UNIVERSITEIT GENT

Academic Year 2012- 2014

CRYOPRESERVATION OF LIPOASPIRATE MATERIAL FOR FUTURE PURPOSES

Sam BRONDEEL

Promoter: Prof. Dr. Phillip Blondeel

Co-promoter: Dr. Filip Stillaert

Dissertation presented in the 2nd Master year in the programme of

MASTER OF MEDICINE IN MEDICINE

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Prologue

In the first place I would like to thank Dr. Stillaert. He presented me this interesting subject en guided me along the way. I want to thank him for reviewing my text and adding improvements, suggestions and corrections.

Prof. Dr. Hilde Beele has been a huge help to send me in the right directions. I would like to thank her for all the help, guidance, reviews, suggestions and improvements.

Further I would like to thank Mrs. Caroline Van Geyt and Mrs. Heidi Declercq for all the help in creating the protocol and standard operating procedures, together with me.

At last I want to thank Mr. Marc Suy, my former English teacher, to review and correct my thesis.

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1. ABSTRACT (ENGLISH)

1.1 Background

Lipofilling is the act of transplantation of own fat harvested with liposuction. This procedure has a huge variety of indications; one of them is breast augmentation. After a lipofilling procedure there will always be partial resorption of the transplanted fat. The degree of survival of the transplanted fat is very unpredictable, one procedure is hardly ever enough to get the wanted results. Patients must get three to four lipofilling procedures dependent on the resorption or wanted enlargement. In case of these successive liposuction procedures there will be an increase of morbidity, despite the low rate. One solution to the problem would be cryopreserving the fat[1-6]. In this way one liposuction procedure would be sufficient. After partial resorption, of the transplanted fat, the preserved fat could be used in order to achieve the intended volume without repeated liposuctions. The aim of this thesis is to create a protocol to cryopreserve and to evaluate fat tissue, based on an extensive review of the literature.

1.2 Methods

Articles were searched in the database 'PubMed', to get a complete image of the available literature. After creating a simple protocol, articles focusing on individual steps were investigated. This resulted in a protocol with a lot of gaps, due to lack of consensus. These gaps were filled by information obtained from experienced scientists, involved in tissue banking and cryopreservation.

1.3 Results

We created a protocol that is both easy, feasible and partially literature-based. After the fat has been removed from the patient by liposuction, it is centrifuged at 300RPM for 3minutes. After transportation, the lipoaspirate is cryopreserved with DMSO in 10ml cryovials. The vials are placed into a styrofoam box. The box is then put in a -80°C freezer for at least 24h (resulting in a cooling at approximately 1°C per minute). The final step is to transfer the

recipient into a -150°C freezer. The thawing procedure is the quick one, in a 37°C waterbath. Viabilitytesting is done before the cryopreservation, to get a starting point, and after cryopreservation to evaluate the decline in viability. Lipoaspirate will be investigated by a cell staining morphometric assay, using HOECHST 33342, Nile Red and propridium iodide, and also with an XTT assay.

1.4 Conclusion

Based on literature and experts' opinion, we have succeeded in creating a protocol that is both easy and feasible, and could be used as a guidance in future trials. A number of aspects need validation before the protocol can be used in a clinical setting.

1. ABSTRACT (NEDERLANDS)

1.1 Achtergrond

Lipofilling is simpelweg de transplantatie van eigen vetweefsel dat bekomen wordt via liposuctie. De mogelijkheden van deze ingreep zijn heel uitgebreid, één ervan is bijvoorbeeld als onderdeel van een borstreconstructie. Het probleem met deze ingreep is de resorptiegraad. De overleving van het getransplanteerde vet is immers onvoorspelbaar. Patiënten moeten meermaals liposuctie en lipofilling ondergaan om het gewenste resultaat te bekomen. Een oplossing die naar voor wordt gebracht is cryopreservatie, hierbij zou één liposuctie voldoende zijn. Het overtollige vet wordt dan ingevroren en nadien ontdooid om bij te spuiten en zo het initiële, afgesproken volume te bekomen. Het doel van deze thesis is om een protocol op te stellen dat zowel de cryopreservatie als de viabiliteitsanalyse beschrijft, gebaseerd op literatuurgegevens.

1.2 Methode

Artikels werden gezocht via de database ‘PubMed’ om zo een volledig beeld van de literatuur te bekomen. Nadat we een ruw protocol hadden opgesteld via de literatuur zijn we op zoek gegaan naar artikels die zich focussen op de verschillende onderdelen van het protocol. Na analyse konden niet veel conclusies gemaakt worden. Deze ‘gaten’ zijn opgevuld via ervaren onderzoekers, betrokken bij de weefselbank en cryopreservatie.

1.3 Resultaten

We zijn erin geslaagd een gemakkelijk en haalbaar protocol op te stellen dat gedeeltelijk is gebaseerd op de literatuur. Nadat het vetweefsel bij de patiënten is verwijderd, wordt het voor 3 minuten gecentrifugeerd aan 3000 RPM. Het lipoaspiraat wordt dan getransporteerd naar de weefselbank waar de cryopreservatie wordt uitgevoerd met DMSO in 10ml cryovials. Deze worden in een isomodoos geplaatst en overgebracht naar een -80°C vriezer voor minstens 24h. Op deze manier bekomen we een koeling van één graad per minuut. Nadien wordt de

cryovial overgebracht naar een -150°C vriezer. De ontdooiing gebeurt via een snelle procedure in een warmwaterbad van 37°C. Viabiliteitsonderzoek is gedaan zowel vóór als ná cryopreservatie, zo hebben we een initiële viabilteit waarop kan vergeleken worden. Het lipoaspiraat wordt dan onderzocht met celkleuringen waar we gebruik maken van HOECHST 33342, Nile Red en propridium iodide. Ook wordt er een XTT assay op het lipoaspiraat uitgevoerd.

1.4 Conclusie

Gebaseerd op de literatuur en de mening van experten, zijn we erin geslaagd om een makkelijk en haalbaar protocol op te stellen, dat kan dienen als leidraad in toekomstige onderzoeken. Een deel van de aspecten hebben nog validatie nodig vooraleer het protocol kan gebruikt worden in klinische toepassingen.

2. METHOD

Articles were searched in the database 'PubMed' to get a complete picture of the available literature. We used this database together with a VPN-connection of Ugent to get more free full articles. In MeSH we only found 'adipose tissue' and 'cryopreservation' as known terms. These two were added to 'search build' and searched on PubMed. Only eight articles were found. To find more articles the following terms were used in different combinations: 'free fat grafts', 'lipofilling', 'cryopreservation', 'adipose tissue', 'adipocytes', 'breast reconstruction', 'liposuction' and 'viability'. These results gave a full image of what was already published about this theme. A very simple protocol was designed, containing all the essential steps. To see if there were articles focusing on these different steps of the protocol we searched them individually. The first step we focused on was the donor site. The articles were found using the terms 'liposuction' AND 'donor site' AND 'viability'. The next step was the liposuction technique, using the terms 'liposuction technique' AND 'viability'. With the next step we focused on the fat processing after the liposuction. Articles were found by using the terms 'fat grafts' AND 'centrifugation'. On 'transport' no results were found. To investigate the ideal cryoprotectans we found articles, using the terms 'cryoprotective agens' AND 'fat' AND 'cryopreservation'. To found out if there is an ideal way of cryopreserving the fat grafts we found articles using the terms 'storage temperature' AND 'fat' AND 'cryopreservation'. There were no specific articles found comparing different types of thawing methods. The last step we focused on were the different methods of viability analyzing, using the terms 'viability' AND 'adipocytes'.

After reading the literature data, just a few conclusions could be made. To fill in the gaps, we asked for the experts' opinion of scientists involved in tissuebanking, cryopreservation and evaluation of cells and tissues. The first part of the protocol was created together with Mrs. Caroline Van Geyt, researcher at Ghent University, Tissue bank, Belgium. The viability analysis was based on the trial done by Suga[7] and created together with the input of Mrs. Heidi Declercq, postdoctoral associate at Ghent University, Belgium, dept. of Histology (Basic Medical Sciences).

The program EndNote X5 included all the references, used from the literature.

3. INTRODUCTION

Liposuction is one of the most frequently performed cosmetic surgeries worldwide. Because of its popularity, the possibilities of the use of autologous fat transplants as a soft tissue substitute are increasingly explored. Free fat transfer, or lipofilling, offers treatment for soft tissue defects caused by trauma, cancer, congenital anomalies, painful scars, irradiation injuries and aesthetic indications[8].

3.1 What is fat tissue?

Adipose tissue is a connective tissue derived from the mesodermal germ layer (mesenchyme), similar to bone marrow. The "mesenchyme" designates the developing loose connective tissue of an embryo, mainly derived from the mesoderm, and giving rise to a large part of the cells of the connective tissue in the adult. Adipose tissue contains a supportive stroma that can easily be isolated. The "panniculus adiposus" is the term for the adipose tissue, which consists of adipocytes and a stromal-vascular fraction (SVF) with connective tissue (figure 1). The combination of various adipose tissue depots in a given organism has been referred to as the "adipose organ".

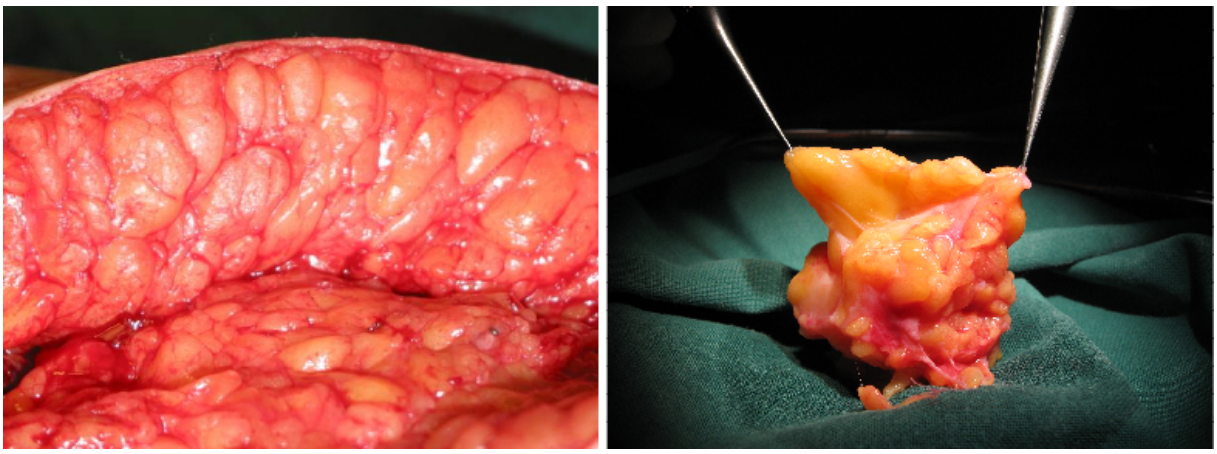


Fig 1: The subcutaneous adipose tissue depots. Adipose tissue is a connective tissue consisting of different cell types in different stages of development[9].

White fat is composed of "preadipocytes" and differentiated adipocytes, interstitial cells, and a microvascular system entwined within an organized extracellular matrix (ECM)[10]. The supportive stroma in adipose tissue consists of a heterogeneous mixture of cells, including endothelial cells, smooth muscle cells, pericytes, fibroblasts, mast cells and pre-adipocytes. Like in the bone marrow, the stromal vascular fraction of adipose tissue contains a population of multipotent adipose tissue-derived mesenchymal stem cells (ASC). One gram of adipose tissue contains approximately 5000 stem cells. The basic organization of a distinct adipose depot shows mature adipocytes, stromal-vascular cells, blood vessels, lymph nodes and nerves. The main cellular component of adipose tissue is the major lipid-filled cell, the adipocyte (figure 2).

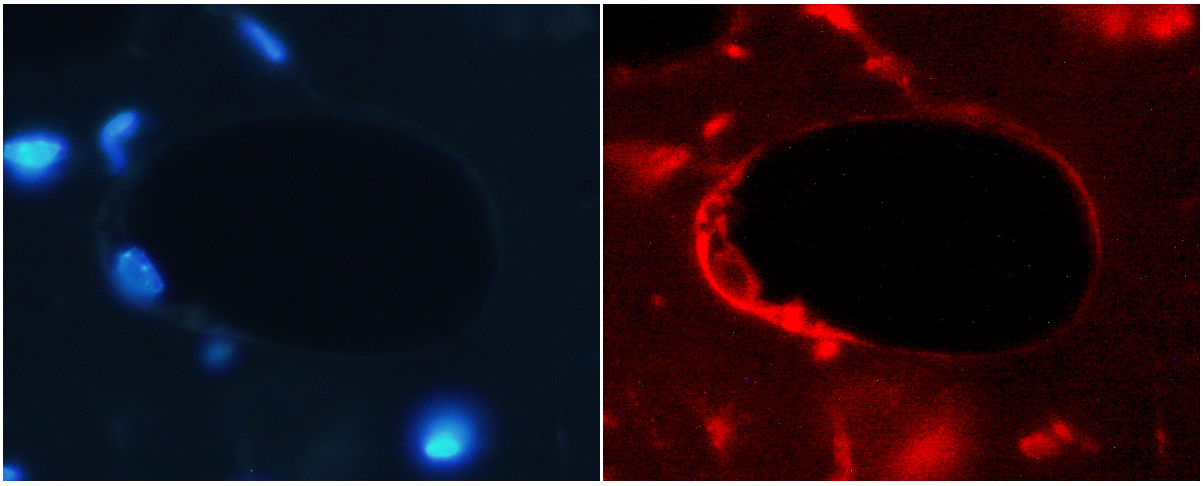


Fig2: The adipocyte is a spherical cell containing a large lipid droplet. An adipocyte has the appearance of a "signet ring": a peripheral nucleus and a small rim of cytoplasm[9].

The blood supply to adipose tissue provides blood for delivery of metabolic substrates and removal of cellular metabolic products[11]. It has been postulated that each adipocyte is in close proximity to a blood capillary. Although blood flow appears to be small in adipose tissue in comparison to other organs – expressed as ml of blood per 100 gr of tissue per min – expressing blood flow per fat cell gives a completely different picture[12],*[13]*. The lean human adipose organ receives 0.2 – 0.6 L of blood $^{-1}$, which corresponds to about 3-7% of cardiac output[14]. With morbid obesity, blood supply to the adipose organ can reach 15-30% of the cardiac output.

3.2 History of free fat grafts

The Swiss naturalist Conrad Gessner was the first to describe adipose tissue in 1551 (figure 3). Physicians have always been interested in fat tissue. This mesenchymal tissue has important physical (protection of underlying neurovascular and musculoskeletal structures) and physiological (metabolic and endocrinological) functions. Surgeons are interested in fat tissue because it is the ideal tissue to cover soft tissue defects. It is a versatile tissue and as a donor tissue it does not inflict an important morbidity at the donor site. Fat transplantation is performed mainly as a microsurgical tissue transfer where the revascularization of the "tissue flap" is established through microsurgical vessel anastomoses (figure 3). Free fat grafting - without microsurgery - has been performed since decades but with poor clinical outcomes. Lipofilling (or free fat grafting) is a well-known surgical technique that already has been introduced in the clinic some decades ago. It uses lipoaspirate material, obtained through liposuction, to treat soft tissue defects (figure 4).

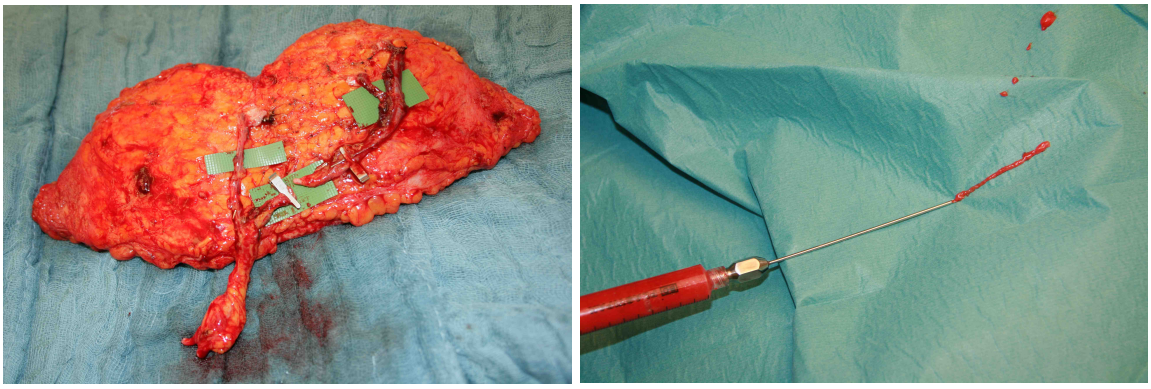


Fig4: A tissue flap ready for microsurgical transfer (left). The flap consists mainly of adipose tissue and will be used to reconstruct a breast. To guarantee a successful outcome a microsurgical vessel anastomosis need to be performed to reestablish the blood flow within the flap. With the introduction of the lipofilling technique, adipose tissue became available as an injectable filler material (right)[9].

The founder of the first aseptic clinic was the German surgeon Gustav Adolf Neuber, but in plastic surgery he will be known as the first to perform an autologous fat graft on a patient in 1893[15] (figure 3). A soft depression, in the face of a twenty-year-old, was filled utilizing blocks of free fat harvested from the arms[16-18].

Fig3: Conrad Gessner was the first to describe fat tissue in 1551 (left)[19]. Neuber was the first to describe free fat grafting[20]. He harvested fat tissue from the upper arm of young male patient to treat a soft tissue defect in the face.

Later in 1895, Czerny used autologous adipose tissue in an attempt to reconstruct a breast[21]. Another great name in the history of autologous fat grafts is Lexer. He modified Neubers' technique and used abdominal harvested fat to treat a malar depression and a receding chin in 1910. He reported excellent short- and long-term results but others have reported significant resorption with his technique[22]. In 1911, Bruning was the first to inject small cubes of surgically harvested adipose tissue with a syringe into a subcutaneous space[23]. In the early 50s, Peer transplanted "en bloc" and concluded a viability rate of 50% after a year of observation. He also stated that the survival of fat grafts was dependent on early neovascular anastomoses, a process called "angiogenesis" (figure 5) [24]. Despite those experimental and clinical reports, the technique wasn't popular because the harvesting had to be done by excisional surgery. `

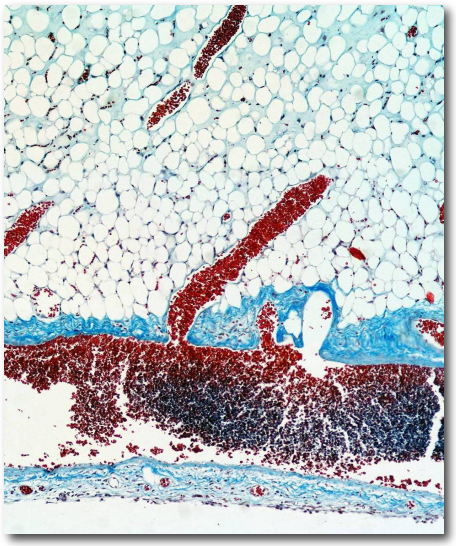


Fig5: Angiogenesis is the process of vessel sprouting from existing vessels. It is a physiological phenomenon that plays a crucial to revascularize the transplanted cell population in fat grafts.

With the liposuction procedure introduced in the clinic, autologous fat was re-introduced into the clinic as an injectable filler material (figure 6). Pioneers were Dr. A. Fischer, Dr. GM.

Fischer and Dr. Yves-Gerard Illouz. The Doctor Fischers were the actual inventors of the technique, which they called 'cellusuctiotome'[25]. The liposuction technique, as we know it today, was invented by Dr. Illouz. But the reawakening began in 1986 when Dr. Illouz re-injected viable fat that had been harvested by his liposuction technique in 1986[26]. The introduction of the syringes to harvest fat for transplantation was the merit of Pierre Fournier. He placed a thirteen gauge needle on a syringe and called it 'microlipoinjection'[27]. The last breakthrough in this area would be the invention of the 'tumescent technique', which came from dermatologist Jeffrey Alan Klein[28] in 1987. This new technique was a combination of a new kind of anesthesia and the use of microcannula. The anesthetic was a solution of highly diluted lidocaine and epinephrine. Infiltrated in the target area, the tissue becomes swollen and firm, or tumescent. Thus there would be no more need of general anesthesia and blood loss would be minimalized by the vasocontrictive effect of epinephrine[29]. Using the microcannula, the liposuction could be done more accurately and uniformly, which gave a better esthetic result and a more rapid postoperative recovery. The liposuction technique revolutionized compared to the previously used methods. The safety of the tumescent technique was confirmed by national survey results executed by Dr. Hanke C. W.[30]. Later Fournier[31] revitalized the idea of using microcannula, Asken[32] also contributed to a refinement. But Mell Bricol was one of the first to describe a series of fat transplantation for both augmentation and reconstruction[33-35].

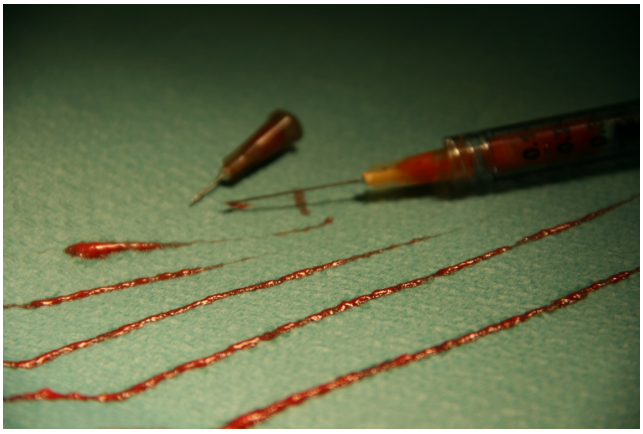


Fig6: Fat obtained through liposuction is some kind of "liquified" material. It can easily be injected using syringes and small needles or cannulas[9].

Although a revolution was started there was a lot of skepticism too. The American Society of Plastic Surgeons ('ASPS') released a position statement about the procedure in 1987. They labeled it "experimental" and considered the possibility that the injected fat could hinder breast cancer detection[36]. European surgeons were undeterred by the American position and began to use the technique. Emmanuel Delay[37] started in 2000 and Gino Rigotti[38] presented

his large series of lipofilling procedures at a congress of the European Association of Plastic, Reconstructive and Aesthetic Surgery in 2007. Baker presented his findings at "the 2006 meeting of the American Society for Aesthetic Plastic Surgery" and concluded there were no issues with breast imaging or difficulties to interpret masses. Sydney Coleman published his landmark study in 2007, making use of several smaller injections of autologous fat, which were also spread in time. This technique - called the structural lipofilling technique - gave very promising esthetic results and was followed up with photography. The identified changes seen on a mammogram were the ones we would expect after any breast procedure[39]. In 2007, the ASPS began to review the new literature and only approved some "Guiding Principles" in 2009. These were no specific recommendations to perform the procedure because of the lack of strong data and literature. The report also indicated that 'there were no reports suggesting an increased risk of malignancy associated with fat grafting and limited data suggested that fat grafts may not interfere with radiologic imaging for breast cancer detection' [33]. They concluded a "B-recommendation", which 'encourages clinicians to employ the available information while remaining cognizant of newer, evidence-based findings'[36]. Because there is no standardized technique, the used ones depend on the performing surgeon, which indicates the differences in safety, efficacy and final outcome. Although there is no standardization the differences are minimal so autologous fat transplantation has become a frequently executed procedure and has huge potential for the future.

3.3 What is lipofilling

Lipofilling is the free transfer or transplantation of the patient's own, autologous, fat harvested with liposuction (figure 7). Essentially, a three-dimensional organ (adipose tissue) is processed into a liquified organ (lipoaspirate). Initially, fat is harvested using tumescent liposuction. Care is taken to avoid major trauma to the lipoaspirate material using a low pressure vacuum system. Subsequently, the lipoaspirate material is collected in a sealed off environment and transferred into syringes. Those syringes are centrifuged for 3 minutes at 3000 rpm. The end-result is a tissue specimen consisting of three layers (figure 8). The top layer is an oily substance containing ruptured adipocytes and triglycerides. The middle layer is the concentration of lipoaspirate material and is used for clinical applications. The bottom layer is a concentration of blood products and cell debris.

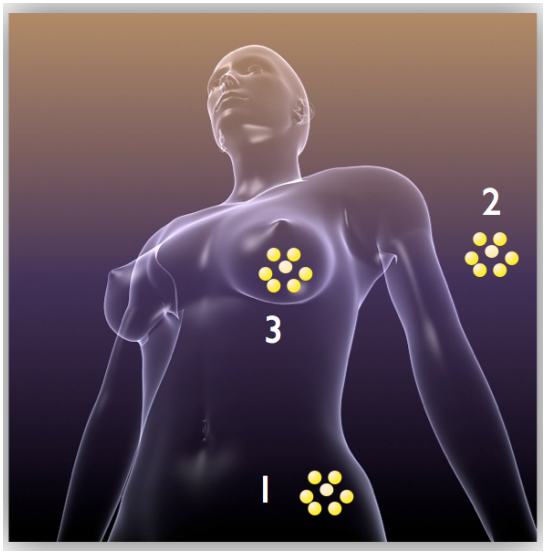
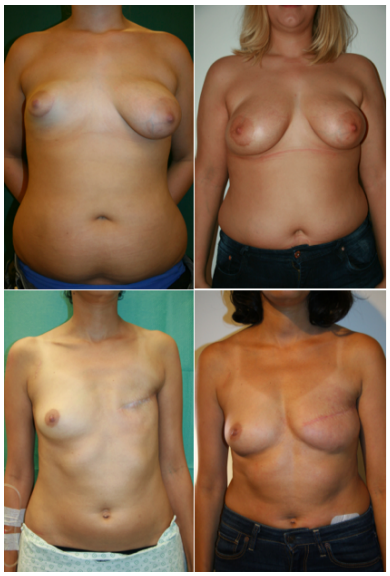


Fig7 (left): Lipofilling is the free transplantation of fat tissue obtained through liposuction. Subcutaneous adipose tissue is harvested from a specific donor site using liposuction (1). The obtained "lipoaspirate material" is processed using centrifugation to separate the adipocytes and other cells from blood debris and infiltration fluid (2). The processed lipoaspirate material is injected at a specific recipient site to treat soft tissue deficiencies (3). [9]

Fig8 (right): The end-result of centrifugating lipoaspirate material. Three layers can be distinguished: 1/ an oily part, concentrating the lipid droplets from traumatized adipocytes, 2/ the concentrated adipocytes and other cells from the stromal-vascular fraction, and 3/ blood debris. The highest concentration of cells are located in the lower third compartment of the concentrated lipoaspirate specimen. [9]

The indication to perform a lipofilling procedure may be based on aesthetic or reconstructive purposes. In most cases, lipofilling will be used to address soft tissue deficiencies. Tissue deficiencies can be the result of aging, trauma, congenital deformities or surgical interventions (figure 9). Fat tissue is the ideal filler as it restores the subcutaneous barrier to protect underlying neurovascular and musculoskeletal structures (figure 10). With the discovery of a stem cell population (ADSC, adipose derived stem cells) within the subcutaneous adipose organ, new therapies have been introduced into the clinic. Lipofilling is currently also used to improve tissue quality as a beneficial effect that has been noted on skin elasticity and skin recovery after being treated with fat injections. Radiotherapy sequellae and scarring of the skin are some examples of disorders that can be treated with lipofilling. The stem cells within the lipoaspirate secrete growth factors, proteins and cytokines that improve the skin quality.



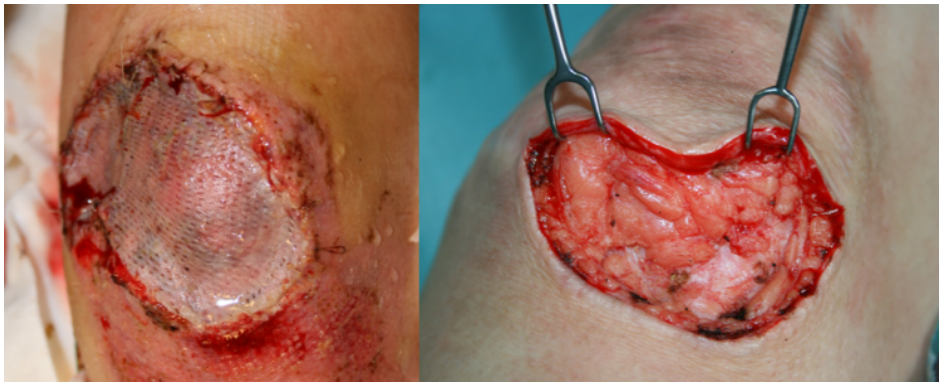


Fig9 (left): Upper image: Congenital deformity of the right breast. Reconstruction has been performed using the lipofilling technique. Four sessions of lipofilling were needed to achieve a pleasing aesthetic outcome. Lower image: Status of the left hemithorax after mastectomy for breast cancer. Reconstruction of the left breast with lipofilling (four sessions) and skin expansion[9].

Fig10 (right): Deglovement injury of the knee. Wound coverage with skin grafting and additional lipofilling procedures to restore the subcutaneous adipose tissue barriers. Intraoperative view showed nicely vascularized and healthy fat tissue.[9]

The advantages of using adipose tissue as a filler are: 1/ it is an *autologous* tissue (immunocompatable), 2/ it is *easily available* within the subcutaneous adipose tissue depots, 3/ it has a population of *postnatal adult stem cells* with no ethical issues to use them in the clinic, 4/ *processing* is easy. Also the current liposuction method makes the whole lipofilling procedure both easy and safe. Although there is a morbidity rate it remains minimal, cases of severe complications and death appear to be extremely rare in the literature[36].

Although there were some concerns about delaying breast cancer diagnosis or hindering the radiography, there is no scientific proof that post-mastectomy breast reconstruction and additional lipofilling does increase the risk of breast cancer recurrence[40-44].

The main disadvantage of lipofilling is the unpredictable resorption rate. Free-fat grafting rarely achieves sufficient tissue augmentation because of delayed neo-vascularization with subsequent cell necrosis, fibrosis, and graft volume shrinkage. It succeeds at a microscale level by providing the appropiate cell type in a suitable environment but fails at a macroscale level, because the majority of the transplanted cells become located more than 100 µm away from blood vessels in the residual tissue, which is the diffusion limit for oxygen. Necrosis of the injected fat[45-47] can lead to calcifications which could be suspicious on photography,

careful monitoring is required [48]. Other complications include hyalinization, fibroplasia, edema, hematoma, infection and granuloma formation[47, 49, 50]. More severe complications could be the development of liponecrotic pseudo cysts [51-53] and life threatening breast abscesses[50]. The absence of more suitable alternatives, such as tissue-engineering approaches, has led to its continued use despite variable resorption and lipid cyst formation.

3.4 Lipofilling: future perspectives

The main challenge of fat grafting is to address the unpredictable clinical outcomes due to the consistent resorption rate. Several lipofilling sessions are often necessary to achieve a pleasing clinical outcome. Despite the fact that the lipofilling technique is a minimally invasive technique, patients do need to undergo repeated general anesthesia to harvest adipose tissue through liposuction. The patient's own motivation is a major prerequisite to continue with a lipofilling strategy to obtain a clinical result. Several hospital admissions and surgery could be very inconvenient for the patient. Survival of the transplanted fat is very unpredictable. This resorption occurs in four to six months after the procedure[39, 54]. The rates of resorption are up to 90% in experimental studies[55-57] but are limited to 40-60% in clinical trials[54, 58, 59]. A possible solution to address the problem of resorption and additional lipofilling procedures, could either be the enhancement of the survival rate of transplanted fat by adding additives to the fat graft, or storage of liposuctioned adipose tissue obtained in one liposuction session[60-62].

Reports have been published where investigators tried to preserve the lipoaspirate, with mostly cryopreservation, [1-6]. When resorption has taken place cryopreserved fat could be used to continue the soft tissue treatment, even on an ambulatory basis.

4. WHAT IS CRYOPRESERVATION?

Cryopreservation is a process where cells or whole tissues are preserved by cooling to sub-zero temperatures. This could be a solution to the shortcoming of lipofilling; a part of the lipoaspirate could be frozen for later purposes.

4.1 Natural cryopreservation

In a cryopreservation procedure, cells, tissues or organs are cooled in order to suspend all the metabolic reactions. In this way we can conserve them. The idea of freezing cells to preserve isn't as artificial as we think. If we take a look at nature we see a category of animals, which we call 'extremophiles', that are immune to extreme conditions[(63)]. Tardigrades, also known as water bears, are extremophiles and have the ability to tolerate freezing body water. They replace most of their internal water with the sugar trehalose, using this ability they prevent it from crystallization that otherwise would damage cell membranes[(64)]. This capacity makes them survive temperatures just above absolute zero, which is the lowest temperature possible and is defined to be −273.15°C. The wood frog is another example of an animal that can tolerate serious freezing temperatures. They can tolerate freezing of their blood and other tissues. In order to give a response to internal ice formation they produce large amounts of the sugar glucose[(65)]. Not only glucose, derived from liver glycogen, helps the frogs to survive the freezing temperatures but also urea which is accumulated in tissues in preparation for overwintering[(66)].

4.2 History of cryopreservation

The first report ever made on the effects of lower temperatures on spermatozoa was done by Spallanzani in 1776[(67)]. He was also the first to have performed the in vitro fertilization in 1780[(68)]. In 1866 Montegazza had the revolutionary idea to suggest banks for frozen human semen[(67)]. But is was Polge in 1949 who successfully cryopreserved spermatozoa for the first time[(69)]. He was the first to solve the mystery of how to preserve living cells at a low temperature. During his trial of freezing fowl sperm, he accidentally discovered glycerol as a

cryoprotectans. After the use of glycerol, pregnancy rates were way higher than before and his work had an enormous impact on the evolution of artificial insemination. In 1952 Lovelock solved important items of cryoprotectants[70], but it was Dr. Jerome K. Sherman's work in cryobiology that led to a method of successfully freezing and thawing sperm[71]. In 1953 he founded the first sperm bank, which led to the first human birth from cryopreserved sperm. In 1964 the term cryobiology was 'invented' and the first meeting of The Society for Cryobiology was held. This society still has an annual meeting to 'bring together those from the biological, medical and physical sciences who have a common interest in the effect of low temperatures on biological systems'[72].

Since the discovery of cryoprotectans the effects could not only be investigated by empirical studies but also by fundamental cryobiology. Although the empirical studies were the basis for a theory of causes why freezing could be a success or a failure, the new variables could be used for the optimization of cryopreservation protocols. Where empirical cryobiology is limited to descriptions of cellular and tissue behavior of many different cell types at subphysiologic temperatures, fundamental cryobiology is a quantitative study of biophysical and biochemical phenomena which occur during cryopreservation of mammalian cells. These include the transmembrane fluxes associated with the addition and removal of CPAs, the change in chemical potentials during cooling and warming, both intracellular and extracellular ice formation, the effects of cooling and warming rates and storage temperatures, heat transfer in solutions and tissues, and, most importantly, the optimization of cryobiological procedures in conjunction with this information. Since then, many methods have been developed for various types of cells, tissues and organs[73].

4.3 Methods of cryopreservation

The two most common methods for cryopreservation of human tissue or cells is slow cooling and vitrification.

4.3.1 Slow cooling

In essence the slow cooling protocol is to gradually reduce the temperature of both the human tissue as the cryoprotectans. When the temperature drops gradually we get an unfrozen

fraction which declines, growing ice mass and an increase of sugar-, salt- and cryoprotectansconcentrations. This results in an increase of osmotic strength that leads to an efflux of water. Because of this gradually cooling, the efflux is really sufficient and the intracellular ice formation will be minimal. Not all the water gets out of the cell but the viscosity of the unfrozen fraction becomes too high for any further crystallization and thus turns into an amorphous solid that contains no ice crystals. A widely used cooling rate is 1°C/ minute and is appropriate for many mammalian cells. This controlled cooling can be achieved by using rate-controlled freezers[74].

4.3.2 Vitrification

Vitrification is actually a rapid freezing protocol. The used medium is added in a very high solute concentration to begin with. The used cryoprotectans works like antifreeze; it decreases the freezing temperature and at sufficiently low temperatures the viscosity is much higher. Before the cooling process the human tissue or cell and cryoprotectans has become an amorphous mass. Thus there cannot be any ice formation. As there is no ice formation, crystallization can't affect the tissue and so there is no need of slow cooling. In fact it may be beneficial to cool very quickly[74, 75] .

4.4 Cryobiology

Cryobiology is derived from the Greek words; "cryo", that means cold, "bios", meaning life, and "logos", meaning science. But if we take the term into a more daily practice we would say that in cryobiology, biological material is studied in environments with temperatures below normal. We have two major types of cryobiology; the empirical and the fundamental cryobiology (cfr. supra). If we take a closer look there are four major problems why a cryopreservation procedure could be a success or a failure.

4.4.1 Solution effects

The solution effects are based on the original work of Lovelock[76], Levitt[77], Karow and Webb[78] and Maryman[79]. They first described this problem in human red blood cells, where the solution effect is caused by salt concentrations. When the temperature falls, extremely high salt concentrations are reached which makes the cell membrane become porous to these molecules. These molecules are trapped in the membrane and cause a difference in osmotic pressure during thawing which leads to an influx of water and the probable lysis of the cell.

4.4.2 Intracellular ice formation

This hypothesis was first published by Mazur in 1972 and with the previous discovered solution effects he defined his 'two-factor hypothesis'. When the temperature reaches the solute below its freezing point, which is called the supercooling phase, water is precipitated out of the extracellular medium as ice. In this way the extracellular water concentration increases, which causes the intracellular water concentration to shift. As a result of this mechanism the cell starts to lose water. When the cooling is done slow enough there is a nice equilibrium between the freezing of the extracellular water concentration and the shift of water derived from the intracellular environment. When the cooling rates are too quick the equilibrium has been disrupted so the intracellular water has no time to leave the cell. This causes intracellular ice formation and is a major factor of cell death in cryopreservation procedures[80].

4.4.3 Dehydration

Dehydration of the cell is a necessity but also here, the line between too much and too little dehydration is small. Too little dehydration, which happens when the cooling is done too quickly, causes intracellular ice formation, which is lethal to the cell. But when the cooling is done too slowly the cell will be totally dehydrated which is also lethal.

4.4.4 Extracellular ice formation

Where intracellular ice formation is lethal to the cell, extracellular ice formation is tolerated. But too much extracellular ice formation has also a downside; this can cause mechanic stress due to crushing[75].

The problems can be solved with permeating cryoprotective agents. Furthermore it is important to let the cooling be slow enough to avoid intracellular ice formation, but not too slow which would lead to a full dehydration of the cell. The ideal situation would be to know the permeability of the membrane of the used cells to adjust the amount of cryoprotective agents. Further also an ideal cooling rate to get a major efflux of water, in this way an intracellular crystallization is avoided. Therefore not all the water gets out of the cell, the viscosity of the unfrozen fraction becomes too high for any further crystallization and thus turns into an amorphous solid mass that contains no ice crystals. In this way we would get an ideal situation with higher rates on cell survival.

5. LITERATURE

5.1 Background

Cryopreservation is, indirectly, one of the solutions to compensate resorption. It would be a major breakthrough when there would be evidence that fat grafts keep their viability after cryopreservation. In order to get a complete and up to date review, literature was searched.

5.2 Adipose tissue harvest

5.2.1 Donor site

Is there a difference between viability and reliability of fat tissue when using lipoaspirate from different donor sites?

Rohrich found no difference between fat removed from abdomen, flank, thigh, and medial knee[81]. Padoin, however, reported a significant difference in cell concentration obtained at the different harvest sites. The cell concentration in the lower abdomen was greater than in other areas, but no significant difference was found in relation to the inner thigh[82]. Li revealed no ideal tissue donor site for fat grafting and SVF isolation[83]. Other studies weren't found on this matter and this lack of literature makes the 'ideal harvest site' undefined. Despite this uncertainty we believe choosing a site should be based on safety of access and the patient's preferences.

5.2.2 The liposuction technique: harvest

Should we prefer one type of liposuction above the other or is there no significant difference between different types of liposuction techniques?

Rohrich et al. analyzed adipose tissue harvested with traditional, ultrasound-assisted, and external ultrasound-assisted lipoplasty. Histological and enzymatical analysis of the different types of liposuction with their effect on cellular disruption revealed no significant effect of

external ultrasound or massage on the adipocytes[84]. Shiffman and Mirrafati used various cannulas, needles, suction pressures and centrifugation for collection and reinjection. No damage to the fat cells was found except for the collection of fat at -700mmHg vacuum[85]. Pump-assisted and hand-held syringe liposuction were compared by Leong in 2005. After testing the metabolic activity and adipogenic potential, no significant differences were observed [86]. In 2006, Smith concluded no unique combination of preparation or harvesting comparing the standard liposuction and syringe aspiration[55]. A 4-mm-diameter cannula seemed to give a higher number of viable adipocytes then 2 and 3-mm-cannulas, as concluded by Oszoy in 2006[87]. Furgeson made a comparative study of adipose aspirates harvested with conventional liposuction and with the LipiVage system[1]. While adipose-aspirate by both modalities maintain normal structure, the LipiVage system yields a greater number of viable adipocytes and sustains a higher level of intracellular enzyme activity within fat grafts and can potentially be a preferred method of choice for large-quantity fat graft harvesting[88]. Another comparison of the conventional liposuction and the Coleman technique was made by Pu in 2008. Both methods maintained a normal histologic structure but the Coleman technique yields a greater number of viable adipocytes and sustains a more optimal level of cellular function within fat grafts [89]. Erdim concluded the same as Oszoy[87], the viability was higher of grafts harvested with 6-mm-cannulas then with smaller ones[90]. Another more sophisticated technique was compared to conventional liposuction by Crawford in 2010. The Viafill system[2] (Lipose Corp., Maitland, Fla.)[91] provided a greater number of viable fat cells[92]. The Shippert technique[3] compared with the Coleman technique gave inconclusive results. Herold concluded a significant higher viability via the WST-8 test with the Coleman technique but with the annexin V/PI analysis, the viability of fat grafts was almost equal with both techniques[93]. Kirkham aimed to found out the perfect size of cannula for use in fat grafting. They concluded that a larger aspiration cannula led to improved graft retention and quality. It should be better using a five mm cannula then a tree mm cannula[94]. The last study found was performed by Lee in 2013. They suggested that shear stress is a more important variable regarding fat graft viability than pressure. Higher aspiration pressure up to -0.83 atm

1 'The LipiVage fat harvest, wash, and transfer system is a disposable, medical device developed by Genesis Biosystems Inc. (Lewisville, TX). This device harvests fat grafts at low vacuum level, washes fat grafts within a closed system, and avoids centrifugation, decant, or other unnecessary handling steps.'[80]

2 'The Viafill™ System uses gentle, manual aspiration rather than mechanical aspiration when harvesting fat cells. A patented transfer system and Viafill™ centrifuge (an apparatus that rotates at high speed and by centrifugal force separates substances of different densities) were designed to minimize damage to fat cells and increase the number of viable cells available for transplantation.'[83]

3 'The Shippert technique uses automatic liposuction with reduced negative pressure and abstains from centrifugation in order not to reduce viability of the graft by exposing it to centrifugal forces'.[85]

or positive pressure up to 6 atm did not affect graft viability. Nevertheless, slowly injected fat grafts with low shear stress had significantly better viability then fat injected with high shear stress[95].

In conclusion, one can say that there is no conclusive method to harvest adipose tissue. Five articles pointed out no significant difference but antoher five articles pointed out a significant difference. The fact these all used different non-conventional techniques made them also not comparable. One conclusion could be made; the use of a larger cannula (5 mm) should be considered as a preferred technique to harvest the fat grafts.

5.2.3 The liposuction technique: fat processing

The techniques, most often used, for preparing grafts are centrifugation, washing with filtration, unit gravity sedimentation and decantation. Fat processing is done to remove blood, debris and free lipid, while the viable adipose tissue is retained. Literature was reviewed in order to find an evidence-based protocol to process fat after liposuction.

Boschert (2002) analyzed the centrifugation times. Lipoaspirate samples were centrifuged at 50 g for 2, 4, 6 and 8 minutes. Centrifugation beyond 2 minutes did not increase the number of viable adipocytes. The study showed that the bottom layer contained the highest concentration of viable cells [96]. Butterwick performed a prospective, randomized, double-blind comparison study that compared centrifuged versus non-centrifuged fat, grafted in hands. After 5 months the centrifuged fat showed a better longevity and aesthetic result[97]. However, Rohrich concluded that centrifugation (500g for 2 minutes) did not appear to enhance immediate fat tissue viability before implantation[81].). Ramon did not found any difference in survival at 16 weeks in vivo when centrifuged fat (1500 rpm for 5 minutes) or lipoaspirate treated with an open method using an operating room cotton towel was used [98]. In 2006 Smith compared different combinations of centrifugation and/or washing the cells with lactated Ringer's solution or normal saline. No unique combination of preparation techniques appeared to be more advantageous on transplanted fat grafts at 3 months[55]. Washing, centrifuging, and sedimentation were compared by Rose. Sedimentation appears to yield a higher proportion of viable adipocytes than does washing or centrifuging[99]. In order to find the best centrifugation technique Kurita compared 400, 700, 1200, 3000 or 4200 g for

3 minutes with a non-centrifugal fat graft. Excessive centrifugation can destroy adipocytes and adipose-derived stem cells, but appropriate centrifugation concentrates them, resulting in enhanced graft take. The authors recommend 1200 g as optimal centrifugal force[100].

In 2009, Khater concluded that transplantation of non-centrifuged adipose tissue, washed by serum, contained more active preadipocytes which could possibly lead to better potential chances of survival[101]. Kim centrifuged fat samples for 1, 3, and 5 minutes at 1500, 3000, and 5000 rounds per minute (RPM), respectively. Cell survival rates were significantly lower for the groups centrifuged at 1500 and 3000 rpm for more than 5 minutes and for the group centrifuged at 5000 RPM for more than 1 minute. They recommended centrifugation at 3000 rpm for 3 minutes[102]. Xie concluded a more harmful effect on the viability of fat grafts with an increase in centrifugal forces, especially when rotation speed is greater than 1145 g[103]. Different methods of fat grafting were investigated by Minn. Centrifugation, metal sieve and cotton gauze were compared suggesting closed centrifugation technique has no advantage over the open cotton gauze technique in terms of fat graft viability. They also concluded that the metal sieve concentration method is deficient as a preparation method because it can cause grafted fat degradation.[104]

Another study comparing the influence of decantation, washing and centrifugation (3000 rpm for 3minutes) was done by Condé-Green. Adipocyte counts were significantly greater in decanted lipoaspirates compared with centrifuged lipoaspirates, which showed a greater majority of altered adipocytes[105]. They also showed that after centrifugation the pellet (the greatest concentration of endothelial cells and mesenchymal stem cells, which play a crucial role in the angiogenic and adipogenic effect of the grafted tissue) and middle (great majority of altered adipocytes and very few mesenchymal stem cells) layer should be used to increase fat graft survival[106]. Botti made the comparison with filtered and washed fat grafts and centrifuged fat grafts (3000rpm for 3minutes). They concluded there was no significant difference between the two fat-processing techniques[107]. The optimal centrifugal force was investigated by Ferraro in 2011. They compared 3000rpm for 3 minutes, 1300 RPM for 5 minutes and simple decantation. In conclusion, a centrifugal force of 1300 RPM resulted in better density of adipose tissue, with good cell viability and increased ability to preserve a significant number of progenitor cells[108]. Also Pulsfort examined the ideal centrifugal force. In contrast to Ferraro[108] they found no effect on the survival rate, even the higher the centrifugation the better the cleansing of oil and cell debris[109]. Hoareau concluded more in line with Ferraro[108] that strong centrifugation (900 g, 1800 g) leads very quickly to greater adipocyte death. Gentle centrifugation (400 g/1 min), preceded by washing, is therefore the

most acceptable method[110]. Another study, done by Condé-Green in 2013, evaluated the outcome of the most common processing techniques for fat grafting - decantation, washing, high-speed centrifugation - and stromal vascular cell-supplemented lipotransfer. Cell-supplemented lipotransfer had optimal outcome for graft retention, viability and vascularity, while washing resulted in high viability with a less intensive process. High-speed centrifugation resulted in consistent volume retention but lower viability. Each of these approaches is ideal under different circumstances and contributes to the versatility and reliability of fat grafting[111]. Zhu also investigated various fat processing methods such as: gravity separation, Coleman centrifugation and simultaneous washing with filtration using a commercially available system (Puregraft; Cytori Therapeutics, Inc., San Diego, Calif.), all compared to a no manipulation control. All test graft preparation methods exhibited significantly less aqueous fluid and blood cell content compared with the control. Though washing with filtration within a closed system produces a fat graft with higher tissue viability and lower presence of contaminants compared with grafts prepared by alternate methods[112].

Reviewing these articles, four articles were found showing no significant difference between centrifugation and other fat processing techniques, four others concluded different. One preferred decantation, two preferred washing and one other preferred sedimentation. Here we can conclude there is no evidence to prefer one technique above the other, although centrifugation is the easiest and mostly used. Three articles were found concluding it's better to centrifuge the fat grafts than to do nothing. The time of centrifugation did not affect the viability but the forces were more important. Five articles described it should be better to use lower forces than high ones. The ideal force is inconclusive but should be below 3000 RPM.

5.2.4 The liposuction technique: transport

There were no specific articles found that studied the best way to transport lipoaspirate. Several used methods are; without precautions[2, 90, 113-116) (117], on ice[1], in insulated containers[118], in RPMI 1640 culture medium supplemented with 100 U penicillin, 100 mg streptomycin and 250 mg amphotericin B / ml[119].
There is also no consensus in this part of the protocol. The method, mostly used is without any precautions.

5.3 Use of cryoprotectans

Is there a difference between fat frozen with or without cryoprotective agens?

The first study was done by Pu in 2004. They made a randomized trial using 3 groups: 1/ the control group (fresh adipose aspirates without preservation); 2/ cryopreservation with liquid nitrogen only and 3/ cryopreservation with CPA consisting of a combination of dimethyl sulfoxide (DMSO) and trehalose. DMSO and trehalose were the most optimal cryoprotective combination, albeit not as ideal as fresh specimens.[116]
In 2005, Wolter investigated the beneficial effect of cryoprotective agents. Fat cells were harvested with the Coleman technique and with various CPA such as DMEM, DMEM + 10% HES, DMEM + 10% glycerol, DMEM + 10% glycerol, 5% dextran, and 5% polyvinyl pyridine. After adding the CPA the samples were cooled down to -20 and -80 degrees. Afterwards they compared the thawed samples to fresh ones. The results were unsatisfying for viability. They concluded cryopreserving at -20°C leads to injecting mostly dead cells. A preservation of up to 54% of baseline activity after adding a CPA was observed, yet no specific CPA was found[120]. Also in 2005, Moscatello investigated several experimental CPAs. The following groups were included, no cryprotective agens, 10% DMSO, 7,5% polyvinylpyrrolidone 40/7,5% DMSO, 10% glycerol, 10%glycerol/10% FBS. The viability tests were performed by Fluorescein Diacetate–Propidium Iodide Staining. They indicated DMSO as the most preferable CPA with the highest viabilityrate[118]. Three groups of specimen were investigated by Pu: the control group, fresh adipose aspirates without preservation; the simple cryopreservation group (no CPA); and the optimal cryopreservation group (with trehalose as a CPA). They concluded that an optimal cryopreservation method using trehalose as a CPA appears to provide better long-term preservation of adipose aspirates than a simple cryopreservation method [121].

A fresh control group, an optimal cryopreserved group (DMSO 0,5 M and trehalose 0,2 M) and a simple cryopreserved group were compared by Pu. They concluded that more maintained volume, weight and fatty tissue structure of injected free grafts were found in the optimal cryopreservation group compared with the simple cryopreservation group, but the results were still less satisfactory than those in the fresh control group[115].

A three-part try-out was done by Cui. In their first research they ^used several combinations of CPAs to cryopreserve free fat grafts, such as 0.2 M Me2SO and 0.1 M trehalose; 0.5 M Me2SO and 0.2 M trehalose; 0.25 M trehalose; 0.5 M trehalose; 1.0 M Me2SO; 1.5 M Me2SO. Compared to a control group of unfrozen fresh adipocytes they concluded that the highest recovered integrated adipocyte count was found in the group using a combination of 0.5M Me2SO and 0.2 M trehalose as CPA[113]. The second study made the comparison using trehalose as a CPA in seven different concentrations and fresh adipose-aspirate. They concluded trehalose as a CPA with a concentration of 0.35 mol/l, appearing to provide the optimal protection of adipose aspirates during cryopreservation. Further in vivo study will be needed to confirm these findings[6]. In the third part of their search, Cui did an in vivo study using nude mice and injecting them with free fat grafts. In the control group, 0.5 mL of fresh fat grafts were injected into the posterior scalp of nude mouse. In the cryopreservation group 1, a combination of dimethyl sulfoxide (in 0.5M) and trehalose (in 0.2M) was used as a CPA. In the cryopreservation group 2, only the optimal concentration of trehalose (in 0.35M) was administered as a CPA. They concluded trehalose, in its optimal concentration, to be similar to the protection provided by DMSO and trehalose as a CPA[3].

Pu made the comparison between fat harvested by the Coleman technique without cryopreservation and fat harvested with the Coleman technique and afterwards cryopreserved with a CPA, consisting of 0.5 M (3.3%) DMSO and 0.2 M (7.6%) trehalose. Their results indicate that autologous fat grafts harvested with the Coleman technique and preserved with our preferred cryopreservation method have a normal histology with near the same number of viable adipocytes as compared with the fresh fat grafts. However, the cryopreserved fat grafts appear to have a less optimal level of adipocyte specific enzyme activity compared with the fresh ones, they may not survive well after they are transplanted [4].

The last study found was executed by Li in 2011. They made the comparison between fat stored in normal saline and stored in hydroxyethyl starch. Afterwards both samples were cryopreserved at -20, -80 and -196 °C. After two and seven days, viability was tested. Two and four weeks after the fat tissue was injected into nude mice. After three months the fat was harvested again for histologic examination. They found no differences in cell viability among the three temperatures and with the use of a cryoprotective agent[1].

Reviewing the literature we could conclude that using a CPA should definitely protect the fat grafts more than without one, only one study concluded otherwise. The preferred CPAs in the literature are DMSO and trehalose. Ideal concentrations are not conclusive and further

investigation is required. Although they are mostly used as a combination, one study showed the viability was similar using DMSO and trehalose as only using trehalose. But further research would also be preferred.

5.4 Method of cooling

Is there an ideal way of cryopreserving the fat grafts? What would be the mostly preferred storage temperature?

The first article reporting on this part of the protocol is executed by Lidagoster in 2000. They stored specimen at -16 degrees C or 1 degree C for a period of 1 or 2 weeks. The control group underwent immediate implantation. A histological comparison, of the experimental and control group, clearly demonstrated a decrease in viable adipocytes and an increase in signs of inflammation and fat cell necrosis in those animals that received stored fat instead of immediate fat implantation[122]. Wolter harvested fat cells using the Coleman technique and tested two other temperatures. Viability analysis was done after 0, 2, 7, 14, and 30 days of cooling to -20 degrees C and -80 degrees C, and after addition of various CPAs. They concluded -80°C as a more preferable cooling temperature, -20°C led to injecting mostly dead cells[120]. Erdim prepared fatty aspirate from the 6 mm cannula. These were stored at temperatures of +4, −20 and −80 °C for 2 weeks. They detected that storing fatty tissue at +4 °C provided higher numbers of viable adipocytes compared to dry frozen specimens (−20 and −80°C)[90]. Comparison of - 15 degrees and - 70 degrees cryopreservation, after abdominal liposuction, was done by Son in 2010. They demonstrated that cryopreservation of lipoaspirated fats at -15°C and -70°C results in a limited number of viable cells. Both seem to be inadequate to maintain the viability of fat cells and thus unusable for cryopreservation of fat tissue[2].

Although there is no evidence of an ideal temperature, we suggest it should be below -130°C because of the stop of metabolic activity at this point[120]. The mostly used manner to cryopreserve fat grafts in published articles is the method of slow cooling. The fat grafts are first cooled down to -30°C with a constant decline of 1°C/ minute, then they let it rest for 10 minutes at -30°C, afterwards its been transferred to -196°C (liquid nitrogen) [1, 113-116], [120], [4].

5.5 Method of thawing

Is there an ideal way to thaw the cryopreserved fat grafts?

There were no specific articles found comparing different types of thawing methods. The recent experimental studies imply two types of thawing methods although there is no scientific proof of one being better than the other. Five studies were found using slow cooling: thawing at room temperature for one or more hours [2, 90, 97, 119, 123]. Seven studies were found using fast cooling; 2 minutes at room temperature, afterwards dropped into a stirred 37°C water bath and removal of the CPA by dilutions and centrifugation[4, 113, 115, 116, 118-120].

5.6 Method of viabilityanalysis

Is there a specific viability test we can use to achieve a reliable result related to the viability of free fat grafts?

In 2005, Lei wanted to establish a new method to accurately measure the viability of fat for grafting, which can lay a foundation for the further study of fat transplantation. They centrifuged 5 groups of fat respectively 1000 RPM, 2000 RPM, 3000 RPM, 4000 RPM and 5000 RPM, took out 12 samples of 5ml from every group of centrifuged fat and put them into dishes to incubate with DMEM containing glucose for one hour. The glucose consistency of DMEM was tested in all dishes and compared with 5 control dishes. The glucose transportation quantities of group 1000 RPM, 2000 RPM, 3000 RPM, 4000 RPM and 5000 RPM decreased gradually. Histopathologic examination didn't show the difference among the five dishes. They concluded the glucose transportation test could be used to accurately measure the viability of fat. The histopathologic examination can reveal fat viability[124]. Investigation of a reliable method to assey viability and number of adipocytes or other cellular components in adipose tissue was done by Suga. The authors assessed cell viability and number of cells, obtained from 1g of suctioned adipose tissue and respective layers (the top, middle, and bottom layers) before and after digestion and centrifugation, using cell staining with HOECHST 33342, Nile Red and propidium iodide, the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino)carbonyl]-2H-tetrazoliumhydroxide-assay (XTT) and

glycerol-3-phosphate dehydrogenase assay. The correlation between the number of prepared cells (adipocytes, adipose stromal cells and white bloodcells) and the resulting values from the XTT and glycerol-3-phosphate dehydrogenase assays were also examined. Both the XTT and glycerol-3-phosphate dehydrogenase assays provided good correlations between the number of viable adipocytes and resulting values, but only the glycerol-3-phosphate dehydrogenase assay was strictly specific for adipocytes. They concluded that the single use or a combination of the viability assays used in this study can appropriately determine the number of adipocytes and other cells, although it remains difficult to assess original cells directly without tissue dissociation[7].

In 2012, Lee demonstrated the effectiveness of an improved method of accurate adipocyte analysis using an automated cell counter. Human lipoaspirate was obtained, centrifuged and digested. Samples were analyzed using a hemocytometer and an automated cell counter with two viability dyes. Results were then optimized by novel methods of preparation using carboxymethyl cellulose and formalin. They concluded that the novel method could more accurately identify the viable adipocyte population without the limitations of traditional cell counting. In addition, the use of carboxymethyl cellulose and formalin in the preparation process can decrease variability and stabilize cell counts over time. This is an efficient, specific, and reliable method of adipocyte analysis[125].

The experimental studies implying testing of adipocytes used the following techniques; eleven times histologic examination[1, 4, 55, 99, 104, 113, 115, 116, 119, 122, 123], four times MTT [1, 117, 119, 120], four times G3PDH activity (cell stability)[2, 4, 116, 120], three times trypan blue viability[4, 116, 120], tree times XTT [2, 55, 104], staining with two times fluorescein diacetate and propidium[2, 118], supravital dye staining[55], weight[55, 104, 113, 115, 123], volume[113, 115, 123],cell count[113], oil red O staining[126], Elisa of leptin secretion[126], PCR adipogenix related genes (adpigenic function)[126] and culture [114, 118].

Although there is no consensus and the methods used in experimental studies are really different, we can conclude it will be impossible to analyze the reliability and viability with only one test. To get all the information we will be obliged to use different tests, which each have their own specific aspect of assessing.

6. RESULTS

6.1 Background

Combining the literature data and experts' opinions we were able to create this protocol. It was arranged in this way to be both feasible and easy. In this protocol the cryopreservation is only elaborated for small amounts of lipoaspirate material. The protocol can easily be adjusted for larger amounts by just adapting all the volumes of used reagentia to the volume of fat you want to cryopreserve. In an experimental setting it is easier to work with small amounts but the actual purpose of this try-out would be cryopreserving larger amounts of fat in the future. So if the protocol is usable for small amounts, the next step would be to try it for larger amounts.

6.2 Prelevation of the lipoaspirate material

6.2.1 Initial selection of potential harvest site

Adipose tissue will be obtained from healthy adult patients with informed consent. Only adipose tissue that would normally be discarded during elective procedures will be used. The area should be based on ease, safety of access, the preference and requests of the patient.

*Remark: the informed consent should be in order BEFORE the procedure.

6.2.2 Prelavation of the fat tissue

The fat will be obtained using the standard liposuction procedure. The patient's donor site will be infiltrated with 1l of lactated Ringer's solution, 30 ml of 2% lidocaine, and 1 ml of epinephrine. Adipose tissue will be harvested with the conventional devices with usage of 5 mm cannulas, a bigger cannula is used to create less damage to the fat grafts (cfr literature conclusions). The liposuction will also be gently executed in order to avoid damage to the fat cells. The purpose is to obtain 50 ml of lipoaspirate material that will be collected in a 50 cc syringe. After the fat has been removed the syringes will be be centrifuged in the OR room at 3000 RPM during 3 minutes (1200 G) as we concluded from the literature. After the

centrifugation three layers appear. The bottom layer, which contains a concentration of blood products and cell debris, is removed by just simply opening the syringe and letting the oil and blood run out. Afterwards only the middle layer will be transported into another syringe. In this way one syringe of 50cc containing the highest amount of adipocytes, is collected.

6.2.3 Temporary preservation of the material at 4°C

In expectation of the surgical logistician, the syringe and the blood sample will be stored into a refrigerator with a stable temperature of 4 °C. The surgical nurse informs the surgical logistician, by telephone, that the sample is ready for collection. We choose this type of preservation to create a stable environment for the material.

6.2.4 Transport of the material

As there was no consensus in the literature we do prefer a transport in a stable environment. The surgical logistician brings a transportation box with him. This box contains melting ice, which will create the stable environment for the lipoaspirate material. The box will be transported to the tissue bank.

6.3 Testing initial viability

This testing is largely based on the 'Numerical Measurement of Viable and Nonviable Adipocytes and Other Cellular Components in Aspirated Fat Tissue'-trial[7]. This trial investigated the ideal way to test lipoaspirate on viability. The used methods were also known with our experiene and seemed to be most reliable and easy.

a) preparation

If we test the viability after freezing we'll have to be able to compare it to an initial viability béfore freezing. Therefore 5ml of initial lipoaspirate material must be pipetted and mixed with 2ml of collagenase 0,075% in phosphate-buffered saline. Afterwards, incubate the recipient

for 30 minutes at 37°C. To terminate the digestion 2ml of phosphate-buffered saline containing 10% fetal bovine serum is added. The final step is to centrifuge the recipient for 5 minutes at 3000 RPM so also here a top, middle and bottom layer is created. Each layer will be investigated by a cell staining morphometric assay and an XTT assay.

6.3.1 Cell staining morphometric assay

6.3.1.1 Distinguishing adipocytes from floating lipid droplets

At first, the adipocytes must be distinguished from floating lipid droplets, released from broken adipocytes. Therefore the NILE RED and HOECHST 33342 staining are used.

a) background

The dye Nile Red, 9-diethylamino-5H-benzo[alpha]phenoxazine-5-one, is an excellent vital stain for the detection of intracellular lipid droplets by fluorescence microscopy and flow cytofluorometry. The dye is very soluble in the lipids it is intended to show and it does not dissolve them. Another reason we choose this dye is because it is strongly fluorescent when it is partitioned in a hydrophobic environment[(127)]. If we can evince intracellular triglycerides we can conclude these cells to be adipocytes[(128)], but Nile Red stains both intracellular triglycerides as floating lipid droplets from raptured adipocytes. This is why also HOECHST 33342 must be used. This stain, (2'-(4-ehtoxyphenyl)-5-54-methyl-1-piperazinyl)-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate), is a cell-permable DNA stain that is excited by ultraviolet light and emits blue florescence at 460-490nm. It is used for specific staining the nuclei of living of fixed cells and tissues. So after both adding these stains we can conclude; only Nile Red staining to be floating lipid droplets from raptured adipocytes, only HOECHST 33342 staining to be cells, but not adipocytes and both the stains to be non raptured adipocytes.

b) procedure

Take each layer of the centrifuged fat and resuspend it in phosphate-buffered saline to a total of 5 ml. Add both stains and conclude which ones are adipocytes, which ones are just droplets and which ones are non-adipocytes under the fluorescence microscoop.

6.3.1.2 Distinguishing viable cells from dead cells:

To determine viable cells from death the different layers must be stained with HOECHST 33342 (cfr. supra) and propridium iodide.

a) Background

Propridium iodide is a membrane impermeant dye that is generally excluded from viable cells. It determines if cells are still intact or broken, therefore it binds to double stranded DNA by intercalating between base pairs. So if PI is bound we know the cell membrane is ruptured and the DNA is free in the suspension. It emits at a maximum wavelength of 617 nm. A combination of HOECHST and propridium iodide has been extensively used for simultaneous flow cytometric and fluorescence imaging analysis of the stages of apoptosis and cell-cycle distribution[(129)].

b) Procedure

Take each layer of the centrifuged fat and resuspend it in phosphate-buffered saline to a total of 5ml. Add both stains; HOECHST 33342 and propridium iodide. After staining next conclusions can be made:

	HOECHST 33342	*Propidium iodide*	*Including lipid ?*
Viable adipocyt	*Positive (blue)*	*Negative*	*Positive*
Dead adipocyte	*/*	*Positive (red)*	*Positive*
Viable non adipocyt	*Positive (blue)*	*Negative*	*Negative*
Dead non adipocyt	*/*	*Positive (red)*	*Negative*

To conclude, three random fields are photographed of each layer and the amount of different cells is counted. Then the total cell number of 1 ml should be calculated.

6.3.2 XTT assay

a) Background

To determine the viability of all the cells in the lipoaspirate, we have chosen for the XTT assay. This was one of the most used in the literature and it seems, in our experience, one of the easiest and most reliable assays. The XTT assay is a colorimetric assay, which analyzes the number of viable cells by the cleavage of tetrazolium salt added to the culture medium. It is easy in use washing and harvesting of the cells is not necessary. The MTT assay was also an option but we prefer the XTT assay because the cleavage product of XTT is soluble in water, so the MTT-solubilization-step is not required.

b) Principle

When the tetrazolium salt XTT is mixed with the cell suspension we get the production of a soluble formazan salt. This formazan production is the result of the cleavage of the tetrazolium salt XTT by the succinate-tetrazolium-reductase-system which belongs to the respiratory chain of the mitochondria. This system is only active in metabolically intact cells.

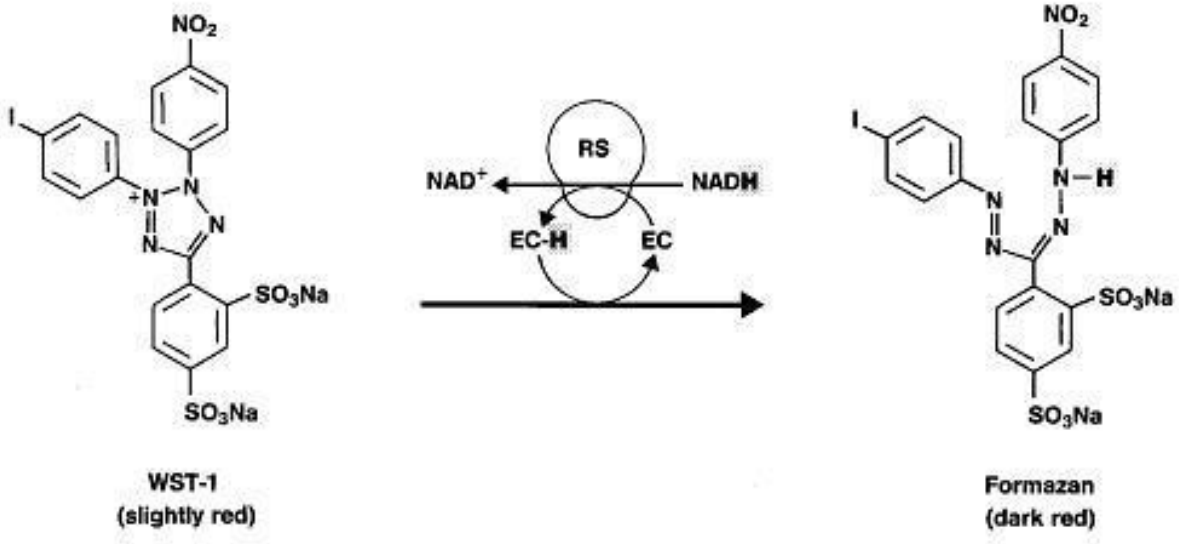


Fig11 : The formazan production is the result of the cleavage of the tetrazolium salt XTT by the succinate-tetrazolium-reductase-system which belongs to the respiratory chain of the mitochondria. [130]

The formed formazan dye is quantitated using a scanning multi-well spectrophotometer (ELISA reader). The measured absorbance directly correlates to the number of viable cells[130].

c) Procedure

Each layer of the centrifuged fat is resuspended in phosphate-buffered saline containing 10% fetal bovine serum, to a total of 5ml. The XTT-labeling reagent and electron-coupling reagent are mixed at a ratio of 50:1. 2,5ml Of this is added to the sample and the mixture should be incubated in a six well plate for four hours at 37°C. After incubation, 150 microliter must be transferred to a 96-well plate. Finally the absorbance should be measured at a test wave-length of 450 nm and a reference wavelength of 650 nm.

6.4 Cryopreservation of the lipoaspirate material

6.4.1 General remarks

All operations are done under a laminar-air-flow-environment in a cleanroom background, class C.

6.4.2 Preparation

We have chosen for DMSO as cryoprotectans. Trehalose was also described in the literature but as we have no experience with trehalose, this wasn't a logical choice. The concentration of freezing fluid is concluded from our own experience. The amount should be 1:1, as concluded from the literature. After the laminar flow is put on, next solution should be prepared:

- In a sterile falcon 15ml: 15% freezing liquid: 2,25ml DMSO + 12,75 ml basic medium

6.4.3 Final Procedure

Pipette 5 ml of lipoaspirate material into a sterile recipient and pipette 5 ml of freezing liquid in the same recipient. Both the substances should be mixed properly by pipetting up and down. After the pipetting the mixture is transported into a self-standing 10ml cryovial. In this way we repeat the procedure another two times so we get two recipients for the staining and one for the XTT assay. After mixing, the recipients should be placed into a styrofoam box. The box is then put in a -80°C freezer for at least 24 h (cooling at 1°C per minute). The final step is to transfer the recipient into a -150°C freezer. Although the literatrue discribed a freezing temperature of -196°C in liquid nitrogen, we have chosen for the -150°C freezer. It is much safer and easer in use. Furthermore it reaches -130°C, so all metabolic activity is stopped[(120)].

6.5 Thawing and viability analysis of the post-cryopreservated lipoaspirate material

As concluded in the literature we chose for the quick thawing procedure. The recipient will be taken out of the fridge and will be rested at room temperature for 2 minutes. Afterwards it must be dropped into a stirred 37°C water bath. When the recipient is fully thawed the CPA must be removed by dilutions and centrifugation.

Afterwards the steps of the initial viability should be repeated. Five mililiters will be used for the morphometric assay with Nile Red and HOECHST 33342, another with HOECHST 33342 and propridium iodide staining and 5ml will be used for the XTT assay.

7. A LOOK INTO THE FUTURE

7.1 Clinical applications

The use of fat tissue isn't limited to plastic surgery only. The fat tissue is very easy to obtain, it contains more mesenchymal cells than the bone marrow and the stroma vascularfraction is able to differentiate and secrete growth angiogenic and antiapoptotic factors. Due to these benefits it has become more attractive in regenerative medicine[131].

Recent developements in the traetement of localized scleroderma has also shown a future role for fat tissue. Localized scleroderma is an autoimmune disease and is characterized by skin and visceral fibrosis and ischemic phenomena. It can lead to severe organ lesions but patients are mostly concerned about the hands and face sclerosis[131]. The appearance of the skin and functional limitation of the hands can cause serious psychological disability[132]. Yet causal therapy isn't available, only symptoms can be treated. Because of the benefits of fat tissue, some trials[132-135] have been done considering reinjecting fat on places that are affected. The principle remains the same as with lipofilling; the depressions are filled for aesthetic purposes but also the regenerative aspects of the fat tissue are really beneficial. Several trials[132-135] have shown some promising results but further investigation is really necessary.
Not only the lipofilling procedure is usefull but also the cryopreservation of the fat tissue would be beneficial. Patients could get more injections spread over time if the first treatments weren't successful, without necessity of repeated liposuction procedures. If the therapy is succesful, storaged fat could be used for future outbreaks of sclerosis with these patients.
But further investigations on both fat as treatment and cryopreservation are necessary.

7.2 Allogeneic transplantation

Just as Montegazza had the revolutionary idea to create banks for frozen human semen[67], this idea could also be useful with fat tissue. The lipoaspirate would always be available; for scientific investigations, cancer patients could store fat for future reconstructions, scleroderma patients for future outbreaks and many other purposes. Also allogeneic transplantation could be an option then. Skinny people, who are not suitable for a lipofilling procedure, could be helped with allogeneic transplantation.

But of course these are all futuristic suggestions, a lot of questions and problems around this theme must be solved first.

7.3 Future viability analysis

Fat tissue will be more and more investigated in the future because of the increasing rates of obesity and diabetes. It is very important to separate adipocytes from other cells to investigate them. Up to now there is no adipocyte specific surface marker available and this has encouraged the industry to create advanced tools to study fat tissue. 'Union Biometrica has developed a method for high-throughput analysis and sorting of intact adipocytes based on the nuclear stain (Hoechst). Sorted cells stay intact and could be used for the downstream applications including imaging and sequencing assays'[136]. The BioSorter® is the first in its kind but it won't take long until other, even more sophisticated tools to make the investigation of fat tissue easier, will be developped.

8. DISCUSSION

The purpose of this thesis was to create a literature-based protocol to cryopreserve lipoaspirate for future purposes. There is a need for a standard protocol as cryopreservation is an interesting application that could have some indications in plastic surgery and other disciplines. The most important application, and the reason we did this thesis, would be to get a solution to the resorption problem during lipofilling.

The lipofilling procedure is the act of transplantation of own fat, harvested with liposuction, into other regions of the body. Free fat transfer offers treatment for soft tissue defects caused by trauma, cancer, congenital anomalies, painful scars, irradiation injuries and aesthetic indications[8]. The major problem with lipofilling is the resorption rate; one procedure is hardly ever enough to the get the wanted results. Patients must get three to four lipofilling procedures dependent on the resorption, wanted enlargement or indication. In case of these successive lipofilling procedures there will be an increase of morbidity despite the low rate. One solution to the problem would be cryopreservation of the fat[1-6]. In this way one liposuction procedure would be sufficient and when resorption has taken place the preserved fat could be used, even on an ambulatory basis, in order to achieve the intended volume without the repeated liposuctions. So in this way, it would be a major breakthrough when there would be evidence that fat grafts keep their viability after cryopreservation.

In order to get a complete and up-to-date protocol, literature was reviewed. We searched literature of every part of the protocol to get a full image of all the different methods. We have investigated the donor site, the liposuction technique, the fat processing, transport, the use of cryoprotectans, method of cooling, thawing and use of viability analysis. In general we can conclude there was not an overdose of articles, few conclusions could be made. To fill in the gaps own experience and the trail done by Suga[7], were used.

In the end the following protocol was created:

In the beginning adipose tissue will be obtained from healthy adult patients with informed consent. Only adipose tissue that would normally be discarded during elective procedures will be used. The choice area should be based on ease, safety of access, the preference and

requests of the patient. The fat will be obtained using the standard liposuction procedure. After the fat has been removed, syringes will be filled and centrifuged in the OR room at 3000 RPM during 3 minutes. This will create three layers, only the middle layer will be transported into another syringe. The syringes and the blood samples will be stored into a refrigerator with a stable temperature of 4 °C. As there was no consensus in the literature we do prefer a transport in a stable environment. The fat will be initially tested with Cell staining morphometric assays. First the lipoasirate will be stained with HOECHST 33342 and Nile Red to distinguish adipocytes from floating lipid droplets. Afterwards with HOECHST 33342 and propridium iodide to distinguish viable cells and non-viable cells. To get an image of the whole viability we have chosen for the XTT-assay. In this way we achieve an initial viability to have a starting point afterwards. The next step is to cryopreserve the remaining samples, these will be mixed with the cryoprotectans DMSO. Afterwards, the recipients should be placed into a styrofoam box. The box is then put in a -80°C freezer for at least 24 h (cooling at 1°C per minute). The final step is to transfer the recipient into a -150°C freezer. Afterwards the thawing will be done with the quick procedure in a 37°C waterbath. When the thawing is completed, the cryoprotectans must be removed. To investigate the post-cryopreserved samples, we repeat the steps of the initial viability. Two times five milliliters will be used for the morphometric assay with Nile Red, HOECHST 33342 and propridium iodide staining and another 5 ml will be used for the XTT assay. In the annex we added the standard operating procedures which are more detailed and step by step.

The aim of this thesis was to create an easy and feasible protocol. Yet trials around this theme are already done, they all use different methods. We have filtered the most effective ones and could use them to conclude different parts of the protocol. Due to the small amount of literature not every single part of the protocol is literature-based. Sometimes it was impossible to draw conclusions. These gaps were filled with experts' opinions and the trial done by Suga[(7)]. This could be considered as a weak point, because some parts are not fully based on evidence in literature. The current protocol is also purely theoretic, further validation is needed.

Despite the lack of validation, I think we succeeded in creating a protocol that is both easy as feasible and could be used as guidance in future trials. It is a great, first step in creating a useful protocol for future trials.

9. REFERENCES

1. Li BW, Liao WC, Wu SH, Ma H. Cryopreservation of fat tissue and application in autologous fat graft: in vitro and in vivo study. Aesthetic plastic surgery. 2012;36(3):714-22. Epub 2011/12/23.
2. Son D, Oh J, Choi T, Kim J, Han K, Ha S, et al. Viability of fat cells over time after syringe suction lipectomy: the effects of cryopreservation. Annals of plastic surgery. 2010;65(3):354-60. Epub 2010/08/25.
3. Cui X, Pu LL. The search for a useful method for the optimal cryopreservation of adipose aspirates: part II. In vivo study. Aesthetic surgery journal / the American Society for Aesthetic Plastic surgery. 2010;30(3):451-6. Epub 2010/07/06.
4. Pu LL, Coleman SR, Cui X, Ferguson RE, Jr., Vasconez HC. Cryopreservation of autologous fat grafts harvested with the Coleman technique. Annals of plastic surgery. 2010;64(3):333-7. Epub 2010/02/25.
5. Pu LL. Cryopreservation of adipose tissue. Organogenesis. 2009;5(3):138-42. Epub 2010/01/05.
6. Cui X, Pu LL. The search for a useful method for the optimal cryopreservation of adipose aspirates: part I. In vitro study. Aesthetic surgery journal / the American Society for Aesthetic Plastic surgery. 2009;29(3):248-52. Epub 2009/07/18.
7. Suga H, Matsumoto D, Inoue K, Shigeura T, Eto H, Aoi N, et al. Numerical measurement of viable and nonviable adipocytes and other cellular components in aspirated fat tissue. Plastic and reconstructive surgery. 2008;122(1):103-14. Epub 2008/07/03.
8. Kauhanen S, Peltoniemi H. [Free fat transfer--a versatile tool in reconstructive surgery]. Duodecim. 2012;128(20):2074-84. Epub 2012/11/22. Rasvansiirto--korjaavan kirurgian yleistyva tyokalu.
9. Property of Dr. Stillaert F. PS, UZGent.
10. Langer R, Vacanti JP. Tissue engineering. Science. 1993;260(5110):920-6. Epub 1993/05/14.
11. Crandall DL, Hausman GJ, Kral JG. A review of the microcirculation of adipose tissue: anatomic, metabolic, and angiogenic perspectives. Microcirculation. 1997;4(2):211-32. Epub 1997/06/01.
12. Crandall DL, DiGirolamo M. Hemodynamic and metabolic correlates in adipose tissue: pathophysiologic considerations. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 1990;4(2):141-7. Epub 1990/02/01.
13. Di Girolamo M, Mendlinger S, Fertig JW. A simple method to determine fat cell size and number in four mammalian species. The American journal of physiology. 1971;221(3):850-8. Epub 1971/09/01.
14. Alexander JK, Dennis EW, Smith WG, Amad KH, Duncan WC, Austin RC. Blood volume, cardiac output, and distribution of systemic blood flow in extreme obesity. Cardiovascular Research Center bulletin. 1962;1:39-44. Epub 1962/01/01.
15. Plagemann K. Zum 150 Geburtstag von Gustav Adolf Neuber (1850-1932). Schleswig-Holsteinisches Ärzteblatt. 2000(12).
16. Billings E, Jr., May JW, Jr. Historical review and present status of free fat graft autotransplantation in plastic and reconstructive surgery. Plastic and reconstructive surgery. 1989;83(2):368-81. Epub 1989/02/01.

17. Mojallal A, Foyatier JL. [Historical review of the use of adipose tissue transfer in plastic and reconstructive surgery]. Annales de chirurgie plastique et esthetique. 2004;49(5):419-25. Epub 2004/11/03. Historique de l'utilisation du tissu adipeux comme produit de comblement en chirurgie plastique.
18. Klein AW, Elson ML. The history of substances for soft tissue augmentation. Dermatologic surgery : official publication for American Society for Dermatologic Surgery [et al]. 2000;26(12):1096-105. Epub 2001/01/03.
19. From Wikipedia tfe. Conrad Gesner; biography. 2014.
20. From Wikipedia tfe. Gustav Adolf Neuber; biography. 2014.
21. Czerny V. Drei Plastische Operationen. III. Plastischer Ersatz der Brustdrüse durch ein Lipom. Verhandlungen der Deutschen Gesellscahft für Chirurgie. 1895;II:216-7.
22. Hinderer U, Del Rio J. Erich Lexer's mammoplasty. Aesth Plast Surg. 1992(16):7.
23. Bruning P. Contribution a l'étude des greffes adipeuses. Bull Acad R Med Belg. 1919(28):5.
24. Peer LA. The neglected free fat graft. Plast Reconstr Surg (1946). 1956;18(4):233-50. Epub 1956/10/01.
25. Fischer A, Fischer G. Revised technique for cellulitis fat. Reduction in riding breeches deformity. Bull Int Acad Cosm Surg. 1977(2):40.
26. Illouz YG. The fat cell "graft": a new technique to fill depressions. Plastic and reconstructive surgery. 1986;78(1):122-3. Epub 1986/07/01.
27. Fournier P. Microlipoextraction et microlipoinjection. Rev Chir Esthet Lang France. 1985(10):36-40.
28. Klein J. The tumescent technique for liposuction surgery. Jorunal of the Amercian Academu of Cosmetic Surgery. 1987;4:263-7.
29. Lillis PJ. Liposuction surgery under local anesthesia: limited blood loss and minimal lidocaine absorption. The Journal of dermatologic surgery and oncology. 1988;14(10):1145-8. Epub 1988/10/01.
30. Hanke CW, Bernstein G, Bullock S. Safety of tumescent liposuction in 15,336 patients. National survey results. Dermatologic surgery : official publication for American Society for Dermatologic Surgery [et al]. 1995;21(5):459-62. Epub 1995/05/01.
31. Fournier P. Body sculpturing through syringe liposuction and autologuous fat re-injection. Paris: Samuel Rolf International. 1987.
32. Asken S. Liposuction surgery and autologous fat transplantation. Norwalk C, editor: Appleton & Lange; 1988.
33. Bircoll M, Novack BH. Autologous fat transplantation employing liposuction techniques. Annals of plastic surgery. 1987;18(4):327-9. Epub 1987/04/01.
34. Bircoll M. Autologous fat transplantation. Plastic and reconstructive surgery. 1987;79(3):492-3. Epub 1987/03/01.
35. Bircoll M. Cosmetic breast augmentation utilizing autologous fat and liposuction techniques. Plastic and reconstructive surgery. 1987;79(2):267-71. Epub 1987/02/01.
36. surgeons Asop. 2012 Post-Mastectomy Fat Graft/Fat Transfer ASPS Guiding Principles. Arlington Heights2012; Available from: http://www.plasticsurgery.org/Documents/medical-professionals/health-policy/guiding-principles/2012Post-MastectomyFatGraft-FatTransferASPSGuidingPrinciples.pdf.
37. Delay E, Garson S, Tousson G, Sinna R. Fat injection to the breast: technique, results, and indications based on 880 procedures over 10 years. Aesthetic surgery

journal / the American Society for Aesthetic Plastic surgery. 2009;29(5):360-76. Epub 2009/10/15.
38. Rigotti G, Marchi A, Galie M, Baroni G, Benati D, Krampera M, et al. Clinical treatment of radiotherapy tissue damage by lipoaspirate transplant: a healing process mediated by adipose-derived adult stem cells. Plastic and reconstructive surgery. 2007;119(5):1409-22; discussion 23-4. Epub 2007/04/07.
39. Coleman SR, Saboeiro AP. Fat grafting to the breast revisited: safety and efficacy. Plastic and reconstructive surgery. 2007;119(3):775-85; discussion 86-7. Epub 2007/02/22.
40. Delay E, Sinna R, Delaporte T, Flageul G, Tourasse C, Tousson G. Patient information before aesthetic lipomodeling (lipoaugmentation): a French plastic surgeon's perspective. Aesthetic surgery journal / the American Society for Aesthetic Plastic surgery. 2009;29(5):386-95. Epub 2009/10/15.
41. Sarfati I, Ihrai T, Kaufman G, Nos C, Clough KB. Adipose-tissue grafting to the post-mastectomy irradiated chest wall: preparing the ground for implant reconstruction. Journal of plastic, reconstructive & aesthetic surgery : JPRAS. 2011;64(9):1161-6. Epub 2011/04/26.
42. Seth AK, Hirsch EM, Kim JY, Fine NA. Long-term outcomes following fat grafting in prosthetic breast reconstruction: a comparative analysis. Plastic and reconstructive surgery. 2012;130(5):984-90. Epub 2012/07/11.
43. Petit JY, Botteri E, Lohsiriwat V, Rietjens M, De Lorenzi F, Garusi C, et al. Locoregional recurrence risk after lipofilling in breast cancer patients. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2012;23(3):582-8. Epub 2011/05/26.
44. Rigotti G, Marchi A, Stringhini P, Baroni G, Galie M, Molino AM, et al. Determining the oncological risk of autologous lipoaspirate grafting for post-mastectomy breast reconstruction. Aesthetic plastic surgery. 2010;34(4):475-80. Epub 2010/03/25.
45. Spear SL, Wilson HB, Lockwood MD. Fat injection to correct contour deformities in the reconstructed breast. Plastic and reconstructive surgery. 2005;116(5):1300-5. Epub 2005/10/12.
46. Missana MC, Laurent I, Barreau L, Balleyguier C. Autologous fat transfer in reconstructive breast surgery: indications, technique and results. European journal of surgical oncology : the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology. 2007;33(6):685-90. Epub 2007/01/24.
47. Mu DL, Luan J, Mu L, Xin MQ. Breast augmentation by autologous fat injection grafting: management and clinical analysis of complications. Annals of plastic surgery. 2009;63(2):124-7. Epub 2009/07/04.
48. Gumucio CA, Pin P, Young VL, Destouet J, Monsees B, Eichling J. The effect of breast implants on the radiographic detection of microcalcification and soft-tissue masses. Plastic and reconstructive surgery. 1989;84(5):772-8; discussion 9-82. Epub 1989/11/01.
49. de Blacam C, Momoh AO, Colakoglu S, Tobias AM, Lee BT. Evaluation of clinical outcomes and aesthetic results after autologous fat grafting for contour deformities of the reconstructed breast. Plastic and reconstructive surgery. 2011;128(5):411e-8e. Epub 2011/10/28.
50. Lee KS, Seo SJ, Park MC, Park DH, Kim CS, Yoo YM, et al. Sepsis with multiple abscesses after massive autologous fat grafting for augmentation mammoplasty: a case report. Aesthetic plastic surgery. 2011;35(4):641-5. Epub 2010/11/09.

51. Maillard GF. Liponecrotic cysts after augmentation mammaplasty with fat injections. Aesthetic plastic surgery. 1994;18(4):405-6. Epub 1994/01/01.
52. Bircoll M. Liponecrotic pseudocysts following fat injection into the breast. Plastic and reconstructive surgery. 2010;126(6):2289. Epub 2010/12/03.
53. Kim H, Yang EJ, Bang SI. Bilateral liponecrotic pseudocysts after breast augmentation by fat injection: a case report. Aesthetic plastic surgery. 2012;36(2):359-62. Epub 2011/08/02.
54. Delay E, Spear SL, Willey SC, Robb GL, M.Y. H. lipomodeling of the reconstructed breast. In: (Eds.) N, editor. Surgery of the breast: principles and art. US: Lippincott Williams & Wilkins; 2005. p. 930-46.
55. Smith P, Adams WP, Jr., Lipschitz AH, Chau B, Sorokin E, Rohrich RJ, et al. Autologous human fat grafting: effect of harvesting and preparation techniques on adipocyte graft survival. Plastic and reconstructive surgery. 2006;117(6):1836-44. Epub 2006/05/03.
56. Nguyen A, Pasyk KA, Bouvier TN, Hassett CA, Argenta LC. Comparative study of survival of autologous adipose tissue taken and transplanted by different techniques. Plastic Reconstructive Surgery. 1990(85):378-86.
57. Kokonas TC, Bucky LP, Hurley C, May JW, Jr. The fate of suctioned and surgically removed fat after reimplantation for soft-tissue augmentation: a volumetric and histologic study in the rabbit. Plastic and reconstructive surgery. 1993(91):763-8.
58. Niechajev I, Sevcuk O. Long-term results of fat transplantation: clinical and histologic studies. Plastic and reconstructive surgery. 1994;94(3):496-506. Epub 1994/09/01.
59. Horl HW, Feller AM, Biemer E. Technique for liposuction fat reimplantation and long-term volume evaluation by magnetic resonance imaging. Annals of plastic surgery. 1991;26(3):248-58. Epub 1991/03/01.
60. Hong SJ, Lee JH, Hong SM, Park CH. Enhancing the viability of fat grafts using new transfer medium containing insulin and beta-fibroblast growth factor in autologous fat transplantation. Journal of plastic, reconstructive & aesthetic surgery : JPRAS. 2010;63(7):1202-8. Epub 2009/06/27.
61. Keck M, Zeyda M, Burjak S, Kamolz LP, Selig H, Stulnig TM, et al. Coenzyme Q10 does not enhance preadipocyte viability in an in vitro lipotransfer model. Aesthetic plastic surgery. 2012;36(2):453-7. Epub 2011/10/04.
62. Marra KG, Defail AJ, Clavijo-Alvarez JA, Badylak SF, Taieb A, Schipper B, et al. FGF-2 enhances vascularization for adipose tissue engineering. Plastic and reconstructive surgery. 2008;121(4):1153-64. Epub 2008/03/20.
63. Rothschild LJ, Mancinelli RL. Life in extreme environments. Nature. 2001;409(6823):1092-101. Epub 2001/03/10.
64. Hengherr S, Reuner A, Brummer F, Schill RO. Ice crystallization and freeze tolerance in embryonic stages of the tardigrade Milnesium tardigradum. Comparative biochemistry and physiology Part A, Molecular & integrative physiology. 2010;156(1):151-5. Epub 2010/02/02.
65. do Amaral MC, Lee RE, Jr., Costanzo JP. Enzymatic regulation of glycogenolysis in a subarctic population of the wood frog: implications for extreme freeze tolerance. PloS one. 2013;8(11):e79169. Epub 2013/11/16.
66. Costanzo JP, Lee RE, Jr. Urea loading enhances freezing survival and postfreeze recovery in a terrestrially hibernating frog. The Journal of experimental biology. 2008;211(Pt 18):2969-75. Epub 2008/09/09.

67. Sherman JK. Historical Synopsis of Human Semen Cryobanking. In: David G, Price WS, editors. Human Artificial Insemination and Semen Preservation: Springers US; 1980. p. pp 95-105.
68. De Felici M, Dolci S. From testis to teratomas: a brief history of male germ cells in mammals. The International journal of developmental biology. 2013;57(2-4):115-21. Epub 2013/06/21.
69. Polge C, Smith AU, Parkes AS. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature. 1949;164(4172):666. Epub 1949/10/15.
70. Lovelock JE. Resuspension in plasma of human red blood-cells frozen in glycerol. Lancet. 1952;1(6721):1238-9. Epub 1952/06/21.
71. Sherman JK. Freezing and freeze-drying of bull spermatozoa. The American journal of physiology. 1957;190(2):281-6. Epub 1957/08/01.
72. Cryobiologie So. About the Society for Cryobiology. Society for Cryobiology Editorial office , University of Bedfordshire, 250 Butterfield, Great Marlings, Luton LU2 8LE, United Kingdom2013; Available from: http://sc.memberclicks.net/about-the-sfc.
73. Walters EM, Benson JD, Woods EJ, Critser JK. The history of sperm cryopreservation. In: Pacey AA, Tomlinson MJ, editors. Sperm Banking - theory and practice. Cambrigde: Cambridge University press; 2009. p. 136.
74. Moore K, Bonilla AQ. Cryopreservation of mammalian embryos: The state of the art. ARBS Annual Review of Biomedical Sciences. 2006;8:19-32.
75. Christiani AA, Curaba M, Van Langendonckt A, Dolmans M, Donnez J. Vitrification as an alternative means of cryopreserving ovarian tissue. Reproductive BioMedicine Online. 2011;23(2):160_86.
76. Lovelock JE. The haemolysis of human red blood-cells by freezing and thawing. Biochimica et biophysica acta. 1953;10(3):414-26. Epub 1953/03/01.
77. Levitt J, Meryman HT. Mechanism of freezing in (plant or animal?) living cells and tissues. Science. 1957;125(3240):194-5. Epub 1957/02/01.
78. Karow AM, Jr., Webb WR. Tissue freezing. A theory for injury and survival. Cryobiology. 1965;2(3):99-108. Epub 1965/11/01.
79. Meryman HT. Observations on the present state of blood preservation by freezing. Cryobiology. 1968;5(2):144-6. Epub 1968/09/01.
80. Mazur P, Leibo SP, Chu EH. A two-factor hypothesis of freezing injury. Evidence from Chinese hamster tissue-culture cells. Experimental cell research. 1972;71(2):345-55. Epub 1972/01/01.
81. Rohrich RJ, Sorokin ES, Brown SA. In search of improved fat transfer viability: a quantitative analysis of the role of centrifugation and harvest site. Plastic and reconstructive surgery. 2004;113(1):391-5; discussion 6-7. Epub 2004/01/07.
82. Padoin AV, Braga-Silva J, Martins P, Rezende K, Rezende AR, Grechi B, et al. Sources of processed lipoaspirate cells: influence of donor site on cell concentration. Plastic and reconstructive surgery. 2008;122(2):614-8. Epub 2008/07/16.
83. Li K, Gao J, Zhang Z, Li J, Cha P, Liao Y, et al. Selection of donor site for fat grafting and cell isolation. Aesthetic plastic surgery. 2013;37(1):153-8. Epub 2012/12/13.
84. Rohrich RJ, Morales DE, Krueger JE, Ansari M, Ochoa O, Robinson J, Jr., et al. Comparative lipoplasty analysis of in vivo-treated adipose tissue. Plastic and reconstructive surgery. 2000;105(6):2152-8; discussion 9-60. Epub 2000/06/06.
85. Shiffman MA, Mirrafati S. Fat transfer techniques: the effect of harvest and transfer methods on adipocyte viability and review of the literature. Dermatologic surgery : official publication for American Society for Dermatologic Surgery [et al]. 2001;27(9):819-26. Epub 2001/09/13.

86. Leong DT, Hutmacher DW, Chew FT, Lim TC. Viability and adipogenic potential of human adipose tissue processed cell population obtained from pump-assisted and syringe-assisted liposuction. Journal of dermatological science. 2005;37(3):169-76. Epub 2005/03/01.
87. Ozsoy Z, Kul Z, Bilir A. The role of cannula diameter in improved adipocyte viability: a quantitative analysis. Aesthetic surgery journal / the American Society for Aesthetic Plastic surgery. 2006;26(3):287-9. Epub 2006/05/01.
88. Ferguson RE, Cui X, Fink BF, Vasconez HC, Pu LL. The viability of autologous fat grafts harvested with the LipiVage system: a comparative study. Annals of plastic surgery. 2008;60(5):594-7. Epub 2008/04/25.
89. Pu LL, Coleman SR, Cui X, Ferguson RE, Jr., Vasconez HC. Autologous fat grafts harvested and refined by the Coleman technique: a comparative study. Plastic and reconstructive surgery. 2008;122(3):932-7. Epub 2008/09/04.
90. Erdim M, Tezel E, Numanoglu A, Sav A. The effects of the size of liposuction cannula on adipocyte survival and the optimum temperature for fat graft storage: an experimental study. Journal of plastic, reconstructive & aesthetic surgery : JPRAS. 2009;62(9):1210-4. Epub 2008/06/24.
91. Surgery RC. Viafill Fat Transfer System. 2008; Available from: http://www.rumercosmetics.com/minimally-invasive-center-philadelphia/fillers-and-injectables/viafill-fat-injections/.
92. Crawford JL, Hubbard BA, Colbert SH, Puckett CL. Fine tuning lipoaspirate viability for fat grafting. Plastic and reconstructive surgery. 2010;126(4):1342-8. Epub 2010/10/05.
93. Herold C, Pflaum M, Utz P, Wilhelmi M, Rennekampff HO, Vogt PM. [Viability of autologous fat grafts harvested with the Coleman technique and the tissu trans system (shippert method): a comparative study]. Handchirurgie, Mikrochirurgie, plastische Chirurgie : Organ der Deutschsprachigen Arbeitsgemeinschaft fur Handchirurgie : Organ der Deutschsprachigen Arbeitsgemeinschaft fur Mikrochirurgie der Peripheren Nerven und Gefasse 2011;43(6):361-7. Epub 2012/01/14. Vitalitatsvergleich von Fetttransplantaten welche mit der Coleman Technik und dem Tissu Trans System (Shippert Methode) gewonnen wurden.
94. Kirkham JC, Lee JH, Medina MA, 3rd, McCormack MC, Randolph MA, Austen WG, Jr. The impact of liposuction cannula size on adipocyte viability. Annals of plastic surgery. 2012;69(4):479-81. Epub 2012/09/12.
95. Lee JH, Kirkham JC, McCormack MC, Nicholls AM, Randolph MA, Austen WG, Jr. The effect of pressure and shear on autologous fat grafting. Plastic and reconstructive surgery. 2013;131(5):1125-36. Epub 2013/02/07.
96. Boschert MT, Beckert BW, Puckett CL, Concannon MJ. Analysis of lipocyte viability after liposuction. Plastic and reconstructive surgery. 2002;109(2):761-5; discussion 6-7. Epub 2002/01/31.
97. Butterwick KJ. Lipoaugmentation for aging hands: a comparison of the longevity and aesthetic results of centrifuged versus noncentrifuged fat. Dermatologic surgery : official publication for American Society for Dermatologic Surgery [et al]. 2002;28(11):987-91. Epub 2002/12/04.
98. Ramon Y, Shoshani O, Peled IJ, Gilhar A, Carmi N, Fodor L, et al. Enhancing the take of injected adipose tissue by a simple method for concentrating fat cells. Plastic and reconstructive surgery. 2005;115(1):197-201; discsussion 2-3. Epub 2004/12/29.

99. Rose JG, Jr., Lucarelli MJ, Lemke BN, Dortzbach RK, Boxrud CA, Obagi S, et al. Histologic comparison of autologous fat processing methods. Ophthalmic plastic and reconstructive surgery. 2006;22(3):195-200. Epub 2006/05/23.
100. Kurita M, Matsumoto D, Shigeura T, Sato K, Gonda K, Harii K, et al. Influences of centrifugation on cells and tissues in liposuction aspirates: optimized centrifugation for lipotransfer and cell isolation. Plastic and reconstructive surgery. 2008;121(3):1033-41; discussion 42-3. Epub 2008/03/05.
101. Khater R, Atanassova P, Anastassov Y, Pellerin P, Martinot-Duquennoy V. Clinical and experimental study of autologous fat grafting after processing by centrifugation and serum lavage. Aesthetic plastic surgery. 2009;33(1):37-43. Epub 2008/11/21.
102. Kim IH, Yang JD, Lee DG, Chung HY, Cho BC. Evaluation of centrifugation technique and effect of epinephrine on fat cell viability in autologous fat injection. Aesthetic surgery journal / the American Society for Aesthetic Plastic surgery. 2009;29(1):35-9. Epub 2009/02/24.
103. Xie Y, Zheng D, Li Q, Chen Y, Lei H, Pu LL. The effect of centrifugation on viability of fat grafts: an evaluation with the glucose transport test. Journal of plastic, reconstructive & aesthetic surgery : JPRAS. 2010;63(3):482-7. Epub 2009/01/09.
104. Minn KW, Min KH, Chang H, Kim S, Heo EJ. Effects of fat preparation methods on the viabilities of autologous fat grafts. Aesthetic plastic surgery. 2010;34(5):626-31. Epub 2010/05/06.
105. Conde-Green A, de Amorim NF, Pitanguy I. Influence of decantation, washing and centrifugation on adipocyte and mesenchymal stem cell content of aspirated adipose tissue: a comparative study. Journal of plastic, reconstructive & aesthetic surgery : JPRAS. 2010;63(8):1375-81. Epub 2009/08/15.
106. Conde-Green A, Baptista LS, de Amorin NF, de Oliveira ED, da Silva KR, Pedrosa Cda S, et al. Effects of centrifugation on cell composition and viability of aspirated adipose tissue processed for transplantation. Aesthetic surgery journal / the American Society for Aesthetic Plastic surgery. 2010;30(2):249-55. Epub 2010/05/06.
107. Botti G, Pascali M, Botti C, Bodog F, Cervelli V. A clinical trial in facial fat grafting: filtered and washed versus centrifuged fat. Plastic and reconstructive surgery. 2011;127(6):2464-73. Epub 2011/05/28.
108. Ferraro GA, De Francesco F, Tirino V, Cataldo C, Rossano F, Nicoletti G, et al. Effects of a new centrifugation method on adipose cell viability for autologous fat grafting. Aesthetic plastic surgery. 2011;35(3):341-8. Epub 2010/11/12.
109. Pulsfort AK, Wolter TP, Pallua N. The effect of centrifugal forces on viability of adipocytes in centrifuged lipoaspirates. Annals of plastic surgery. 2011;66(3):292-5. Epub 2010/08/28.
110. Hoareau L, Bencharif K, Girard AC, Gence L, Delarue P, Hulard O, et al. Effect of centrifugation and washing on adipose graft viability: a new method to improve graft efficiency. Journal of plastic, reconstructive & aesthetic surgery : JPRAS. 2013;66(5):712-9. Epub 2013/01/30.
111. Conde-Green A, Wu I, Graham I, Chae JJ, Drachenberg CB, Singh DP, et al. Comparison of 3 techniques of fat grafting and cell-supplemented lipotransfer in athymic rats: a pilot study. Aesthetic surgery journal / the American Society for Aesthetic Plastic surgery. 2013;33(5):713-21. Epub 2013/05/31.
112. Zhu M, Cohen SR, Hicok KC, Shanahan RK, Strem BM, Yu JC, et al. Comparison of three different fat graft preparation methods: gravity separation, centrifugation, and simultaneous washing with filtration in a closed system. Plastic and reconstructive surgery. 2013;131(4):873-80. Epub 2013/04/02.

113. Cui XD, Gao DY, Fink BF, Vasconez HC, Pu LL. Cryopreservation of human adipose tissues. Cryobiology. 2007;55(3):269-78. Epub 2007/10/19.
114. Pu LL, Cui X, Fink BF, Gao D, Vasconez HC. Adipose aspirates as a source for human processed lipoaspirate cells after optimal cryopreservation. Plastic and reconstructive surgery. 2006;117(6):1845-50. Epub 2006/05/03.
115. Pu LL, Cui X, Li J, Fink BF, Cibull ML, Gao D. The fate of cryopreserved adipose aspirates after in vivo transplantation. Aesthetic surgery journal / the American Society for Aesthetic Plastic surgery. 2006;26(6):653-61. Epub 2006/11/01.
116. Pu LL, Cui X, Fink BF, Cibull ML, Gao D. Long-term preservation of adipose aspirates after conventional lipoplasty. Aesthetic surgery journal / the American Society for Aesthetic Plastic surgery. 2004;24(6):536-41. Epub 2004/11/01.
117. MacRae JW, Tholpady SS, Ogle RC, Morgan RF. Ex vivo fat graft preservation: effects and implications of cryopreservation. Annals of plastic surgery. 2004;52(3):281-2; discussion 3. Epub 2004/05/26.
118. Moscatello DK, Dougherty M, Narins RS, Lawrence N. Cryopreservation of human fat for soft tissue augmentation: viability requires use of cryoprotectant and controlled freezing and storage. Dermatologic surgery : official publication for American Society for Dermatologic Surgery [et al]. 2005;31(11 Pt 2):1506-10. Epub 2006/01/19.
119. Atik B, Ozturk G, Erdogan E, Tan O. Comparison of techniques for long-term storage of fat grafts: an experimental study. Plastic and reconstructive surgery. 2006;118(7):1533-7. Epub 2006/11/15.
120. Wolter TP, von Heimburg D, Stoffels I, Groeger A, Pallua N. Cryopreservation of mature human adipocytes: in vitro measurement of viability. Annals of plastic surgery. 2005;55(4):408-13. Epub 2005/09/28.
121. Pu LL, Cui X, Fink BF, Cibull ML, Gao D. Cryopreservation of adipose tissues: the role of trehalose. Aesthetic surgery journal / the American Society for Aesthetic Plastic surgery. 2005;25(2):126-31. Epub 2005/03/01.
122. Lidagoster MI, Cinelli PB, Levee EM, Sian CS. Comparison of autologous fat transfer in fresh, refrigerated, and frozen specimens: an animal model. Annals of plastic surgery. 2000;44(5):512-5. Epub 2000/05/11.
123. Shoshani O, Ullmann Y, Shupak A, Ramon Y, Gilhar A, Kehat I, et al. The role of frozen storage in preserving adipose tissue obtained by suction-assisted lipectomy for repeated fat injection procedures. Dermatologic surgery : official publication for American Society for Dermatologic Surgery [et al]. 2001;27(7):645-7. Epub 2001/07/10.
124. Lei H, Li q F. [Study on viability measurement of fat for grafting]. Zhonghua zheng xing wai ke za zhi = Zhonghua zhengxing waike zazhi = Chinese journal of plastic surgery. 2005;21(5):375-8. Epub 2005/12/13.
125. Lee JH, Kirkham JC, McCormack MC, Medina MA, Nicholls AM, Randolph MA, et al. A novel approach to adipocyte analysis. Plastic and reconstructive surgery. 2012;129(2):380-7. Epub 2012/01/31.
126. Kim M, Kim I, Kim SH, Jung M, Han S, Lee J, et al. Cryopreserved human adipogenic-differentiated pre-adipocytes: a potential new source for adipose tissue regeneration. Cytotherapy. 2007;9(5):468-76. Epub 2007/09/06.
127. Greenspan P, Mayer EP, Fowler SD. Nile red: a selective fluorescent stain for intracellular lipid droplets. The Journal of cell biology. 1985;100(3):965-73. Epub 1985/03/01.
128. Greenberger JS. Corticosteroid-dependent differentiation of human marrow preadipocytes in vitro. In vitro. 1979;15(10):823-8. Epub 1979/10/01.

129. Company BDa. Flow Cytometry Protocol for Analysis of Cell Viability using Propidium Iodide. R&D Systems, Inc., 614 McKinley Place NE, Minneapolis, MN 554132013; Available from: http://www.rndsystems.com/literature_flow_cytometry_analysis_propidium_iodide.aspx.
130. Diagnostics R. Cell Proliferation Kit II (XTT) - Principle. Roche Diagnostics Nederland B.V., Postbus 1007, 1300 BA Almere, Netherlands2014; Available from: http://www.roche-applied-science.com/shop/products/cell-proliferation-kit-ii-xtt- -tab-3.
131. Scuderi N, Ceccarelli S, Onesti MG, Fioramonti P, Guidi C, Romano F, et al. Human adipose-derived stromal cells for cell-based therapies in the treatment of systemic sclerosis. Cell transplantation. 2013;22(5):779-95. Epub 2012/04/25.
132. Daumas A, Eraud J, Hautier A, Sabatier F, Magalon G, Granel B. [Interests and potentials of adipose tissue in scleroderma]. La Revue de medecine interne / fondee par la Societe nationale francaise de medecine interne. 2013;34(12):763-9. Epub 2013/09/21. Potentialites et interet du tissu adipeux dans la sclerodermie.
133. Zanelato TP, Marquesini G, Colpas PT, Magalhaes RF, de Moraes AM. Implantation of autologous fat globules in localized scleroderma and idiopathic lipoatrophy--report of five patients. Anais brasileiros de dermatologia. 2013;88(6 Suppl 1):120-3. Epub 2013/12/19.
134. Del Papa N, Caviggioli F, Sambataro D, Zaccara E, Vinci V, Di Luca G, et al. Autologous Fat Grafting in the Treatment of Fibrotic Perioral Changes in Patients with Systemic Sclerosis. Cell transplantation. 2013. Epub 2013/10/25.
135. Consorti G, Tieghi R, Clauser LC. Frontal linear scleroderma: long-term result in volumetric restoration of the fronto-orbital area by structural fat grafting. The Journal of craniofacial surgery. 2012;23(3):e263-5. Epub 2012/05/26.
136. Shinya A, Pedersen D, Malinouski M. Method for Analysis and Sorting of Live Adipocytes. Union Biometrica, Holliston, MA; 2014; Available from: http://www.unionbio.com/biosorter/.

10. ANNEX

	Naam	Datum	Handtekening
Auteur	Sam Brondeel		
Autorisatie			
Revisor 1			

Distributie

Personeel weefselbank
Stafmedewerkers Plastische Heelkunde
Verpleegkundigen OK
Logistiek medewerkers OK

Overzicht van alle versies

SOP ID	Datum effectief
NVT	

Veranderingen sinds vorige versie:

- NVT

1. DOEL:

Deze procedure beschrijft de organisatie van activiteiten met betrekking tot de prelevatie van lipoaspiraat materiaal voor cryopreservatie in het kader van plastische heelkunde (vb lipofilling). De cryopreservatie staat nu nog in teken van wetenschappelijk onderzoek maar kan in de toekomst leiden tot klinische toepassingen voor autologe en misschien zelfs allogene.

2. LOKALEN:

OK K12 C
Atelier biomedisch technische diensten OK K12C

3. VERANTWOORDELIJKEHEID

Arts verantwoordelijk voor de plastische ingreep is de arts verantwoordelijk voor de prelevatie van het vetweefsel (Geneesheer Specialist plastische heelkunde)
Verpleegkundigen OK
Logistiek medewerkers OK

4. MATERIALEN

4.1. Toestellen

Koelkast 4°C (Toestel 105)
Centrifuge (Toestel)

4.2. Instrumenten

OK-instrumentarium
50cc syringes voor transport

4.3. Reagentia

1l gelacteerde Ringers oplossing
30ml 2% lidocaïne
1ml epinephrine

4.4. Humaan materiaal

Lipoaspiraat
Bloodstaal (één buis voor gestolde bloedafname)

4.5. Wegwerpmateriaal

Syringes

4.6. Andere : Documentatie

- Etiketten “LIPOASPIRAAT voor cryopreservatie” met de vermelding van de greffecode
- Bruine enveloppe (door Weefselbank aangemaakt). In deze enveloppe zijn volgende materialen/ documenten aanwezig:
    - Donorformulier (FORM_Pih_K_004)
    - Plastiek zakje met daarin:
        - 1 buis (met daarop de greffecode) voor gestolde bloedafname

5. REGISTRATIE

5.1. Formulieren

FORM_Pih_021: Wegnemingsformulier autologe greffes

5.2. Logboek

Register: Binnengebracht materiaal OK 1K12C

6. PROCEDURE

Algemene opmerkingen met betrekking tot het toepassingsgebied:
NVT

6.1. Voorbereiding

NVT

6.2. Eigenlijke procedure

6.2.1. Initiële selectie van de potentiële vetdonor door de verantwoordelijke arts

- Het vetweefsel wordt weggenomen bij gezonde volwassenen waarbij vooraf een informed consent is bekomen. Het gaat hier om vetweefsel dat normaal nadien zou worden weggegooid.
- De plaats van wegname wordt gebaseerd op bereikbaarheid, veiligheid en voorkeur van de patiënt.

***Opmerking:** de informed consent moet VÓÓR de procedure in orde gebracht zijn.

6.2.2. Identificatie van de patient in het OK

- De patiënten worden in het kader van de ingreep (liposuctie van vooraf bepaalde regio) geïdentificeerd volgens de op dat ogenblik vigerende procedures in het OK (zie ook Time-out procedure OK)

6.2.3. Prelevatie van het vetweefsel door de verantwoordelijk arts

- Het vetweefsel zal bekomen worden door gebruik te maken van dE liposuctie methode. De donorsite van de patiënt zal eerst geïnfiltreerd worden met 1l gelacteerde Ringers oplossing, 30ml van 2% lidocaïne en 1ml epinephrine. De liposuctie zal zachtjes uitgevoerd worden met de 3mm canules, dit om schade aan de vetcellen zoveel mogelijk te vermijden. Het doel is om 50 ml lipoaspiraat te bekomen.

- Na verwijdering wordt het vetweefsel in een 50cc syringe verzameld. Dit zal in het OK gecentrifugeerd worden aan 3000 rpm (of 1200G) voor drie minuten volgens het protocol.
- Na centrifugatie krijgen we drie lagen. De onderste laag bevat een concentratie aan bloed producten en celdebris en wordt verwijderd door simpelweg deze laag uit de syringe te laten lopen. De middelste laag, die het effectieve lipoaspiraat bevat wordt overgespoten in een andere syringe. Op deze manier bekomen we het meest kwaliteitsvolle lipoaspiraat welke dan ook wordt gebruikt om in te vriezen.

6.2.4. Bloedname door de anesthesist

- De anesthesist neemt 1 buis bloed af voor gestolde bloedafname.

6.2.5. Registratie van het menselijk materiaal

Nadat de syringes zijn gevuld vullen we het wegnemingsformulier in met volgende gegevens:

- Adremasticker van de donor
- Identificatie van de donor
- Naam van de preleverend chirurg
- Aanwezigheid bereidheidsverklaring: OK of niet OK
- Aanwezigheid medische vragenlijst: OK of niet OK
- Datum en uur start van de prelevatie
- Beschrijving en indicatie van het weggenomen menselijk lichaamsmateriaal

6.2.6. Tijdelijke preservatie van het materiaal bij 4°C

In afwachting van ophaling door de logistiek medewerkers van het OK, worden de syringes, de bloedbuis en de documentatie (opnieuw in de bruine enveloppe), onmiddellijk in de koelkast bij 4°C (toestel 105; ter hoogte van het atelier biomedisch technische dienst) geplaatst.

- De OK-verpleegkundige verwittigt telefonisch de logistiek medewerkers voor ophaling van de materialen en transport naar -1P4 lokaal 102

6.2.7. Transport van het materiaal

- De logistiek medewerker brengt een transport box met zich mee. Deze bevat smeltend ijs, om op deze manier een stabiele omgeving te creëren voor de bloedbuis en het lipoaspiraat tijdens het transport.

7 REFERENTIES

7.1. Wetgeving

- Wet inzake het verkrijgen en het gebruik van menselijk lichaamsmateriaal met het oog op de geneeskundige toepassing op de mens of het wetenschappelijk onderzoek (19 december 2008) en bijhorende uitvoeringsbesluiten
- EU Directive 2004/23/EC

7.2. Literatuur

NVT

8 Verwante documentatie

8.1. Verwante work-flows

WF_Pih_003: Interne, levende weefseldonoren

8.2. Verwante SOP's

Time-out procedure OK (identificatie van patiënten in het OK UZ Gent)

8.3. Verwante documenten

DOC_Pp_004: Informatiebundel voor logistiek medewerkers met betrekking tot het transport van menselijke weefsels en cellen binnen het UZ Gent

8.4. Verwante formulieren

NVT

	Naam	Datum	Handtekening
Auteur	Sam Brondeel		
Authorisatie			
Revisor 1			

Distributie

Personeel weefselbank

Overzicht van alle versies

SOP ID	Datum effektief
NVT	

Veranderingen sinds vorige versie:

- NVT

1. DOEL:

Deze procedure beschrijft de organisatie van activiteiten met betrekking tot cryopreservatie van lipoaspiraat in het kader van wetenschappelijk onderzoeken en in de toekomst, autologe en zelfs allogene toepassingen. (vb. lipofilling)

2. LOKALEN

Cleanroom labo D (Lokaal 203)
Koude opslag (Lokaal 102, -1P4)

3. BEVOEGDE PERSONEN

Laboranten weefselbank
Wetenschappelijk medewerker weefselbank

4. MATERIALEN

4.1. Toestellen

Diepvriezer -80°C (Toestel 10)
Diepvriezer -150°C (Toestel28)
LAF (Toestel 23)

4.2. Instrumenten

Pipetman
Pipetboy
Gesteriliseerde pincet
10ml cryovial

4.3. Reagentia

DMSO
DMEM (basismedium)

4.4. Humaan materiaal

Lipoaspiraat

4.5. Wegwerpmateriaal

Steriele pipetten
Steriele tips
Steriele recipiënten
Isomodoos

5. REGISTRATIE

5.1. Formulieren

FORM_Pp_002: Attestformulier greffes voor autoloog gebruik

5.2. Logboeken

Kaft "levende donor – Quarantaine – kraakbeen/amnion/autoloog"

6. PROCEDURE

Algemene opmerkingen:
Alle labo-verwerkingen gebeuren onder LAF- omstandigheden in Cleanroom achtergrond omgeving Klasse C.

6.1. Voorbereiding

- Zet minstens 15minuten op voorhand de laminaire flow aan
- Reinig werkoppervlaktes en LAF-kast volgens SOP_Mw_003
- Maak volgende oplossing klaar:
  - In een steriel Falcon 15mL: 15% vriesvloeistof: 2,25ml DMSO + 12,75 ml basis medium

6.2. Testen initiële viabiliteit

- Verdeel drie keer 5ml vers aspiraat in aparte 15ml Falcon buisjes
- Volg nadien de stappen voor de initële viabiliteitstesten via de 'SOP_ Viabiliteitsanalyse lipo-aspiraat vóór cryopreservatie en na ontdooiing'.

6.3. Eigenlijke procedure

- Neem het resterende lipo-aspiraat en pipetteer 5ml in een 10ml cryovial
- Pipetteer 5ml van de DMSO oplossing in diezelfde cryovial
- Pipetteer op en neer zodat beide stoffen met elkaar vermengd geraken
- Schroef het dopje op de cryovial en label het met een unieke code en de datum
- Herhaal dit 2 maal, zodat 3 stalen worden bekomen die nadien kunnen getest worden met de kleuringen en XTT-assay
- Plaats de recipiënten na het mengen, in een isomodoos
- Plaats de doos met het recipiënt in bij – 80°C voor minstens 24h (voor afkoeling aan 1°C per minuut)
- Na minstens 24h moet het recipiënt, zonder isomodoos, verplaatst worden in een -150°C vriezer

6.4. Microbiologie

- Cfr SOP_Pp_K_008_03

6.5. Registratie

- Per greffe moet een attestformulier aangemaakt worden (FORM_Pp_002)
- Volgende gegevens moeten vermeld worden
  - Donor gegevens
  - Invriesprocedure (verwijzing naar voorliggende SOP)
  - Datum van invriezen en lotnummers van gebruikte media (DMSO & DMEM) en van het bewaarrecipiënt

6.6. Afwerken procedure

- Verwijder alle gebruikte wegwerpmaterialen en ontsmet het werkblad van de laminaire flow zoals beschreven in SOP_Mw_003
- Sluit de laminaire flow en andere gebruikte toestellen af en schakel het Uv-licht in de flowkast aan
- Verwijder de gebruikte wegwerpmaterialen

7 REFERENTIES

7.3. Wetgeving

- Wet inzake het verkrijgen en het gebruik van menselijk lichaamsmateriaal met het oog op de geneeskundige toepassing op de mens of het wetenschappelijk onderzoek (19 december 2008)
- EU Directive 2004/23/EC
- EU Directive 2006/17/EC
- EU Directive 2006/56/EC

7.4. Literatuur

NVT

8 Verwante documentatie

8.5. Verwante work-flows

/

8.6. Verwante SOP's

- CRG-SOP-001: afvalverwerkingsvoorschriften
- CRG-SOP-0002: Toegangs- en kledijvoortschriften
- CRG-SOP-0003: Werkvoorschriften

8.7. Verwante documenten

/

8.8. Verwante formulieren

	Naam	Datum	Handtekening
Auteur	Sam Brondeel		
Authorisatie			
Revisor			

Distributie

Personeel weefselbank

Overzicht van alle versies

SOP ID	Datum effektief
NVT	

Veranderingen sinds vorige versie:

- NVT

1. DOEL

Deze procedure beschrijft hoe de viabiliteit van lipo-aspiraat wordt gemeten vóór de invriesprocedure en na de ontdooiing.

2. LOKALEN

Lokaal 102 (Koude opslag -1P')
Lokaal 203 (Cleanroom Labo D)
Lokaal 214 (Cleanroom Labo C - celkweek)
Lokaal 200 (Koude opslag cleanroom)

3. BEVOEGDE PERSONEN

Laboranten weefselbank
Wetenschappelijk medewerker weefselbank

4. MATERIALEN

4.1. Toestellen

LAF (Toestel CRG-T-241)
Centrifuge (Toestel 114)
Diepvriezer van -150°C
Warmwaterbad (Toestel 49)

4.2. Instrumenten

15ml Flacon
Pipetman

4.3. Reagentia

Cultuurmedium
HOECHST 33342; (Dojindo, Kumamoto, Japan)
NILE RED (AdipoRed, Cambrex, Walkersville, Md.)
PROPRIDIUM IODIDE (Sigma-Aldrich, St-Louis, Mo.)
XTT-assay kit (Roche Diagnostics, Indianapolis, Ind.)
Elektron-koppelend reagens
Collagenase
PBS
FBS

4.4. Humaan materiaal

Spuit van 50cc met het lipoaspiraat
Cryovials van 10ml met daarin het lipoaspiraat

4.5. Wegwerpmateriaal

Steriele cryovals
Steriele pipetten

6well plaat
96well plaat

5. REGISTRATIE

5.1. Formulieren

FORM_PP_ADIPO_002_01: Cryopreservatie van lipoaspiraat materiaal

5.2. Logboeken

Donorregister van het betrokken jaar bewaard in folder \\ai\werkgroepen\WEEWeefselcoordin\Keratinocytenbank\Jaargege\Donorregister
Zwarte kaft: "Levende donor/Quarantaine/Kraakbeen+Amnion+Keratinocyten+Autoloog"

6. PROCEDURE

Algemene opmerkingen:
Vooraleer het vet in te vriezen is het nodig een initiële viabilteitspercentage te weten. Deze methode is identiek aan de methode die wordt gebruikt om de viabiliteit te onderzoeken na het invriezen.

6.1. Voorbereiding

- Zet minstens 15 minuten op voorhand de laminaire flow aan
- Reinig werkoppervlaktes en LAF-kast volgens SOP_Mw_003
- Neem de 50 cc syringe met vetweefsel en breng 5ml over in een Falcon-buis van 15ml. Herhaal dit een tweede keer en een derde keer in aparte Falcon-buisjes.
- Meng elke 5ml vetweefsel met 2ml collagenase 0,075% in fosfaatgebufferd zoutoplossing
- Incubeer de mengsel gedurende 30minuten aan een temperatuur van 37°C
- Voeg 2ml fosfaat-gebufferde zoutoplossing toe, die 10% foetaal runderserum bevat, om de digestie te eindigen.
- Centrifugeer af gedurende 5 minuten bij 430g
- Hierdoor krijgen we 3 lagen die elke worden onderzocht met kleuringen en met een XTT-assay.

6.2. Eigenlijke Procedure: initiële viabiliteitsanalyse, kleuringen

a. Kleuring met Nile Red en HOECHST 33342

- Scheid de drie lagen door ze elke afzonderlijk in een nieuwe 15ml Falconbuis te deponeren
- Leng aan met fosfaatgebufferde zoutoplossing tot 5ml
- Voeg 5mg HOECHST 33342 (blauw) en 400 microliter Nile Red (rood) toe
- Bekijk onder fluorescentiemicroscoop welke cellen vetcellen zijn en welke drijvende vetdruppels zijn, volgens volgend principe

  * Opmerking:

  - Alleen Nile Red kleuring = drijvende vetdruppel vrijgekomen door ruptuur adipocyt
  - Alleen HOECHST 33342 kleuring = non-adipocyten
  - Beiden aangekleurd = niet-gerupteerde adipocyten
- herhaal dit voor elke laag
- Voer voor elke laag een celtelling uit en bereken de hoeveelheidcellen per ml (zie SOP_Pp_K_012)

b. Kleuring met HOECHST 33342 en propridium iodide

- Scheid de drie lagen door ze elke afzonderlijk in een nieuwe 15ml Falconbuis te deponeren
- Leng elke laag aan met fosfaatgebufferde zoutoplossing tot 5ml
- Voeg 5mg HOECHST 33342 (blauw) en 5mg Propridium iodide (rood) toe
- Bekijk onder fluorescentiemicroscoop volgens volgend principe

  * Opmerking:

	HOECHST 33342	*Propidium iodide*	*Geïncludeerd vet ?*
Viabele adipocyt	*Positief*	*Negatief*	*Positief*
Dode adipocyte	*/*	*Positief*	*Positief*
Viabele non-adipocyt	*Positief*	*Negatief*	*Negatief*
Dode non-adipocyt	*/*	*Positief*	*Negatief*

- Herhaal dit voor elke laag
- Voer voor elke laag een celtelling uit en bereken de hoeveelheidcellen per ml (zie SOP_Pp_K_012)
-

6.3. Eigenlijke Procedure: initiële viabiliteitsanalyse, XTT-assay

- Scheid de drie lagen door ze elke afzonderlijk in een nieuwe 15ml Falconbuis te deponeren
- Leng aan tot 5ml met fosfaat-gebufferde zoutoplossing, die 10% foetaal runderserum bevat
- Meng het XTT-labeling reagens en het elektron-koppelend reagens volgens een ratio van 50:1.
- Voeg 2,5ml van dit mengsel toe aan elke laag
- Incubeer de mengeling, in een 6-well plaat, voor vier uur aan 37°C
- Transfereer 150microliter, na de incubatie, naar een 96-well plaat
- Meet de absorbatie met een test golflengte van 450nm en een referentie golflengte van 650nm.

6.4. Afwerken procedure

- Verwijder alle gebruikte wegwerpmaterialen en ontsmet het werkblad van de l**amina**ire flow zoals beschreven in SOP_Mw_003
- Sluit de laminaire flow af en andere gebruikte toestellen af
- Verwijder de gebruikte wegwerpmaterialen

7. REFERENTIES

7.5. Wetgeving

- Wet inzake het verkrijgen en het gebruik van menselijk lichaamsmateriaal met het oog op de geneeskundige toepassing op de mens of het wetenschappelijk onderzoek (19 DECEMBER 2008) en uitvoeringsbesluiten
- Kwaliteitsnormen voor verschillende types van menselijk lichaamsmateriaal die voor toepassing op de mens bestemd zijn: weefsels (HGR 8716)
- EU Directive 2004/23/EC
- EU Directive 2006/17/EC

7.6. Literatuur

NVT

8. VERWANTE DOCUMENTATIE

a. Verwante work-flows

WF_Pih_K_001: Allogene keratinocyten
WF_Pih_K_002: Autologe keratinocyten

b. Verwante SOP's

CRG-SOP-001: Afvalverwerkingsvoorschriften
CRG-SOP-0002: Toegangs- en kledijvoorschriften
CRG-SOP-0003:Werkvoorschriften

SOP_Pp_K_012: Celtelling m.b.v een Bürker telkamer

c. Verwante documenten

DOC_Mw_002: Werken met vloeibare stikstof-Algemeen

d. Verwante formulieren

NVT

	Naam	Datum	Handtekening
Auteur	Sam Brondeel		
Authorisatie			
Revisor			

Distributie

Personeel weefselbank

Overzicht van alle versies

SOP ID	Datum effektief
NVT	

Veranderingen sinds vorige versie:

- NVT

1. DOEL

Deze procedure beschrijft het lipoaspiraat moet ondooid worden na cryopreservatie met het oog op wetenschappelijk onderzoek met eventueel in de toekomst klinische toepassingen.

2. LOKALEN

Lokaal 102 (Koude opslag -1P4)
Lokaal 203 (Cleanroom Labo D)
Lokaal 214 (Cleanroom Labo C - celkweek)
Lokaal 200 (Koude opslag cleanroom)

3. BEVOEGDE PERSONEN

Laboranten weefselbank
Wetenschappelijk medewerker weefselbank

4. MATERIALEN

4.1. Toestellen

LAF (Toestel CRG-T-241)
Diepvriezer van -150°C
Warmwaterbad (Toestel 49)
Centrifuge (toestel 120)

4.2. Instrumenten

Pipetboy

4.3. Reagentia

Geen

4.4. Humaan materiaal

Drie cryovials van 10ml met daarin het lipoaspiraat

4.5. Wegwerpmateriaal

Steriele pipetten

5. REGISTRATIE

5.1. Formulieren

FORM_PP_ADIPO_002_01: Cryopreservatie van lipoaspiraat materiaal

5.2. Logboeken

Donorregister van het betrokken jaar bewaard in folder \\ai\werkgroepen\WEEWeefselcoordin\Keratinocytenbank\Jaargege\Donorregister

Zwarte kaft: "Levende donor/Quarantaine/Kraakbeen+Amnion+Keratinocyten+Autoloog"

6. PROCEDURE

6.1. Algemene opmerkingen:

Na het vet in te vriezen moet het dus ontdooid worden en het cryoprotectans moet verwijderd worden. Daarna gebeurt natuurlijk de viabiliteitstesting die wordt vergeleken met de initiële viabiliteit.
Zie form; SOP_Pp_Adip_002_01, Viabiliteitsanalyse lipo-aspiraat vóór cryopreservatie

6.2. Voorbereiding

NVT

6.3. Eigenlijke Procedure

- Zet minstens 15 minuten op voorhand de laminaire flow en het warm waterbad aan
- Reinig werkoppervlaktes en LAF-kast volgens SOP_Mw_003
- Haal de te ontdooien cryotubes met daarin het lipoaspiraat uit de diepvriezer (3 x 10ml cryovials)
- Laat twee minuten rusten
- Leg de 5ml tubes hierna in een warmwaterbad van 37°C tot zo volledig ontdooid zijn
  **Opmerking: dit duurt hooguit enkele tientallen seconden!*
- Reinig de buitenzijde van de cryovials met ethanol 70%
- Breng het lipoaspiraat over in een Falcon-buis van 15ml
- Leng aan met standaardmedium tot een volume van 10ml
- Centrifugeer af gedurende 5 minuten bij 1200 toeren per minuut
- Pipeteer het supernatans af
- Herhaal na spoeling alle bovenbeschreven stappen om de viabiliteit te testen

6.4. Afwerken procedure

- Verwijder alle gebruikte wegwerpmaterialen en ontsmet het werkblad van de l**amina**ire flow zoals beschreven in SOP_Mw_003
- Sluit de laminaire flow af en andere gebruikte toestellen af
- Verwijder de gebruikte wegwerpmaterialen

7. REFERENTIES

7.7. Wetgeving

- Wet inzake het verkrijgen en het gebruik van menselijk lichaamsmateriaal met het oog op de geneeskundige toepassing op de mens of het wetenschappelijk onderzoek (19 DECEMBER 2008) en uitvoeringsbesluiten
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- EU Directive 2004/23/EC
- EU Directive 2006/17/EC

7.8. Literatuur

NVT

8. VERWANTE DOCUMENTATIE

8.9. Verwante work-flows

WF_Pih_K_001: Allogene keratinocyten
WF_Pih_K_002: Autologe keratinocyten

8.10. Verwante SOP's

SOP_Pp_Adip_002_01: Viabiliteitsanalyse lipo-aspiraat vóór cryopreservatie
SOP_Pp_Adip_002_01: Cryopreservatie van lipoaspiraat materiaal
CRG-SOP-001: Afvalverwerkingsvoorschriften
CRG-SOP-0002: Toegangs- en kledijvoorschriften
CRG-SOP-0003:Werkvoorschriften

8.11. Verwante documenten

NVT

8.12. Verwante formulieren

NVT