cells frozen in liquid nitrogen at -35°C for 6 months. The use of fat cells frozen in liquid nitrogen showed no significant difference with fresh transplanted cells, but histological analysis showed a higher proportion of vacuolar and fibrotic areas, which caused volume loss (18).

When Cui et al. (2007) compared fat cells cryopreserved with CPA and fresh fat, the same results of viability, volume and histology were found (34).

A randomized double-blind comparison study by Butterwick et al. (2006) revealed that the result of aesthetics, vein prominence and depth of metacarpal space of frozen fat (for maximum 2 weeks) were superior to fresh fat. This two-hand study also showed that the side effect profile was less in the hand injected with frozen fat. Discoloration and painful swelling was less present in the hand injected with frozen fat, compared to the hand injected with fresh fat. Butterwick et al. (2006) also claimed that the size of swelling was proportional to the amount of fat injected (33).

When studying fat viability before and after freezing, fat viability decreases by freezing in all clinical studies where viability was mentioned, except for one (table 7) (22). Li et al. (2012) show that the lower the freezing temperature and the shorter the period of freezing time, the higher the viability of fat cells (12). Son et al. (2010) partially agree with this. When fat cells are frozen for a long period, fat viability decreases. But they noticed lower viability of fat cells when fat cells were frozen at -70°C compared to those frozen at -15°C (48). Atik et al. (2006) concluded that freezing in liquid nitrogen is the best way to cryopreserve fat cells (18). MacRae (2004) also prefer cryopreservation in liquid
nitrogen instead of cryopreservation at -20°C, because they observed lower viability in the last group (42). Pu et all. (2004) and Moscatello (2005) compared viability of fat cells frozen with or without CPA. They both concluded that CPA increase viability (32, 47) (table 7).

Table 7 Analysis of viability decline: comparison of viability (in percentage) of fresh and frozen adipocytes (with specification of the relevant process or freezing conditions).

<table>
<thead>
<tr>
<th>Viability fresh adipocytes (%)</th>
<th>Viability freeze-thawed adipocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(12) /</td>
<td>-20°C, 2 days: 78 % of fresh ones</td>
</tr>
<tr>
<td></td>
<td>-80°C, 2 days: 88 % of fresh ones</td>
</tr>
<tr>
<td></td>
<td>-196°C, 2 days: 92 % of fresh ones</td>
</tr>
<tr>
<td></td>
<td>-20°C, 7 days: 65,6 % of fresh ones</td>
</tr>
<tr>
<td></td>
<td>-80°C, 7 days: 74 % of fresh ones</td>
</tr>
<tr>
<td></td>
<td>-196°C, 7 days: 81% of fresh ones</td>
</tr>
<tr>
<td>(52) /</td>
<td>63,7 (cotton gauze)</td>
</tr>
<tr>
<td></td>
<td>57,1 (metal sieve)</td>
</tr>
<tr>
<td></td>
<td>63,1 (centrifugation)</td>
</tr>
<tr>
<td>(48) 79,7 ± 8,4</td>
<td>-15°C, 1 day: 13,3 ± 7,4</td>
</tr>
<tr>
<td></td>
<td>-70°C, 1 day: 12,6 ± 6,3</td>
</tr>
<tr>
<td></td>
<td>-15°C, 56 days: 5,8 ± 6,8</td>
</tr>
<tr>
<td></td>
<td>-70°C, 56 days: 4,5 ± 4,0</td>
</tr>
<tr>
<td>(34) /</td>
<td>87,1 % of fresh ones</td>
</tr>
<tr>
<td>(22) 96,2</td>
<td>96,3</td>
</tr>
<tr>
<td>(49) /</td>
<td>90 % of fresh ones</td>
</tr>
<tr>
<td>(18) 78,3</td>
<td>66,4 (liquid nitrogen)</td>
</tr>
<tr>
<td></td>
<td>47,3 (dry-freeze)</td>
</tr>
<tr>
<td></td>
<td>38,1 (glycerol)</td>
</tr>
<tr>
<td></td>
<td>47,9 (dead control)</td>
</tr>
<tr>
<td>(32) /</td>
<td>-20°C: 30 % of fresh ones</td>
</tr>
<tr>
<td></td>
<td>-80°C with glycerol: 50 % of fresh ones</td>
</tr>
<tr>
<td></td>
<td>-80°C with DMSO: 64 % of fresh ones</td>
</tr>
<tr>
<td>(65) 94,3</td>
<td>81,03</td>
</tr>
<tr>
<td>(47) /</td>
<td>with CPA: 74 % of fresh one</td>
</tr>
<tr>
<td></td>
<td>without CPA: 37 % of fresh one</td>
</tr>
<tr>
<td>(42) /</td>
<td>in liquid nitrogen: 86%</td>
</tr>
<tr>
<td></td>
<td>-20°C: 75 %</td>
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</table>

The effects of cryopreservation should be further investigated. Five studies showed no significant difference between frozen and fresh fat. Four studies showed that frozen fat was less viable than fresh fat and two studies even indicated that fat was dead because of the freezing. Most studies show no significant difference in appearance of the receptor site.
5.7 METHODS
In the following pages, a description is made of the various methods that can be used to measure viability or functionality of adipocytes.

The number of mature viable adipocytes is difficult to measure, mainly because of their fragility and the presence of adjacent progenitors. Lee et al. (2012) underline that adipocytes are metabolically less active than other cell lines, which makes it difficult to interpret results of metabolic assays. Moreover adipocytes are buoyant. They almost immediately separate out of aqueous solution, leading to a tremendous amount of sampling variability, which can lead to inconsistent and inaccurate results. These characteristics make it challenging to study adipocyte viability (71).

Xie et al. (2010) claim that viability can be tested in two ways: a histological stain examination and an evaluation of the metabolic function of cells (59).

5.7.1 ADENOSINE TRIPHOSPHATE ASSAY
Adenosine triphosphate (ATP) is an adenine nucleotide containing three phosphate groups esterified to the sugar moiety. It plays a crucial role in metabolism (72). ATP assay detects cellular adenosine triphosphate in active cells (73). In study of Pohjala et al. ATP assay is carried out using the luminescent luciferase reaction. ATP assay showed to have an excellent signal quality, but a small number of cells could be used (74).

5.7.2 ADHERENCE
Pu et al. (2006) studied the length of time until adipocytes became adherent to a surface of a culture plate. The length of time until adherence would be an indication for cell growth. A slower cell growth may be a sign of loss of function of the adipocytes (49). Before the procedure, washing of the adipocytes is important, because adherence can be influenced by the amount of fibrin content in the extracellular matrix. Fibrin ameliorates adherence (16).

5.7.3 BLOOD SAMPLING
In mice retro-orbital blood sampling in ethylene diaminetetra-acetic acid (EDTA) can be performed after tissue grafting. Samples are centrifuged at 9000 rpm for 5 minutes to isolate plasma and are then frozen at -20°C for enzyme-linked immunosorbent assay (ELISA). By using ELISA, inflammation parameters can be quantified. The inflammation parameters used are mouse interleukin-6 and monocyte chemotactic protein-1 (MCP-1). Increased levels of MCP-1 could be an indication of macrophage infiltration inside the graft and in long term tissue necrosis (60).

5.7.4 COMBINED CELL STAINING
To distinguish adipocytes from floating lipid droplets, cells are stained with Nile Red and Hoechst 33342. Nile Red staining is used to detect the presence of intracellular lipid-filled droplets (table 9). Cell are fixed, washed and stained. Analysis is performed using a fluorescence microscope (31).
To distinguish viable cells from dead cells, cells are stained with Hoechst 33342 and propidium iodide. Hoechst 3342 stains the nuclei in all cells (live and dead). Propidium iodide only stains the nuclei in dead cells (table 10).

Another option is a combination of fluorescein diacetate and propidium iodide, which is a test to control cell membrane integrity. It distinguishes living cells from non-living cells. Using the fluorescence microscope, living cells turn green when the intracellular absorbed non-fluorescent fluorescein diacetate is converted to fluorescent by a nonspecific esterase. Dead cells stain red when propidium acetate penetrates the cells through a damaged membrane and incorporates into the nuclei (48). Cell staining is a simple method but it is indirect. Cell viability cannot be quantified and can only be compared (59).

Table 8 (73): Cell morphometric assay with Nile Red and Hoechst 33342 staining.

<table>
<thead>
<tr>
<th></th>
<th>Nile Red</th>
<th>Hoechst 33342</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipocyte</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Non-adipocyte</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Floating lipid droplets</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Detected non-adipocytes could be adipose-derived stem/stromal cells and WBC.

Table 9 (73): Cell morphometric assay with Propidium iodide and Hoechst 33342 staining.

<table>
<thead>
<tr>
<th></th>
<th>Hoechst 33342</th>
<th>Propidium iodide</th>
<th>Including lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viable adipocyte</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Dead adipocyte</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Viable cell other than adipocyte</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dead cell other than adipocyte</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

CAVE! Viability does not only depend on an intact membrane, but also intact cellular functions must be evaluated by assessing metabolic functions.

5.7.5 CULTURE

The total number of viable adipocytes is counted with a hemocytometer under a phase-contrast microscope with 100 X magnification after culture (16, 49).

CAVE! Human adipocytes do not proliferate or adhere well to a culture. So cell integrity and viability cannot be tested properly with proliferation tests. Because of the little cell turnover of fat cells, normal proliferation tests such as culture are ineffective. Culture is not preferred as a method to test viability or function of adipocytes (3, 65).
5.7.6 EXTRACELLULAR GPDH ASSESSMENT
Glycerol-3-phosphate-dehydrogenase (G3PDH) is an intracellular enzyme in normal circumstances. The extracellular amount of enzyme present in solution of cells is proportional to the amount of cell destruction. The G3PDH activity is measured by the reduction of NADH to NAD+. The amount of NADH and NAD+ is measured by use of a spectrometer at 340 nm. This test measures the integrity of the cell membrane. This is a rapid and efficient method, quantifying the amount of damage without relying on individual counting. The G3PDH assay is superior to the XTT assay because of its strict specificity for adipocytes (47, 48).

Xie et al. (2010) emphasize that this assay is too complicated to be used clinically and can only be used in experimental settings (59).

5.7.7 FLOW CYTOMETRY
To determine the presence of non-adipocytes, multicolor flow cytometry can be used. The following antibodies conjugated to fluorochromes can be used: anti-CD31, anti-CD34 and anti-CD45 (73).

Flow cytometry fulfills many criteria of the ideal endpoint, but it can be problematic when dealing with fragile and adherent adipocytes. Flow cytometry is also labor intensive and expensive (71). The ideal endpoint would result in

1. Data instead of indirect markers for adipocytes viability.
2. Reliable quantitative and qualitative data and results
3. Differentiation between living and dead cells
4. The ability to identify a specific cell population (71).

5.7.8 GLUCOSE TRANSPORTATION TEST
The glucose transport test is a method to evaluate the viability of fat grafts. Viable adipocytes normally transport glucose into the cell. Transported glucose over the cell membrane is quantized, which reflects the metabolic activity of adipocytes. Fat cells are incubated with DMEM containing glucose for one hour. After incubating, the glucose consistency of DMEM is measured (24, 59). Xie et al. (2010) show similar findings on viability in the MTT assay and glucose transport test. But since the glucose transport test measures the viability of adipose tissue, it is not specific for adipocytes. The glucose transport test is simple and can be performed by an analyzer (59).

5.7.9 HISTOLOGICAL EXAMINATION
After fat grafting, fat grafts can be dissected. For histological examination this dissected tissue is fixed in 4 % formaldehyde, embedded in paraffin and processed for hematoxylin and eosin staining. Using the microscope, a pathologist examines the histological slides. Areas occupied by vacuolar, fibrotic, inflammatory and normal fat tissue are measured. The result is expressed as percentage (18, 47, 64). Simple hematoxylin and eosin staining outlines the anatomy of the fat cell, but it does not indicate the viability of the stained cells (32, 60).
5.7.10 IMMUNOSTAINING FOR PPARγ

PPARγ (Peroxisome Proliferator-Activated Receptors) is a nuclear transcription factor that is activated by ligands and heterodimerizes with retinoid receptors and binds to peroxisome proliferator response elements in the promoter regions of target genes (72). Fixed cells are incubated with PPARγ rabbit antibody and anti-rabbit antibody (31).

5.7.11 LEPTIN ASSAY

Leptin is a peptide hormone secreted from white adipocytes. Leptin serves as a feedback signal from fat cells to the central nervous system in regulation of food intake, energy balance, and fat storage (72). The secreted leptin is estimated using leptin ELISA assay kits and a 72 hour conditioned medium. Values are expressed as pictograms of leptin per milliliter of the conditioned medium (22).

5.7.12 LIQUID AND OIL MEASUREMENT AFTER CENTRIFUGATION

After centrifugation the release of oil and liquid is measured. Quantification of the volume of oil release can be made. Oil release results from adipocyte cell death. Quantification of the volume of the extracted liquid gives an idea of the tissue compactness (60).

5.7.13 MTT TEST/XTT TEST

Measurement of cell viability is performed using a colometric test with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT test or XTT test. These 2 tests measure intact mitochondrial function as a function of cellular activity by reduction of water-soluble tetrazolium salts into an insoluble purple formazan salt. Formazan salt absorbs light at 570 nm. Values of cell viability are given as a percentage of the optical density of fresh cells. The XTT test purely reflects mitochondrial activity, whereas the MTT test also reflects some intracellular activity. Dead cells can be used as a control. Because the MTT test is a dissolving procedure, it may impair reproducibility. Normally fat cells are rich in mitochondria. The MTT test determines if fat cells have actively producing mitochondria, but it doesn’t test other activities of the fat cells. So this assay provides a good correlation between the number of viable cells and the resulting formazan absorbance values, but it is not specific for adipocytes. CAVE! A lot of the cellular components can be damaged, but that doesn’t mean that the mitochondria are damaged too (29, 42, 48, 73).

5.7.14 OIL RED O STAINING AND QUANTIFICATION OF LIPID ACCUMULATION

Monolayer cultures of cells are analyzed under a phase-contrast microscope at X 400. Adipocytes are fixed in 10% neutral-buffered formalin, stained with a 60% Oil red O stock solution in distilled water. To remove the background Oil red O adipocytes are washed with distilled water. Then cells are treated with 100% isopropanol. Spectrophotometric absorbance of the resulting solution is measured at 540 nm. Oil red O stains triglycerides and lipids and concentrates in lipid droplets. Mature adipocytes are characterized by many lipid droplets. This Oil red O staining can be identified by his bright red color (22).
5.7.15 RT-PCR
RNA is isolated from cells. Total RNA is reverse-transcribed. Synthesized first-strand cDNA is used as a template for PCR. The primer is an adipogenic-related gene, such as PPARγ (peroxisome proliferating antigen receptor gamma). PPARγ is a nuclear hormone receptor that regulates adipogenesis. PCR products are separated in an agarose gel containing ethidium bromide. PCR product intensities are visualized using a UV transilluminator. The expression of adipogenic-related genes, such as PPARγ, determines the presence of functional adipocytes. This is based on gene expression levels. Adipogenic-related genes are only expressed after differentiation in adipocytes. This makes sure that stem cells or other cell lines are not detected and cannot disrupt the result of the RT-PCR (22).

5.7.16 SUPRAVITAL DYE STAINING
Viability is measured with brilliant cresyl supravital dye staining which is a basic dye that stains the deoxyribonucleic acid of the viable cells (16).

5.7.17 TRYPAN BLUE VITAL STAINING
The trypan blue vital staining is a procedure for detecting viable cells. Cells are incubated with trypan blue and flavin adenine dinucleotide (FAD)/ethidium bromide (EB). A noticeable uptake of color is defined as stained. However, this technique is difficult because of the scant cytoplasm of mature adipocytes. CAVE! A fat cell without mitochondrial activity might look normal in a histological stain (47, 64, 73).

Manual cell counting using trypan blue creates a high degree of variability between observers and nonspecificity toward certain cell lines. Furthermore, this method is very labor-intensive (71).

So Lee et all. (2012) promote the use of an automated cell counter. An automated cell counter machine is similar to a hemocytometer in that it identifies cells within a certain area and calculates cell counts. But in contrast to a hemocytometer, it uses a software algorithm to count the cells. This minimizes human error. In addition, an automated cell counter machine can read both visual lights and fluorescent channels, which makes it possible to identify specific cell populations. This novel method utilizing automated cell counters accurately identifies the viable adipocyte population. Many samples can be compared in a short period of time, which decreases labor intensity and the warm ischemic time of samples (71).

In addition, by using of carboxymethyl cellulose and formalin in the preparation process, variability and stability of cell count decreases. Carboxymethyl cellulose is a natural anionic polysaccharide used as an additive in food, adhesives, lubricants and pharmaceuticals. Carboxymethyl cellulose is viscous, which allows adipocytes to remain in a homogenous cell suspension. This reduces sampling error. Formalin is used for its cross-linking abilities as a tissue fixative (71).
5.7.18 TUNEL ASSAYS
TUNEL assays are used to detect the presence of fragmented DNA as an indication of cell death/apoptosis. This test is an assessment of the presence of dead cells in a fat graft (70).

5.7.19 WEIGHT AND VOLUME
Grafts can be dissected and weight and volume can be compared (figure 7) (17, 29).

![Figure 7](image_url)

Figure 7 (41): Dissection of fat grafts twelve weeks after transplantation in mice.

Attention must be paid to tests that are not specific for adipocytes and to tests in which other cellular components can contribute to the observed result. Results of these tests should be interpreted very carefully. Also keep in mind that a positive test result of a test that only measures the function of a particular cellular component (such as mitochondria) does not mean that other cellular components are intact and/or functioning. Also reproduction of the results of the test is necessary, so results can be objectively compared. Interobserver-variability, labor-intensity, specificity for certain cell lines, complexity and ease of use are important properties of a test.

Staining can only detect intact adipocytes and fat cells without any cellular activity may look normal in histological stains. Adenosine triphosphate test is not specific for adipocytes. Also the glucose transportation test is not adipocyte specific. On the contrary, G3PDH is an adipocyte specific method to test viability by detecting the integrity of the cell membrane. MTT/XTT tests mitochondrial/intracellular activity but is not adipocyte specific.

In clinical studies a combination of staining, histological examination, XTT/MTT and G3PDH can be used. The glucose transport test can also be useful. But the ideal method with the perfect endpoint has not been found yet.
5. Discussion

Autologous fat grafting is a technique with growing interest in medicine. Although numerous studies have been carried out, there is no consensus or agreement on what the best technique/protocol is to obtain the results as wanted. In literature a large numbers of case reports can be found that show clinical success with fat grafting. Unfortunately few objective data have been found to explain these successes. In general, failures are not likely to be published. Publication bias is excessive. So despite more than a century of reports in medical literature, the efficacy and longevity of autologous fat transplantation is still controversial.

The main problem of the disappointing results of autologous fat transplantation is resorption of fat. Although resorption is the crucial problem of lipofilling, no consensus is found about the underlying mechanism of resorption. There are two theories to explain resorption: the cell survival theory and the theory of cell replacement by phagocytes. But still no obvious answer is found and the crucial question remains: what is happening in a fat graft when fat is injected in the body? So, more studies will have to be done to outline the processes that occur in transplanted fat grafts.

If fat grafting is due to the introduction of fat cells that will die and be replaced by phagocytes, is there then a need for high viability in fat grafts? All clinical studies about measuring viability in fat grafts would not be necessary. But no studies have been done in which dead cells or other cell lines, beside fat cells, were used for lipofilling. The results of such a study would be very instructive.

Because of resorption, results of autologous fat grafting are disappointing. Cryopreservation of fat creates an opportunity to obtain the outcome as wanted without additional liposuction.

Literature on cryopreservation of fat cells is limited in number. Also notice the small amount of participants in these studies, which makes it difficult to interpret the results. Furthermore in the clinical practice and in the literature many inconsistencies regarding cryopreservation of fat can be found. Controlled studies are infrequent. Results differ and contradict each other. Fat viability after cryopreservation is variable and may be questionable. A lot of unsolved issues can be noticed (3).

It’s also difficult to compare studies of fat grafting with cryopreservation because in these studies different techniques for fat grafting and cryopreservation are used. Different instruments are used. One step of the process was left out or another step was added, which may influence the outcome. The different methods to measure viability also result in different outcomes.

In this review no attention has been made to the presence of adipose-derived stem cells and preadipocytes in the fat graft. These cells have a self-renewing capacity and the ability to produce daughter cells. Fatty tissue has the highest percentage of adult stem cells compared to any other tissue in the body (27). Preadipocytes and ASCs are more resistant to trauma and therefore more likely to
survive harvesting and cryopreservation than fat cells. Introduction of ASCs causes an enhanced production of VEGF. The long-term survival of fat grafts is likely to be enhanced by improving their vascularization within the recipient. Observations showed that ASCs can survive much longer without nutrition or in an environment with low oxygen content (70).

Stem cells enhance the viability and effectiveness of fat grafts when mixed with cryopreserved tissue. They secrete multiple bioactive angiogenic and anti-apoptotic factors (68). Stem cells produce VEGF, FGF, TGF-beta and probably a number of factors that are not yet known. VEGF is produced in hypoxia and increases angiogenesis. TGF-beta increases the proliferation capacity of stem cells and therefore reduces cystic and fibrotic transformation. FGF promotes pre-adipocyte migration and neovascularization. Insulin and IGF-1 also showed evidence of being involved in adipocyte differentiation.

Ohnishi et al. (2012) showed that stem cells can be frozen and stocked for a very long period of time (75). Therefore a major effect of fat transplantation is probably caused by the survival of stem cells in the stromal cell fraction.
In the following paragraphs I will review the results of this paper. The results are written down in chronological order as in execution of a fat graft with cryopreservation.

A clear preference for a particular donor site has not been established, although the choice of donor site seems to be important with respect to the outcome. Most studies have shown no difference in viability between fat grafts harvested from different donor sites (1, 13, 39-41). But because of increased ASCs, higher amount of processed lipoaspirate cells, easy accessibility and safety, lower abdomen and medial thighs are most commonly chosen as the preferred donor site, compared to other regions (8, 9, 27, 36, 37). But also take into account the wishes of the patient and the preference and experience of the surgeon.

Depending on the volume of fat graft needed, epidural, local and general anesthetics or combinations can be used. In general tumescent technique is not used in the liposuction procedure.

In the existing literature, no significant differences were found between the different harvest techniques. Because of the ease of the technique, manual syringe liposuction is the best option. Most authors also conclude that large cannulas should be preferred for harvest.

Evidence-based literature did not clear out one ideal processing technique. But it is obvious that centrifugation is the most commonly used technique.

Concerning cryopreservation, the majority of the authors suggest slow cooling and fast thawing in order to minimize damage to adipocytes.

The process of slow cooling includes several steps. The adipose tissue is put in a cryovial, which is immersed in a methanol bath. The temperature of the bath will be reduced with 1-2°C/ minute from room temperature to -30°C. Once the adipose tissue reaches the ending temperature, it is held at -30°C for 10 minutes. Then the adipose tissue is transferred into liquid nitrogen (-196°C). Below -85°C is considered to be the optimal temperature for storage.

The process of fast thawing consists of two steps. First adipose tissue is adapted to room temperature for 2 minutes. These will enable the liquid nitrogen to vapor out of the cryovial. Then the cryovial is dropped into a stirred 40°C water bath until the adipose tissue is thawed.

There is no consistency in results of cryopreservation comparing storage temperature. Most commonly used is cryopreservation in liquid nitrogen, because temperature below -85°C is considered to stop metabolism.

Storage time should not influence adipocytes viability. But a lot more clinical studies are needed to make evidence-based conclusions, based on literature. In four of the selected clinical studies storage time was twenty minutes. This time was considered to be equivalent to long-term-cryopreservation.
five studies two weeks was used as storage time, but no motivation was mentioned for this amount of time.

Cryopreservation should decrease adipocytes viability, but by adding a cryoprotectant, viability increases. When using a cryoprotectant, a preference is made for 0, 5 M (3, 3 %) DMSO and 0, 2 M (7, 6 %) trehalose. In general membrane-permeating protectants seem to be beneficial. But because of the toxicity of DMSO, there is a need to develop DMSO-free methods.

Reinjection of fat should involve washing with sterile saline to remove oily components. Reinjection is performed in multiple small-volume sessions with small, blunt needles to increase vascularization of the fat graft which decreases the reabsorption rate.

Clinical studies show a positive effect of the use of anabolics on the survival of fat grafts. More clinical studies are needed to confirm this data. The use of an anabolic agent could decrease fat reabsorption and thus the need for repeated liposuction procedures. Investigations should be able to identify which anabolics can be used and how it should be integrated in the process of lipofilling. This could boost the possibilities of fat grafts.

To determine the effect of the cryopreservation or parts of the process of liposuction-lipofilling, viability of the fat cells is measured. Attention must be paid to tests that are not specific for adipocytes, and to tests in which other cellular components can contribute to the observed result. Results of these tests should be interpreted very carefully. Also keep in mind that a positive test result of a test, that only measures the function of a particular cellular component (such as mitochondria), does not mean that other cellular components are intact and/or functioning. Also reproduction of the results of the test is necessary, so results can be objectively compared. Interobserver-variability, labor-intensity, specificity for certain cell lines, complexity and ease of use are important properties of a test.

Staining can only detect intact adipocytes and fat cells without any cellular activity may look normal in histological stains. Adenosine triphosphate test, the glucose transportation test and MTT/XTT are not specific for adipocytes. On the contrary, G3PDH is an adipocyte specific method to test viability by detecting the integrity of the cell membrane. MTT and XTT tests respectively mitochondrial and intracellular activity but these are not adipocyte specific.

In clinical studies a combination of staining, histological examination, XTT/MTT and G3PDH can be used. The glucose transport test can also be useful. But the ideal method with the perfect endpoint has not been found yet.
To conclude, I would like to propose a protocol that can be used in an experimental study, based on evidence based medicine and based on the practice and equipment of the laboratory of the University Hospital in Ghent.

1. Preparation of patient and explanation of procedure with informed-consent. People with major systemic metabolic diseases or lipid disorders are excluded.

2. Liposuction using general anesthetics with in situ infiltration of local anesthetic with or without adrenaline (0.1% lidocaine, 0.001 % epinephrine), preferable donor site is abdomen or medial thighs.

3. Harvest with blunt-tipped cannula (> 3 mm) connected to a 10 cc gentle manual syringe aspiration at low vacuum pressure.

4. Centrifugation for three minutes at 3000 rpm in 10 cc tubes. Top supernatant, bottom blood cells and debris are removed.

5. Transport immediately to laboratory at room temperature.

6. Cell count and viability measurement using trypan blue staining. MTT (XTT) and G3PDH. Viability should be over 90 % to ensure the cells to be healthy enough for freezing. Start viability is written down.

7. Addition of cryoprotective agent by mixing into a vial (preference for 3, 3 % DMSO and 7, 6 % trehalose, but this is not used in the University Hospital of Ghent so normal saline and 10 % HES is preferred). One cc of adipose aspirate is mixed with one cc of cryoprotectant in a three cc vial.

8. Slow freezing: 1°C/ minute temperature drop till a temperature of -30°C is reached. Then fat is held for 10 minutes at -30°C. Subsequently fat is placed in liquid nitrogen in -196°C. In Ghent a minicooler is used for this process. Liquid nitrogen conservation is not practical, because of the small amount of volume of vials, high costs and safety reasons. A freezer with a temperature below -85°C can be used.

9. Recording of positions of vials in computer.

10. Cryopreservation for 20 minutes.

11. Fast thawing at room temperature for two minutes. Then the vials are placed in a 37°C water bath which allows fat cells to heat 35°C each minute.

12. Washing with sterile saline to remove oil.

13. Cell count and viability measurement using trypan blue staining. MTT (XTT) and G3PDH.


15. Fat graft is dissected out: histological examination is performed, weight and volume is measured.

When this study would be carried out, results will show if this protocol is useful for a later clinical use in the context of lipofilling.
The main purpose of this paper was to determine if fat cells can be frozen and if this can be done, to look for the best procedure of fat cryopreservation. The best procedure based on evidence based medicine is mentioned above. Below I would like to discuss the effects of cryopreservation on the fat cells, comparing them to fresh fat cells (see table 7 and 8.5 table of comparative studies of fresh fat and frozen fat).

When studying the seventeen selected clinical studies, five clinical studies showed no significant difference between frozen and fresh fat. Four studies showed that frozen fat was less viable than fresh fat and two studies even indicated that fat was dead because of the freezing. But most studies show no significant difference in appearance of the receptor site.

When studying fat viability before and after freezing, fat viability decreases by freezing in all clinical studies where viability was mentioned, except for one (22). Li et all. (2012) show that the lower the freezing temperature and the shorter the period of freezing time, the higher the viability of fat cells (12). Son et all. (2010) partially agree with this. When fat cells are frozen for a long period, fat viability decreases. But they noticed lower viability of fat cells when fat cells were frozen at -70°C compared to those frozen at -15°C (48). Atik et all. (2006) concluded that freezing in liquid nitrogen is the best way to cryopreserve fat cells (18). MacRae (2004) also prefer cryopreservation in liquid nitrogen instead of cryopreservation at -20°C, because they observed lower viability in the last group (42). Pu et all. (2004) and Moscatello (2005) compared viability of fat cells frozen with or without CPA. They both concluded that CPA increase viability (32, 47) (table 7).

Lidagoster et all. (2000) performed a study with 96 mice. They compared histological sections of normal fat, fat after lipofilling, fat frozen at -16°C after fat transplant and fat stored at 1°C after fat transplant. Lidagoster et all. found a significant decrease in viable adipocytes and an increase in signs of inflammation and necrosis in the histological sections of the fat graft injected by frozen fat compared to the sections of the fat grafts injected with fresh fat (66).

Wolter et all. (2005) found a significant decrease of metabolic activity after freezing. 92, 7% of metabolic activity was lost by freezing. By adding a CPA a preservation of up to 54% of baseline activity was observed. Wolter et all. also concluded that lower freezing temperature (-80°C <= -20 °C) results in more cell destruction, but yields higher viability of surviving cells (65).

Pu et al. (2006) compared fresh adipocytes and adipocytes cryopreserved for 20 minutes with 0, 5 M DMSO and 0, 2 M trehalose. Storage temperature was not mentioned. A nonsignificant difference in cell count and in culture was observed between fresh fat and fat frozen with CPA (49).

Another study of Pu et al. (2006) showed a significant higher volume of the fat graft in fresh fat versus cryopreserved fat (with slow cooling and fat thawing for 20 minutes at -196°C). All results were less
satisfactory in the cryopreserved group. These results were based on the gross appearance of the fat graft, the volume and weight of the graft and histological examination (50).

Butterwick et all. (2006) performed a side-by-side two-hand comparison pilot study. Lipofilling was done in both hands. One hand was injected with fresh fat, one hand with frozen fat. They found a superior result in the hand that was injected with frozen fat, compared to the hand that was injected with fresh fat at 1, 3, 5 month follow-up. Both hands were evaluated by a physician and scored on aesthetic appearance, vein prominence and depth of metacarpal space. Butterwick et all. (2006) also observed less side effects in the hand injected with frozen fat. Fat was frozen for two weeks at -40°C (33).

Another important study was performed by Atik et all. (2006). They compared fat originating from four groups: fat that was immediately transplanted in the scalp of mice, dry-frozen fat, fat frozen immersed in glycerol and fat frozen in liquid nitrogen by histological examination and MTT. The frozen fat was cryopreserved at -35°C for six months. A nonsignificant difference in viability between fresh fat and fat frozen in liquid nitrogen was observed. Viability of dry-frozen fat was the same as the viability of dead control cells (18).

Cui et al. (2007) compared three different groups: one group of fresh fat which served as a control group, one group of fat cryopreserved without CPA and one group cryopreserved with CPA (0, 5 M DMSO and 0, 2 M trehalose). Cryopreservation was performed using slow cooling and fast thawing. Fat cells were frozen for one hour at -196°C in liquid nitrogen. Outcome was measured using weight and volume of the graft, histological examination and cell count. Cui et al concluded that cell count and the volume of fresh adipocytes was significant higher then count and volume of cryopreserved fat. A nonsignificant difference was found in histology between fresh and frozen fat with CPA (34).

Kim et al. (2007) compared frozen fat and fresh fat by Oil red O staining, ELISA of leptin secretion, PCR of adipogenic-related genes (adipogenic function). A nonsignificant difference in fresh and freeze-thawed adipocytes was found (22).

Son et all. (2010) froze fat at -15°C and -70 °C for 1,3,7,14,28 or 56 days. Viability was measured using staining with fluorescein diacetate and propidium iodide by fluorescence microscopy, G3PDH activity and XTT reduction assay. A significant decrease of viability, G3PDH activity and XTT measurement was observed between fresh and frozen adipocytes (48).

So while Cui et al. (2007) and Kim et al. (2007) show a nonsignificant difference in fat cell histology between fresh and frozen fat cells, Lidagoster et all. (2000), Wolter et all. (2005), Pu et al. (2006) and Atik et all. (2006) conclude that there is more necrosis in grafts composed from frozen adipocytes.
Comparing metabolic activity, Wolter et al. (2005) and Son et al. (2010) conclude that metabolic activity of fat cells is decreased by freezing. Atik et al. (2006) found a nonsignificant difference in metabolic activity between fresh fat and fat frozen in liquid nitrogen.

But most studies show a nonsignificant difference in appearance of the receptor site. It is not clear if this result maintains or if the fat graft with frozen fat loses volume by absorption over time. If appearance of the receptor site does not differ between frozen and fresh fat, no more studies have to been done. This is the main endpoint for patients and doctors.

So I can state that the effects of cryopreservation on fat cells should be further investigated. But probably a portion of the fat cells dies and a portion of the fat cells loses metabolic activity because of cryopreservation. It is certain that viability decreases by cryopreservation. Addition of a cryoprotective agent could decrease this lose of viability.

It is sure that liposuction-cryopreservation-lipofilling is a promising technique with a lot of opportunities. There is a widespread belief that because the ease of the lipofilling technique, it can be used worldwide. In the future, liposuction followed by cryopreservation and lipofilling would enable us to reach high success rates in the clinical results of patients. By using this technique even large volume of fat grafts should be able to be reached. This promising technique could be used in broad indications and can help people all over the world. But first more clinical studies are needed to clear the issues mentioned in this paper.

6. References
72. Medical Subject Headings. the NLM controlled vocabulary thesaurus used for indexing articles for PubMed. MeSH.


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8.3 LIST OF ABBREVIATIONS

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<tbody>
<tr>
<td>ASC</td>
<td>Adipose derived Stromal/Stem Cell</td>
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<tr>
<td>bFGF</td>
<td>Basic Fibroblast Growth Factor</td>
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<tr>
<td>BMI</td>
<td>Body Mass Index</td>
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<tr>
<td>CAVE</td>
<td>Caution</td>
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<tr>
<td>cc</td>
<td>Cubic Centimeter</td>
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<tr>
<td>CPA</td>
<td>Cryoprotective Agent</td>
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<tr>
<td>(c) DNA</td>
<td>(Complementary) Deoxyribonucleic Acid</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<tr>
<td>EB</td>
<td>Ethidium Bromide</td>
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<tr>
<td>EDTA</td>
<td>Ethylene Diaminetetra-acetic Acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
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<tr>
<td>FAD</td>
<td>Flavine Adenine Dinucleotide</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<td>FGF</td>
<td>Fibroblast Growth Factor</td>
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<td>g</td>
<td>Gravitational force</td>
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<tr>
<td>G</td>
<td>Gauge</td>
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<tr>
<td>G3PDH</td>
<td>Glyceraldehyde 3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>H &amp; E</td>
<td>Hematoxylin and Eosin stain</td>
</tr>
<tr>
<td>HES</td>
<td>Hydroxyethyl Starch</td>
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<tr>
<td>IGF-1</td>
<td>Insulin-like Growth Factor</td>
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<td>Interleukin</td>
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<td>Monocyte Chemoattractant Protein-1</td>
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<td>MeSH</td>
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<tr>
<td>mm Hg</td>
<td>Millimeter of mercury</td>
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<tr>
<td>MTT</td>
<td>Methylthiazol Tetrazolium: 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide</td>
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<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
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<td>Nicotinamide Adenine Dinucleotide (Hydrogen)</td>
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<td>Nerve Growth Factor</td>
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<tr>
<td>PCR</td>
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<td>PPARY</td>
<td>Peroxisome Proliferator-Activated Receptor Gamma</td>
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<tr>
<td>PVP</td>
<td>Polyvinylpyridone</td>
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<tr>
<td>(m) RNA</td>
<td>(Messenger) Ribonucleic Acid</td>
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<tr>
<td>rpm</td>
<td>Revolutions Per Minute</td>
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<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor Beta</td>
</tr>
<tr>
<td>TRIP-database</td>
<td>Turning Research Into Practice-database</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP Nick End Labeling</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>XTT</td>
<td>Sodium 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt</td>
</tr>
</tbody>
</table>
8.4 GLOSSARY OF TERMS

**Adipocyte**  
Cell in the body that store fats. (MeSH)

**Centrifugation**  
Process of using a rotating machine to generate centrifugal force to separate substances of different densities, remove moisture, or simulate gravitational effects. (MeSH)

**Cryopreservation**  
Preservation of cells, tissues, organs, or embryos by freezing. (MeSH)

**Cryoprotectant**  
Substances that provide protection against the harmful effects of freezing temperatures. (MeSH)

**Freeze-drying**  
Freezing the material and then reducing the surrounding pressure to allow the frozen water in the material to sublimate directly from the solid phase to the gas phase. ( = lyophilisation, lyophilization, cryodesiccation) (Wikipedia)

**Hypesthesia**  
Absent or reduced sensitivity to cutaneous stimulation. (MeSH)

**Lipofilling**  
The surgical transfer of autologous fat removed by liposuction to areas of the body that need filling. (Wikipedia)

**Poland’s syndrome**  
A syndrome which is characterized by symbrachydactyly and aplasia of the sternal head of pectoralis major. (MeSH)

**Romberg’s disease**  
A syndrome characterized by slowly progressive unilateral atrophy of facial subcutaneous fat, muscle tissue, skin, cartilage, and bone. The condition typically progresses over a period of 2-10 years and then stabilizes. ( = facial hemiatrophy) (MeSH)

**Tumescent technique**  
A technique that provides local anesthesia to large volumes of subcutaneous fat and thus permits liposuction by local anesthesia. (Wikipedia)
### 8.5 TABLE OF COMPARATIVE STUDIES OF FRESH AND FROZEN FAT

<table>
<thead>
<tr>
<th>Title</th>
<th>Author</th>
<th>Publication year</th>
<th>Number of participants</th>
<th>Compared groups</th>
<th>Cooling</th>
<th>Freezing time</th>
<th>Freezing temperature</th>
<th>Thawing</th>
<th>Evaluation</th>
<th>Conclusion of authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryopreservation of human adipose tissue</td>
<td>Cui</td>
<td>2007</td>
<td>36 persons, 45 mice</td>
<td>1. Fresh control. 2. Cryopreservation without CPA. 3. cryopreservation with CPA: 0.5 M DMSO + 0.2 M trehalose with 3 X centrifugation 100 g to remove CPA (15 mice each group)</td>
<td>Slow cooling (first 22°C =&gt; -30°C (1°C/min), 10 minutes at -30°C, transferred in -196°C (below -85°C = optimal)</td>
<td>1 hour</td>
<td>Freezing at -196°C</td>
<td>Fast thawing: 2 minutes at room temperature, 5 minutes in 40°C water bath (35°C/minute)</td>
<td>Weight, volume, histological examination, cell count</td>
<td>SIGNIFICANT HIGHER CELL COUNT AND VOLUME IN FRESH ADIPOCYTES / NONSIGNIFICANT DIFFERENCE IN HISTOLOGY BETWEEN FRESH AND FROZEN WITH CPA</td>
</tr>
<tr>
<td>Cryopreserved human adipogenic-differentiated pre-adipocytes: a potential new source for adipose tissue regeneration</td>
<td>Kim</td>
<td>2007</td>
<td></td>
<td>1. Freeze-thawed fat. 2. Fresh fat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Oil red O staining, ELISA of leptin secretion, PCR of adipogenic-related genes (adipogenic function)</td>
<td>NONSIGNIFICANT DIFFERENCE IN FRESH AND FREEZE-THAWED ADIPOCYTES</td>
</tr>
<tr>
<td>Adipose aspirates as a source for human processed liposarpirate cells after optimal cryopreservation</td>
<td>Pu</td>
<td>2006</td>
<td>12 persons</td>
<td>1. Fresh adipocytes: 1cc + 1cc normal saline at room T 2. Cryoprotectant (DMSO 0.5M or 3.3% and trehalose 0.2M or 7.6%) 1cc + 1cc adipocytes in 3 cc vial =&gt; room T 10 min =&gt; methanol bath</td>
<td>Slow cooling (first 22°C =&gt; -30°C (1°C/min), 10 minutes at -30°C, transferred in -196°C (below -85°C = optimal)</td>
<td>20 minutes</td>
<td></td>
<td>Fast thawing: 2 minutes at room temperature, 5 minutes in 37°C water bath</td>
<td>Culture: length of time until cells become adherent to culture plate and number of cells after a 2-week-culture</td>
<td>NONSIGNIFICANT DIFFERENCE IN CELL COUNT IN CULTURE BETWEEN FRESH AND FROZEN WITH CPA</td>
</tr>
<tr>
<td>Fat transplantation using fresh versus frozen fat: a side-by-side two-hand comparison pilot study</td>
<td>Butterwick</td>
<td>2006</td>
<td>10 persons</td>
<td>1. Fat augmentation of 10 cc of fresh fat 2. Fat augmentation of 10 cc of frozen fat</td>
<td>Rapidly frozen at -40°C in an upright freezer</td>
<td>Maximum 2 weeks</td>
<td>Freezing at -40°C</td>
<td>Slow thawing over a couple of hours</td>
<td>Aesthetics, vein prominence, depth of metacarpal space</td>
<td>SIGNIFICANT HIGHER RATE OF AESTHETICS, VEIN PROMINENCE AND DEPTH OF METACARPAL SPACE WHEN INJECTED WITH FROZEN FAT / LESS SIDE EFFECTS WHEN FROZEN</td>
</tr>
<tr>
<td>Study Title</td>
<td>Author</td>
<td>Year</td>
<td>Subjects</td>
<td>Methods</td>
<td>Results</td>
<td></td>
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<tr>
<td>The fate of cryopreserved adipose aspirates after in vivo transplantation</td>
<td>Pu</td>
<td>2006</td>
<td>1 woman, 60 mice</td>
<td>1. Fresh control group 2. Simple cryopreservation group 3. Optimal cryopreservation group: with CPA (DMSO 0.5 M and trehalose 0.2 M) 4. Slow cooling (first 22°C =&gt; -30°C (1°C/min), 10 minutes at -30°C, transferred in -196°C (below -85°C = optimal)) 5. Freezing at -196°C 6. Fast thawing: 2 minutes at room temperature, dropped into a stirred 37°C water bath, remove of CPA by dilutions and centrifugation</td>
<td>Gross appearance of fat graft over their posterior scalps, volume, weight, histological examination</td>
<td>SIGNIFICANT HIGHER VOLUME IN FRESH FAT VERSUS CRYOPRESERVED / ALL RESULTS ARE LESS SATISFACTORY IN CRYOPRESERVED GROUP THAN IN FRESH GROUP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Comparison of autologous fat transfer in fresh, refrigerated, and frozen specimens: an animal model</td>
<td>Lida gost</td>
<td>2000</td>
<td>96 mice</td>
<td>1. Normal mice 2. Immediate fat transplant 3. Frozen at -16°C 4. Fat stored at 1°C</td>
<td>1 or 2 weeks</td>
<td>Freezing at -16°C and 1°C</td>
<td>Histological examination</td>
<td>SIGNIFICANT DECREASE IN Viable adipocytes and increase in signs of inflammation and necrosis in frozen fat compared to fresh fat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryopreservation of mature human adipocytes: in vitro measurement of viability</td>
<td>Wol ter</td>
<td>2005</td>
<td>24 persons</td>
<td>1. DMEM 2. DMEM + 10% HES 3. DMEM + 10% glycerol 4. DMEM + 10% glycerol + 5% dextran + 5% PVP (polyvinylpyridone) 5. Slow cooling to -20°C/-80°C (1°C/min)</td>
<td>2,7,14,30 days</td>
<td>Freezing at -20°C and -80°C</td>
<td>Thawing in a water bath at 37°C</td>
<td>Trypan blue staining, MTT, G3PDH</td>
<td>SIGNIFICANT DECREASE OF METABOLIC ACTIVITY WHEN FROZEN</td>
<td></td>
</tr>
<tr>
<td>Viability of fat cells over time after syringe suction lipectomy: the effects of cryopreservation</td>
<td>Son</td>
<td>2010</td>
<td>16 persons</td>
<td>Viability measurement after 1, 3, 7, 14, 28, 56 days of freezing</td>
<td>Cooling by placing in a deep freezer</td>
<td>1,3,7,14,28,56 days</td>
<td>Freezing at -15°C and -70°C</td>
<td>Thawing at room temperature</td>
<td>Staining with fluorescein diacetate and propidium iodide by fluorescence microscopy, G3PDH activity (cell stability), XTT reduction assay (metabolic activity)</td>
<td>SIGNIFICANT DECREASE OF VIABILITY; G3PDH ACTIVITY AND XTT MEASUREMENT BETWEEN FRESH AND FROZEN ADIPOCYTES</td>
</tr>
<tr>
<td>Comparison of techniques for long-term storage of fat grafts: an experimental study</td>
<td>Atik</td>
<td>2006</td>
<td>40 mice</td>
<td>1. Immediately transplanted to donor 2. Dry-frozen 3. Immersed in glycerol 4. Frozen in liquid nitrogen</td>
<td>In freezer: -35°C</td>
<td>6 months</td>
<td>Freezing at -35°C</td>
<td>Thawing at room temperature for 1 hour</td>
<td>MTT (viability), histological examination</td>
<td>NONSIGNIFICANT DIFFERENCE IN VIABILITY BETWEEN FRESH FAT AND FAT IMMERSED IN LIQUID NITROGEN</td>
</tr>
</tbody>
</table>

Notes:
- DMEM: Dulbecco's Modified Eagle's Medium
- HES: Human Erythrocyte Solvent
- CPA: Cryoprotectant Agent (DMSO, Trehalose)
- G3PDH: Glucose-6-phosphate dehydrogenase
- MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
- XTT: 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt
### 8.6 TABLE OF COMPARATIVE STUDIES OF FAT FROZEN WITH OR WITHOUT CRYOPROTECTANT AND DIFFERENCE IN CRYOPROTECTANTS

<table>
<thead>
<tr>
<th>Title</th>
<th>Author</th>
<th>Publication year</th>
<th>Number of participants</th>
<th>Compared groups</th>
<th>Methods</th>
<th>Conclusion of authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryopreservation of fat tissue and application in autologous fat graft: in vitro and in vivo study</td>
<td>Li</td>
<td>2011</td>
<td>10 persons</td>
<td>1. In normal saline 2. In 10% HES</td>
<td>MTT (viability), histological examination</td>
<td>NON SIGNIFICANT DIFFERENCE WHEN ADDING HES AS CPA</td>
</tr>
<tr>
<td>Cryopreservation of autologous fat grafts harvested with the Coleman technique</td>
<td>Pu</td>
<td>2010</td>
<td>8 persons</td>
<td>1. Mixed with CPA (DMSO 0,5M or 3,3% and trehalose 0,2M or 7,6%): 1ml fat graft + 1 ml CPA in 3 cc vial, incubated for 10 min at room temperature =&gt; methanol bath 2. No CPA: 1 ml fat + 1 ml normal saline at room T</td>
<td>Trypan blue viability, G3PDH activity (cell stability), routine histology</td>
<td>NON SIGNIFICANT DIFFERENCE IN ADIPOCYTE COUNT AND HISTOLOGY BETWEEN CRYOPRESERVATION WITH AND WITHOUT CPA/ SIGNIFICANT DIFFERENCE (P&lt;0,001) IN G3PDH ACTIVITY</td>
</tr>
<tr>
<td>Cryopreservation of human adipose tissue</td>
<td>Cui</td>
<td>2007</td>
<td>36 persons, 45 mice</td>
<td>1. Fresh control 2. Cryopreservation without CPA 3. cryopreservation with CPA: 0,5 M DMSO + 0,2 M trehalose with 3 X centrifugation 100 g to remove CPA (15 mice each group)</td>
<td>Weight, volume, histological examination, cell count</td>
<td>SIGNIFICANT DIFFERENCE IN ADIPOCYTE COUNT BETWEEN FAT FROZEN WITH AND WITHOUT CRYOPROTECTANT / GROUP WITH 0.5 DMSO AND 0.2 TREHALOSE SIGNIFICANT HIGHER CELL COUNT THAN OTHER CRYOPROTECTANT GROUP</td>
</tr>
<tr>
<td>Comparison of techniques for long-term storage of fat grafts: an experimental study</td>
<td>Atik</td>
<td>2006</td>
<td>40 mice</td>
<td>1. Immediately transplanted to donor 2. Dry-frozen 3. Immersed in glycerol 4. frozen in liquid nitrogen (10 mice each group)</td>
<td>MTT (viability), histological examination</td>
<td>SIGNIFICANT DIFFERENCE IN VIABILITY BETWEEN FROZEN WITH OR WITHOUT GLYCEROL</td>
</tr>
<tr>
<td>The fate of cryopreserved adipose aspirates after in vivo transplantation</td>
<td>Pu</td>
<td>2006</td>
<td>1 woman, 60 mice</td>
<td>1. Fresh control group 2. Simple cryopreservation group 3. optimal cryopreservation group: with CPA (DMSO 0,5 M and trehalose 0,2 M)</td>
<td>Gross appearance of fat graft over their posterior scalps, volume, weight, histological examination</td>
<td>SIGNIFICANT INCREASE IN VOLUME,WEIGHT AND HISTOLOGY WHEN CRYOPRESERVED WITH CPA</td>
</tr>
<tr>
<td>Cryopreservation of mature human adipocytes: in vitro measurement of viability</td>
<td>Wolter</td>
<td>2005</td>
<td>24 persons</td>
<td>1. DMEM 2. DMEM + 10% HES 3. DMEM + 10% glycercol 4. DMEM + 10% glycercol + 5% dextran + 5% PVP (polyvinylpyridone)</td>
<td>Trypan blue staining, MTT, G3PDH</td>
<td>NON SIGNIFICANT DIFFERENCE IN MTT, XTT</td>
</tr>
<tr>
<td>Ex vivo fat grafts preservation: effects and implications of cryopreservation</td>
<td>MacRae</td>
<td>2004</td>
<td>1. 2 ml of DMEM and 10% FBS: mixed and incubated at 37°C 2. No medium 3. -20°C freezer, no medium 4. “Snap” frozen by immersion in -196°C, without medium 5. Fresh adipocytes</td>
<td>MTT (viability)</td>
<td>SIGNIFICANT DIFFERENCE IN ADIPOCYTE VIABILITY: SIGNIFICANTLY LESS IN GROUP INCUBATED WITH MEDIA</td>
<td></td>
</tr>
</tbody>
</table>
## 8.7 ANALYSIS OF VIABILITY DECLINE: COMPARISON OF VIABILITY OF FRESH AND FROZEN ADIPOCYTES

<table>
<thead>
<tr>
<th>Title</th>
<th>Author</th>
<th>Publication year</th>
<th>Viability fresh adipocytes (%)</th>
<th>Viability freeze-thawed adipocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryopreservation of fat tissue and application in autologous fat graft: in vitro and in vivo study</td>
<td>Li</td>
<td>2011</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td>Effects of fat preparation methods on the viabilities of autologous fat grafts</td>
<td>Minn</td>
<td>2010</td>
<td>/</td>
<td>63.7 (cotton gauze)</td>
</tr>
<tr>
<td>Viability of fat cells over time after syringe suction lipectomy: the effects of cryopreservation</td>
<td>Son</td>
<td>2010</td>
<td>79.7 ± 8.4</td>
<td></td>
</tr>
<tr>
<td>Cryopreservation of human adipose tissue</td>
<td>Cui</td>
<td>2007</td>
<td>/</td>
<td>87.1 % of fresh ones</td>
</tr>
<tr>
<td>Cryopreserved human adipogenic-differentiated pre-adipocytes: a potential new source for adipose tissue regeneration</td>
<td>Kim</td>
<td>2007</td>
<td>96.2</td>
<td>96.3</td>
</tr>
<tr>
<td>Adipose aspirates as a source for human processed lipoaspirate cells after optimal cryopreservation</td>
<td>Pu</td>
<td>2006</td>
<td>/</td>
<td>90 % of fresh ones</td>
</tr>
<tr>
<td>Comparison of techniques for long-term storage of fat grafts: an experimental study</td>
<td>Atik</td>
<td>2006</td>
<td>78.3</td>
<td>66.4 (liquid nitrogen)</td>
</tr>
<tr>
<td>Cryopreservation of human fat for soft tissue augmentation: viability requires use of cryoprotectant and controlled freezing and storage</td>
<td>Moscatello</td>
<td>2005</td>
<td>/</td>
<td>47.3 (dry-freeze)</td>
</tr>
<tr>
<td>Cryopreservation of mature human adipocytes: in vitro measurement of viability</td>
<td>Wolter</td>
<td>2005</td>
<td>94.3</td>
<td>38.1 (glycerol)</td>
</tr>
<tr>
<td>Long-term preservation of adipose aspirates after conventional lipoplasty</td>
<td>Pu</td>
<td>2004</td>
<td>/</td>
<td>47.9 (dead control)</td>
</tr>
<tr>
<td>Ex vivo fat grafts preservation: effects and implications of cryopreservation</td>
<td>MacRae</td>
<td>2004</td>
<td>/</td>
<td></td>
</tr>
</tbody>
</table>