“OVIDIUS” UNIVERSITY, CONSTANŢA

FACULTY OF MEDICINE

DOCTORAL SCHOOL OF MEDICINE

PHD THESIS SUMMARY

NEW APPROACHES IN SOFT TISSUE REGENERATIVE MEDICINE BASED ON LIPOTRANSFER ASSISTED OR NOT WITH STEM CELLS

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CONSTANTA, 2017

Content

I.INTRODUCTION

The resection of various tumors of the head, neck, breast, as well as various traumas, congenital abnormalities, aging associated changes, and many other medical conditions result in deformation of the skin contour due to defects in the dermis and subcutaneous tissue (Patrick 2000, 2001, 2004; Patel et al., 2003) [1-4]. These defects can have aesthetic and psychological effects and alter the function of the affected anatomical formation. Plastic surgery has developed increasingly effective strategies for solving these defects in parallel with the development of knowledge in the field.

Autologous fat transplantation is one of the promising treatments for soft tissue augmentation and facial rejuvenation, due to the lack of incisional scarring and complications associated with the transplantation of foreign materials. However, unpredictable problems and a low rate of survival of the graft due to partial necrosis can appear after surgery. (Coleman and Saboeiro, 2007; Yoshimura et al., 2008 a.) [5, 6].

Recent research has shown that the most effective body repairing system is represented by stem cells. Stem cells are non-specialized cells capable of self-renewal by successive mitosis, while maintaining their ability to differentiate into different cell types (Niemela et al., 2008) [7]. The discovery of adult stem cells has been of crucial importance for the field of regenerative medicine, as these cells do not raise major ethical issues and are easier to harvest than embryonic stem cells. In 2002 a group of researchers from the University of California, Los Angeles (*Regenerative Bioengineering and Repair Laboratory, Department of Surgery*), led by Patricia Zuk described for the first time the isolation of adipose tissue derived stem cells (ADSC) as multipotent stem cells (Zuk et al., 2002) [8]. Since then, ADSC research and therapeutic applications have known an explosive development.

White adipose tissue (WAT) is an ideal source for adult stem cells, as large amounts are easily accessible through liposuction techniques. Adipose tissue contains adipogenic progenitor cells in the stromal vascular fraction (SVF) (Van et al., 1976) [9]. However, Matsumoto et al., (2006) [10] have shown that liposuction negatively affects the viability of adipogenic progenitor cells, which explains the poor survival rate of these preparations and the atrophy of the transplanted lipoaspirate over time.

The SVF fraction contains a mixture of cells, including adipocyte progenitors and endothelial cells (Riordan et al. 2009) [11], which is a great advantage for regenerative medicine. Freshly isolated SVF cells can induce the formation of new blood vessels by dynamic reassembly of endothelial cells and can thus be used in therapeutic neovascularization in cases of ischemia. Furthermore, SVF co-transplantation prevented the resorption of adipose tissue grafts in a subcutaneous cellular graft model (Koh et al., 2011) [12].

The cells in the SVF fraction can be cultured *in vitro*, leading to the enrichment in ADSCs as the passages increase. Zuk et al., (2001, 2002) [13, 8] pointed out that ADSCs act as multipotent stem cells, which can differentiation into adipocytes as well as cartilage, bone, pericardial or myocyte cells under particular conditions.

The discovery of ADSCs and their angiogenesis-stimulating and inflammatory-suppressing properties has stimulated the interest of using them along with adipose tissue grafts in a number of tissue reconstruction clinical trials (Locke et al., 2011) [14]. For most applications, ADSCs are isolated from subcutaneous adipose tissue by minimally invasive techniques and are subsequently cultured *in vitro* (Murohara et al., 2009; Zuk, 2010) [15, 16]. ADSC proliferation and cell population doubling times depend on the surgical procedure performed, with superior results reported for tumescence liposuction and resection techniques compared to ultrasound assisted liposuction (De Ugarte et al., 2003) [17]. ADSC cell therapy is currently used in breast augmentation (replacing silicone implants) and facial rejuvenation procedures.

ADSC cell suspensions have been employed in tissue engineering techniques after being incorporated into scaffold structures and transplanted to fill soft tissue defects (Stosich and Mao, 2006) [18]. When using ADSCs, biodegradable injectable hydrogels have played an important role in facilitating a homogeneous distribution of cells in the affected area, regardless of its size and shape. Hydrogels may include biologically active compounds such as insulin, growth factors or adipogenesis-inducing factors such as dexamethasone (Tan et al., 2010) [19].

Matsumoto et al. (2006) [10] reported that the transfer of ADSCs combined with fat preserves the injected adipose tissue volume. The process, called cell-assisted lipotransfer (CAL), represents a novel approach for soft tissue augmentation, including breast (ASAPS, 2007) [20]. In CAL, half of the harvested fat is used to isolate the ADSCs, which are subsequently mixed with the other half, and reinjected.

II. AIM AND OBJECTIVES

Aim: The current study aims to investigate the feasibility of using adipose stem cells enriched lipoaspirates as a superior material for tissue engineering in reconstructive, aesthetic and corrective surgical procedures.

For the achievement of our goal, the following objectives were pursued:

Objective 1: Isolation of adipose derived stem cells (ADSC) from clinical lipoaspirates in culture followed by their *in vitro* differentiation into adipocytes: proof of principle study confirming the adipose tissue as a source of adipose stem cells.

Objective 2: The use of autologous fat enriched in ADSC in clinical applications: Cell Assisted Lipotransfer (CAL) technique applied to:

- Reconstructive procedures following oncological resections, complex trauma and procedures meant to alleviate congenital abnormalities.
- Aesthetic augmentation procedures (breast, cheek, chin).
- Rejuvenating procedures (wrinkles, buttocks), aiming to correct soft tissue defects

Objective 3: Postoperative evaluation of graft stability and appearance over a 12-month period.

III. LITERATURE ANALYSIS

III.1 White adipose tissue – "filler" in soft tissue plastic surgery

The adipose tissue (AT) is a specialized connective tissue which forms deposits that differ based on gene expression profiles, distribution profiles (often influenced by gender), quantity, size, species, etc. Macroscopically, there are several types of AT. Major types include white and brown AT and minor ones (from bone marrow, mammary glands) (Bjørndal et al., 2011) [21]. Considering that all experiments and procedures described in this thesis employed white AT, further discussions will only refer to this type of tissue.

White AT is not richly vascularized, but each adipocyte is in contact with at least one capillary, providing sufficient support for active metabolism. Blood flow through white AT varies with body weight and nutritional status.

III.1.1 Adipose tissue components

The cellular composition of AT is not homogeneous. The human adult AT contains about 50-70% adipocytes, ~ 20-40% vascular stromal cells and 1-30% infiltrated macrophages.

Adipocytes represent the main cell type of the adipose tissue. Although they have a variable volume, mature adipocytes are large cells with diameters of ~ 20 - 200 μm. The AT mass is determined by the number and volume of these adipocytes. Increased AT volume is achieved by increasing the size of preexisting adipocytes (adipose hypertrophy) and/or by generating new adipocytes (hyperplasia).

The stromal vascular fraction (SVF) represents an insufficiently characterized cellular population, including pericytes, fibroblasts, endothelial cells, vascular smooth muscle cells, progenitor cells for adipocytes and endothelial cells. The presence of progenitor cells for adipocytes in the SVF fraction explains the high turn-over rate of adipocytes in adult AT deposits. Approximately a tenth of all adipocytes are renewed annually through two antagonistic processes: the death of "old" cells and the adipogenesis – or birth – of "young" cells.

Additionally, AT contains a connective tissue matrix (collagen and crosslinked fibers – known as Extra Cellular Matrix or ECM), nerve fibers, lymph nodes and vascular stroma. The ECM provides structural support, tension resistance, cell surface receptor attachment sites and is a source of signaling factors that modulate a number of processes such as: cell migration, proliferation, differentiation, angiogenesis, immune response modulation, etc.

III.1.2. Stem cells from the adipose tissue

Because in the adult body, progenitor cells of adipocytes were highlighted in the SVF fraction of white adipose tissue (WAT), SVF cells were considered primary preadipocytes. Most frequently, the SVF fraction is isolated from lipo-aspirates and is referred to as *processed lipoaspirate* (PLA), whereas component cells are called SVF cells or PLA cells (Zuk et al., 2002) [8].

It has been demonstrated that cells from the SVF fraction can be cultured under special conditions, leading to an *in vitro* enrichment of culture in cells resembling mesenchymal stem cells (MSCs). After 2001, adipose tissue was found to be one of the richest sources in MSCs (Zuk et al., 2001) [13]. Compared to the bone marrow, one gram of fat contains 500 times more stem cells than 1 gram of bone marrow aspirate.

An important step in the characterization of ADSCs was the establishment of cell surface markers known as *clusters of differentiation* (CDs), which allowed the comparison of ASCs with bone marrow derived MSCs and other SVF cells of the adipose tissue. The ADSC cell surface immunophenotype is similar to that of bone marrow derived MSCs (Pittenger et al., 1999) [22] and skeletal muscle derived cells (Young et al., 1999) [23]. One major difference is that the CD34 glycoprotein does not appear on MSC cell surface but is present on human ASCs in early *in vitro* passages (Pittenger et al., 1999) [22].

Unlike freshly isolated SVF cells, human ADSCs cultured for more than one passage express low levels of histocompatibility antigens and no longer render allospecific proliferative responses in T cells (Puissant et al., 2005; McIntosh et al., 2006) [24, 25]. Therefore, ADSCs isolated from allogeneic healthy donors, grown *in vitro*, can represent an exceptional source of stem cells for therapeutic use, with a reduced risk of generating graft rejection immune responses.

Adipose tissue is an ideal source of autologous stem cells because it is easily obtained with minimal discomfort from the patient, and allows the isolation of a large number of cells through cell culture expansion (Zuk et al., 2001) [13].

III.2. Soft tissue augmentation procedures

III.2.1. Autologous fat transfer

Although fat grafting (FG) was first described 120-years ago, the interest in its development and use in plastic surgery has grown considerably after the liposuction technique was developed in the early 1980s. The presentation of *autologous fat grafting* technique (AFT) in 1985 at a meeting of the California Society of Plastic Surgeons as a breast augmentation procedure, has sparked controversy. Thus, in 1987, the *American Society of Plastic and Reconstructive Surgeons Ad-Hoc Committee on New Procedures* recommended prohibiting this procedure, since most injected fat cells do not survive and fat cell necrosis leads to scarring and macrocalcifications that reduce the sensitivity of mammography, impairing the detection of breast carcinomas. It was subsequently acknowledged that all breast surgery involves the risk of nodules and mammographic changes, and that it is possible to distinguish benign post-operative calcifications from carcinomas (Coleman 2001, Coleman and Saboeiro, 2007, Smith 2006) [26, 5, 27]. In recent years, the interest for FG has been rekindled (Bucky and Kanchwala, 2007) [28]. However, our knowledge of the fat graft behavior remains insufficient. A better understanding of developmental biology, regulation of adipogenesis at the molecular level and adipocyte survival is critical to optimizing the FG technique.

III.2.2. Use of Stromal Vascular Fraction (SVF) for soft tissue augmentation

The SVF fraction is a cell population resulting from AT manipulation. This manipulation includes: homogenization, enzymatic digestion, differential centrifugation, red blood cell lysis and washing.

SVF can be isolated either by block excision of fat (e.g. from abdominoplasty or breast reduction) or from liposuction aspirates. Most researchers separate SVF fraction from lipoaspirates (LA) obtained through liposuction procedures. If the SVF is derived from LA, the SVF cells are also referred to as PLA (*processed lipoaspirates*) cells.

In recent years, there has been a growing number of publications reporting the use of SVF cells for the repair of lesions caused by radiotherapy following mastectomy (Rigotti et al., 2007) [29], breast augmentation (Yoshimura et al., 2008 b) [30], breast reconstruction after mastectomy (Rigotti et al., 2010) [31], facial lipoatrophy (Yoshimura et al., 2008 b) [6], etc.

III.2.3. The use of PRP (*Platelet Rich Plasma*) in plastic surgery

Healing of tissue lesions is a complex process involving many cellular and humoral components, including blood platelets (BP). BP are anucleated formations in the blood derived from megakaryocyte fragmentation (Gobbi and Vitale, 2012) [32].

PRP (*Platelet-Rich Plasma*) concentrates were originally obtained from blood transfusion units and were used for hemostasis during surgery. PRP is an autologous suspension obtained by centrifugation of blood with a BP concentration 4-6 times higher that of the normal blood (200,000/µl). BP remain viable in suspension and theoretically preserve their ability to release growth factors for up to seven days (Everts et al., 2006) [33].

PRP is used in many clinical areas of surgery to stimulate wound healing and reduce bleeding during surgery. PRP is also used in aesthetic surgery for facial rejuvenation by stimulating the dermis, with minor side effects.

Autologous PRP treatment is generally considered safe in appropriately selected patients. However, the clinical use and validation of PRP is still in its infancy. The results of preclinical studies have not been confirmed by controlled clinical trials (Borzini and Mazzucco, 2007) [34].

III.2.4. Combined treatments (FG + PRP) for soft tissue augmentation

Autologous fat transfer (AFT) is a process that allows the grafting of an ideal "filler" for soft tissue augmentation. However, there is a large observed variation in cell survival rates (10-90%). Recently, several research groups have proposed the co-application of fat harvested mainly through the Coleman technique (1995, 1997) [35, 36] and PRP – a natural reservoir of growth factors that stimulates tissue repair and regeneration. Adding PRP to fat is a way to provide adequate nutrients at every transplant moment, improving fat graft survival. The release of growth factors stimulates angiogenesis, differentiation and proliferation of cells, resulting in the reconstruction of an appropriate three-dimensional matrix for the transplanted adipocytes.

Since the adipocyte's life in the graft is short, Modarressi (2013) [37] recommends the grafting method described by Coleman. In the Coleman method, a small amount of fat is injected in fine layers to increase contact surface with receptors (Coleman, 2001) [26], which enhances the chance of survival and revascularization.

In a series of *in vitro* studies, PRP has been shown to increase both the survival rate of AT cells and stem cell differentiation (Natsuko et al., 2008) [38]. Although the effect of PRP on AT has been studied *in vitro* and in model animals, there is still a lack of randomized controlled trials for clinical applications.

III.2.5. Use of ADSC (A*dipose Derived Stem Cells*) in plastic surgery

The AT mononuclear fraction, called SVF, was originally described as a source of mitotically active adipocyte precursors by Hollenberg and Vost in 1969 (Rangwala and Lazar, 2000) [39, 40]. The concept of adipose derived stem cells (ADSC) was recognized only after 2001, when Zuk et al. demonstrated that SVF contains a large number of MSC-type cells, that are able to differentiate into adipocytes, chondrocytes, myocytes and osteoblasts. The authors highlighted for the first time that AT stem cells are a promising option for future tissue engineering strategies.

Most researchers (Gregoire, 2001; Rosen and MacDougald, 2006; Cawthorn et al., 2012) [41-43] describe two phases of adipogenesis:

Phase 1 – determination phase, which includes the transformation of mesenchymal stem cells (MSC) into preadipocytes;

Phase 2 - terminal differentiation phase, which characterizes the transformation of preadipocytes into adipocytes, thus distinguishing between ADSCs and preadipocytes. The main difference between the two types of mesenchymal stem cells is that ADSCs are multipotent stem cells and differentiate into adipocytes, chondrocytes, myocytes and osteoblasts, whereas preadipocytes are unipotential cells capable only of adipogenic differentiation (Laudes, 2011) [44].

It is now widely acknowledged that ADSC cells can be easily extracted from adipose tissue, are capable of expanding *in vitro*, have the ability to differentiate into many cell types and can be used in regenerative medicine (Yuksel et al., 2005; Stosich and Mao, 2005; Schäffler and Büchler, 2007; Choi et al., 2010) [45-48]. Clinical trials have already been published, some of which have demonstrated the success of ADSC treatment in tissue reconstruction. However, there are still problems related to the practical difficulties of extending the isolation protocols of these cells to clinically required volumes, that cellular preparations

isolated in different laboratories differ in their degree of purity, molecular markers, etc. (Locke et al., 2011) [14].

Additional studies are needed to evaluate the safety of their use in humans, as there are questions as to the isolation and purification of ADSC, their effect on tumor growth, etc. Clinical trials are underway using ADSC therapy and their partial results are promising not only in plastic surgery but also in other medical specialties. Thus, Herreros et al. (2012) [49] conducts a randomized clinical trial in 200 adult patients on the autologous ADSC cells involved in the management of complex anal fistulas, partial data demonstrating that this procedure is safe and demonstrates the acceleration in the re-epithelisation process of open wounds. It is necessary to standardize protocols and carry out larger randomized trials to ensure the safety of applications in accordance with FDA (*U.S. Food and Drug Administration*) guidelines (Gir et al., 2012) [50].

The International Stem Cell Institute in San Diego, the world leader in stem cell therapy, consider that adult stem cells are a real possibility for curing diseases and medical conditions, being a future direction of medicine compared to embryonic stem cells studies that led to negative results. They announced the launch of the ADSC cell therapy program to treat some diseases and degenerative conditions. For therapeutic purposes, from peripheral blood, about 10,000 stem cells can be separated, from bone marrow - 50,000 stem cells, and from adipose tissue up to 60×10^6 ADSC cells.

III.2.6. Cell-Assisted Lipotransfer (CAL) technique for correcting soft-tissue defects

In order to overcome the practical drawbacks of autologous fat transplantation, a new strategy called Cell Assisted Lipotransfer (CAL) was developed. The technique employs autologous ADSCs in combination with autologous fat transplantation.

Many authors have not used *in vitro* purified ADSCs, but the freshly isolated SVF fraction, which is a heterogeneous cell population containing also ADSCs. The procedure is especially used for breast augmentation by grafting autologous fat enriched with SVF cells.

In autologous fat transplantation, the implanted AT survives by a simple diffusion mechanism until the blood circulation is restored. Thus, graft survival, particularly in the case of a high graft volume, is the result of the balance between this process and hypoxia-induced cell death (Lin et al., 2008) [51]. Factors favoring cell survival, induce long-term retention and therefore an extended graft life.

SVF favors neoangiogenic vasculature and the fibrinogenic activity of fibroblasts that improve AT survival and induce the proper 3-D tissue organization. The results are: enhanced graft survival compared to traditional FG technique and reduced AT necrosis due to neovascularization of the transplanted tissue (Gentile et al., 2012) [52]. Riordan et al. (2009) [11] considers that the CAL procedure using the SVF fraction leads to the best results in plastic surgery, because it provides ADSCs that can differentiate and regrow the adipose tissue, while also providing the graft with other cell types that induce angiogenesis and have immunomodulatory effects. In addition, the SVF fraction is also a source of soluble factors that facilitate the wound healing process, and the cost of the procedure is less then when employing *in vitro* propagated ADSCs.

IV. MATERIALS AND METHODS

Part of the research performed in the current PhD thesis is the result of previous collaboration with the Department of Biochemistry and Molecular Biology at the University of Bucharest. The collaborative project to which I contributed as research-partner was funded by the Exploratory Research Project PCCE 248 (2010-2013) entitled "New Concepts and Strategies for Developing the Knowledge of New Biocompatible Structures in Bioengineering".

IV.1. Isolation of the SVF fraction

Lipoaspirates were obtained from subcutaneous adipose tissue of patients who have undergone cosmetic liposuction. All medical procedures were performed with patient consent, in accordance with the Helsinki Declaration and with the approval of the Ethics Commission (Decision No. 3076 / 10.06.2010). Patients affected by neoplasia, infections, inflammations and autoimmune diseases were excluded from the study.

All steps were performed under aseptic working conditions using sterile reagents and media. Lipoaspirates were stored at room temperature (maximum 4 hours) or at 2 - 8º C (maximum 24 hours) before processing. The lipoaspirate processing technique has been optimized following the protocol described by Gimble et al. (2007) [53]. The procedure includes the following steps:

• washing the fraction to remove red blood cells and leukocytes;

• AT dispersion by collagenase digestion and

• centrifugation of the collagenase digest, which allows the separation of two populations: the mature adipocyte population (floating in the medium) and the SVF fraction which precipitates at the bottom of the tube.

IV.2. Isolation and multiplication of adipocyte stem cells (ADSC) *in vitro*

The protocol used by the Department of Biochemistry and Molecular Biology at the University of Bucharest is a variation of the already published protocol by Aust et al. (2004) [54] on the isolation of human ADSCs from lipoaspirates after collagenase digestion.

Following the last centrifugation step of the LA / SVF cell-isolation protocol, the cell pellet was resuspended in a low glucose DMEM: F12 medium (Sigma-Aldrich) supplemented with 40% fetal bovine serum (FBS), 1 % ABAM, 13.8 mM $NaHCO_3$ and 0.5 mM sodium pyruvate. The medium was changed every 24 hours until the cells reach 70-80% confluency, after which they were detached and transferred to new culture vessels. Cellular passages were numbered consecutively.

Cell counting was performed using the hemocytometer - a classic method for monitoring cell viability and proliferation. Cell viability assessment was performed using Trypan Blue - a dye that penetrates dead cells when membrane structure and permeability are compromised. Dead cells are thus stained blue, whereas viable cells do not incorporate the dye.

The senescence of cells isolated from the lipoaspirate, grown over 10 passages, was evaluated by the detection of β-galactosidase (β-Gal) activity using the Senescence Cells Histochemical Staining kit (Sigma-Aldrich).

IV.3. Adipogenic differentiation of ADSCs

Passage III ADSCs were evaluated for adipogenic differentiation. The induction of adipogenesis in ADSCs was performed with a hormonal cocktail containing insulin, dexamethasone and 1-methyl-3-isobutylxanthine (Rosen and Spiegelman, 2000) [55].

To promote adipogenic differentiation, ADSC cells were grown in culture medium supplemented with 0.5 mM 3-Isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich), 1 μM dexamethasone (DEX), 1 μg / ml (2.26 μM) Triglitazone, 1 μM Insulin, 10 μg / ml (~ 41 μM) Biotin, 200 μM Indomethacin, 0.1 mM Hydrocortisone, 0.2 nM 3,3 ', 5- Triiodothyronine and 10 μg / mL Transferrin (all reagents purchased from Sigma-Aldrich). Cells were maintained in the above mentioned supplemented growth medium for 21 days.

The adipogenic differentiation process was subsequently monitored using oil red staining with Oil Red O and immunofluorescence observations.

As adipogenic differentiation advances, the cells accumulate intra-cytoplasmic lipids in parallel with cell morphology alterations. At the mature adipocyte stage, about 90% of the cell volume will be occupied by lipids. Lipid accumulation was observed by phase contrast microscopy after staining with Oil Red O (Sigma-Aldrich).

The following MSC specific surface markers were investigated by fluorescence microscopy: CD44, CD73 and CD90. The hematopoietic stem cells (HSC) specific antigen CD34 was chosen as negative control. Primary and secondary antibodies were purchased from Santa Cruz Biotechnology, INC.

IV.4. Clinical study

IV.4.1. Preparing the surgery

All patients were advised to quit smoking prior to surgery. Certain drug treatments were discontinued (such as those that increase the risk of bleeding or thrombosis). Patients were advised not to eat or drink 6 hours prior surgery. After investigating the patient's medical history, all patients were clinically evaluated, with an emphasis on detecting areas with excess fat deposits and good skin quality (parameters of interest: degree of hydration, elasticity, local infection, etc.).

IV.4.2. Harvesting the fat through Coleman technique

Surgical interventions were performed under general or epidural anesthesia. The most commonly used donor areas were those recognized by Padoin et al. (2008) [56] as most likely to render a high yield of adult stem cells – namely abdomen and inner thighs (especially in the upper third of the thigh). As far as possible, donor areas were accessed through incisions performed in folds, pre-existing scars, stretch marks or hairy areas (Rohrich et al., 2004) [57]. All interventions were performed under maximum sterility conditions.

In all cases, to ensure hemostasis, donor areas were infiltrated with a mixture containing adrenaline 1:1,000,000 (Coleman, 2007) [5]. We used a 15 or 23 cm long blunt Coleman cannula with a 1 - 2.5 mm diameter, having two holes close to the tip, attached to a 10 ml Luer syringe. The cannula's distal holes are sized and shaped to allow the harvest of the largest intact AT fragments that can pass through the lumen of the Luer-Lock type syringe.

IV.4.3. Obtaining ADSCs enriched adipose tissue graft

IV.4.3.1. Coleman technique for fat processing

Half of the aspirated fat was processed according to the technique proposed by Coleman. To ensure sterility, we used a centrifuge with a sterilizable central rotor, fitted with chambers in which the 10 ml syringes were placed. The syringes were centrifuged at 700 g for 3 minutes, allowing the components to separate based on density. The less-dense upper layer has an oily composition. The middle layer consists of potentially viable portions of AT and the most dense, pelleting layer, contains blood, water and lidocaine. The oily layer was decanted from the syringe with an absorbent pad. After changing the absorbent pad 2-3 times, the plunger was inserted back into the syringe and pushed forward, allowing the fat to slide toward the tip of the syringe and obliterate the "dead space".

IV.4.3.2. Isolation of the stromal vascular fraction (SVF)

To isolate the stromal vascular fraction (SVF) we used the protocol proposed by Yoshimura (Yoshimura et al., 2008 a) [6]. The other half of the adipose tissue harvested was dispersed using 0.075% type I collagenase dissolved in saline phosphate buffer, followed by agitation at 37 ° C for 30 minutes. The mixture was then dispensed into 10 ml syringes and centrifuged for 5 minutes at 1,200 g. The SVF was located in the pellet derived from centrifugation, at the bottom of the lipoaspirate. The stromal vascular fraction (SVF) was mixed with the previously purified fat and transferred into 10 ml syringes.

IV.4.4. Infiltration of ADSC enriched adipose tissue graft

The AT grafts were placed in the recipient site so that they were either separated from each other as much as possible by the host tissue, which created a greater contact surface between the harvested fat and the recipient tissues, thus facilitating diffusion and respiration Piasecki et al., 2007) [58].

For the infiltration stage we used local and/or general anesthesia. The infiltration was performed using a blunt Coleman infiltration cannula, having a single hole close to the tip, in order to reduce the risk of hematomas. The use of the blunt cannula allowed the placement of AT grafts in a stable manner. Sharp-tipped cannulae, which offer better control for subdermal, fibrous and scar tissue, were used only to remove adhesions. Caution was employed when using sharp cannulae, in order to avoid damaging nerves and other underlying structures. For the face, the maximum tissue volume placed with each retraction of the cannula, was 0.1 ml and in some areas, such as eyelids, the volumes were as small as 0.02 - 0.03 ml.

IV.4.5. Post-operative monitoring

Patients were monitored at 3, 6 and 12 months after surgery, respectively.

V. RESULTS AND DISCUSSIONS

Unfractionated autologous fat is commonly used in reconstructive and aesthetic procedures as a filler for the repair of soft tissue defects. Adipose tissue offers several advantages to the plastic surgeon including: ease of harvesting and injection and significant versatility. Unfortunately, mature adipocytes from the transplanted tissue frequently degenerate, leading to partial resorbtion of the graft and formation of cysts and calcifications (Frye et al., 2005; Stillaert et al., 2007) [59-60].

Recent studies have highlighted that the mature adipose tissue contains pluripotent stem cells, called adipocyte derived stem cells (ADSC) (Sterodimas et al., 2009) [61]. The purpose of this thesis was to evaluate the benefits of using ADSC-enriched lipoaspirates in reconstructive, aesthetic and corrective procedures, as an alternative to current tissue engineering techniques. To achieve this goal, we focused our attention on the ADSC-enriched stromal vascular fraction (SVF).

V.1: The SVF fraction is enriched in ADSCs, which can be amplified in culture and differentiated into mature adipocytes

ADSCs isolated from the SVF are scarce and are estimated to represent less than 0.02% of this fraction (Stillaert et al, 2007) [60]. Therefore, if a pure population of adipocyte stem cells is to be used for clinical applications, it is first necessary to considerably expand this population. Culturing ADSCs *in vitro*/*ex-vivo* allows not only their amplification, but also the characterization of the isolated cell population, while offering the possibility to differentiate the cells into mature adipocytes by manipulating the culture medium composition.

Therefore, the current study sought firstly to isolate adherent preadipocyte stem cells in culture, expand them, and identify the biochemical factors necessary for their adipocytary differentiation.

Lipoaspirates obtained from subcutaneous AT of patients who required cosmetic liposuction procedures were processed according to the protocol described by Gimble et al (2007) [53] for the isolation of the SVF fraction. Isolated cells were placed in culture dishes and incubated in culture media containing nutrients and fetal serum, thus providing them with the essential ingredients for maintaining cell viability. (Lodish, 2013; Campisi & d'Adda di Fagagna, 2007) [62, 63].

The senescence of the primary cells isolated from the SVF fraction was interrogated by performing a cytotoxic staining for the marker Senescence Associated-β-Galactosidase (SA-β-Gal). Our analysis revealed that cells in the incipient passages (I-VII) did not become senescent. These passages can therefore be used in potential tissue engineering procedures.

Analysis of cell surface markers by immunofluorescence confirmed the existence of a population of cells expressing MSC specific markers: CD44, CD73 and CD90. The CD34 HSC specific marker was not detected, thus confirming the purity of the *in vitro* isolated stem cell population.

In addition, we were interested in assessing whether cultured stem cells can be differentiated into mature adipocytes. For this purpose, the culture medium was supplemented with insulin, dexamethasone, glucocorticoids and 1-methyl-3-isobutylxanthine (IBMX) (Rosen and Spiegelman, 2000) [55]. After supplementing the culture medium with the above-mentioned factors, we observed the gradual modification of cell morphology and the intracytoplasmic accumulation of lipids. These observations confirm the differentiation of stem cells into mature adipocytes, which can later be used in surgical and tissue engineering applications.

V.2: The use of CAL (Cell Assisted Lipotransfer) technique for grafting ADSC enriched adipose tissue leads to satisfactory clinical results

Autologous fat grafting (FG) is a procedure used for 120-year, but the interest in its development and use in plastic surgery has increased considerably since the liposuction technique was developed in the early 1980^{s}. The main complications reported after autologous FG are: partial graft resorption (leading to the necessity of repeating the surgical procedure) and partial necrosis of the transplanted adipose tissue (Toyserkani et al., 2016) [64].

Transplanted adipose tissue (AT) survives by a simple diffusion mechanism until blood circulation is restored. Thus, the survival of the graft, particularly in the case of a large grafted volumes, is the result of reaching a balance between this process and hypoxia-induced cell death (Lin et al., 2008) [65]. Factors favoring cell survival induce long-term retention and hence enhance the life span of the graft.

In order to overcome the inconveniences associated with lipoinjection, a new strategy, called cell assisted lipotransfer (CAL), was developed, in which autologous ADSCs were used in combination with adipose tissue graft. Many authors have not used *in vitro* purified ADSCs, but the freshly isolated SVF fraction, which is a heterogeneous cell population enriched in ADSCs (Yoshimura et al., 2008. b; Matsumoto et al., 2006) [6, 10].

Riordan et al. (2009) [11] considers that using the SVF fraction for the CAL procedure leads to the best results in plastic surgery, since the fraction contains both ADSCs, which can be differentiated into mature adipose tissue, and other cell types that induce angiogenesis and have immuno-modulating effects. In addition, the SVF fraction is a source of soluble factors that facilitate wound healing (Riordan et al., 2009) [11].

In the current study, the CAL procedure was applied to 5 patients presenting with various congenital or acquired soft tissue defects, including: Parry-Romberg Syndrome, buttock lipodystrophy, Poland syndrome, or breast asymmetry. Additionally, 18 patients requested aesthetic procedures - facial rejuvenation and hand rejuvenation. The use of the CAL procedure has led to a significant improvement in the aesthetic aspect of the treated soft tissue. In addition, the post-operative aspect has been shown to be stable for a minimum of 12 months.

The current study did not include a control cohort of patients assigned to the control grou(for example, patients treated using classical FG technique). However, the results observed previously in patients treated with classical lipofilling revealed a higher degree of resorption of the transplanted fat and more frequent calcifications, the results of the transplant being thus less stable over time.

For the study group, the end results were appreciated by all patients as good or very good, the transplanted tissue having a soft, natural consistency. No significant complications were reported, and only in two cases there was partial necrosis of the transplanted fat reported.

In the case of patients treated with other fillers (hyaluronic acid, collagen, etc.) the procedure need to be repeated due to the resorption of the filling material. CAL has the advantage that adipose tissue recovers and continues to proliferate and therefore does not need to be reinjected.

V.3: Integrating the current study's data into the data presented in the literature to address the effectiveness of the CAL procedure

Reports published over the past decade have not reached a consensus regarding the superiority of the CAL procedure compared to autologous fat transplantation (AFT) technique. In their retrospective report, Toyserkani et al [64] concluded that most of the published CAL procedures seem to favor the supplementation of transplanted adipose tissue with ADSCs. However, the authors draw attention to the fact that future studies require better standardization of the reporting mode, allowing for: (i) an objective evaluation of the observed clinical outcomes and (ii) identifying the potential risks associated with this new procedure (Toyserkani et al., 2016) [64].

It is important to remember that the AFT technique was also met with reluctance at the time of its introduction. Resorption and necrosis of transplanted adipose tissue are two of the complications associated with classical AFT technique. It is known that the formation of functional vasculature governs the survival of mature adipose tissue (Fukumura et al., 2003) [66]. By supplementing ECM/adipose tissue residing ADSCs to the transplanted fat, the CAL technique allows the protection of mature adipocytes from hypoxia (Strawford et al., 2004) [67].

Both the stem-cells-enriched SVF fraction and ADSCs isolated from autologous fat can be supplemented to the transplanted adipose tissue. It is important to note that high volume lipoaspirates (~ 500 ml) contain a sufficient number of adipose stem cells, so their *in vitro* amplification is not necessary (Yoshimura et al., 2010) [68]. By eliminating the intermediate ADSC amplification steps, ethical objections and potential immunological complications are avoided (Toyserkani et al., 2016) [64], and at the same time the cost and duration of the surgical procedure is reduced to the benefit of the patients. For these reasons, the use of the SVF fraction is most often preferred in the CAL procedure.

Current literature provides both examples that support the superiority of the CAL procedure compared to the classic AFT technique, as well as examples in which supplementing ADSCs to transplanted fat did not render better results than the classical transplantation method.

Toyserkani et al. (2016) [64] summarized the observations of seven clinical trials that compared the CAL technique with the classic AFT procedure. Six of the seven studies underpinned the superiority of CAL, although the controls, reporting, or statistical evaluation of these studies is generally not satisfactory (Toyserkani et al., 2016) [64]. Five of the studies performed opted for supplementing the fat injected with the SVF fraction; two of these studies reported cases of breast augmentation, while the rest of the studies focused on facial aesthetic procedures. In most cases, the survival of the fat graft following the CAL procedure was superior to that observed for classic AFT transplantation, although the observed difference was modest (Toyserkani et al., 2016) [64]. The reported clinical trials did not generally evaluate post-transplantation graft histology. Preclinical studies in animals suggest that generally, the CAL procedure leads to the formation of a lower number of cysts, the maturation of a larger number of adipocytes, and the formation of denser vasculature compared to classical AFT (Toyserkani et al., 2016) [64].

Sterodimas et al. (2009; 2011) [61, 69] also compared the CAL procedure with AFT. Their 2011 study tracked 20 patients with various facial soft tissue defects (Sterodimas et al., 2011) [69]. The authors concluded that patients who were transplanted with ADSC-enriched fat reported a superior degree of satisfaction 6 months after surgery compared to patients undergoing the classic procedure. However, the satisfaction rate 18 months after the completion of the procedure was similar for both arms of the study (Sterodimas et al., 2011) [69]. The authors have admitted that the CAL procedure is more expensive compared to classical fat grafting (justified by the need to use additional medical and laboratory equipment and the need for hiring specialized staff) and that the process of enriching the transplanted fat

with stem cells leads to the prolongation of the surgical procedure. However, considering that patients undergoing CAL procedure required a single transplant to achieve satisfactory aesthetic results, it can be concluded that both the cost and the final time needed to complete the surgery are in most cases inferior to classic AFT procedures (Sterodimas et al., 2011) [69]. Although the author's conclusions seem encouraging, the data presented do not support claims of superior satisfaction to patients undergoing CAL 6 months after surgery compared to those undergoing the classic AFT procedure. In addition, the authors mistakenly tried to statistically assess the differences in patient satisfaction based on subjective patient evaluation. It is well known that statistical tests assessing the significance of a study cannot be based on arbitrary parameters, such as self-evaluation of satisfaction on an arbitrary scale (Motulsky, 2013) [70]. The statistical evaluation of the volume of resorbed fat, or an evaluation of the percentage of surviving fat by objective procedures (MRI, tomography, ultrasound) would be statistically superior to those reported in the published study.

Yoshimura et al. reported in 2010 [68] a summary of a set of studies comparing CAL with AFT. Between 2003-2008, the authors treated 188 patients, the majority (164) undergoing breast augmentation procedures. 26 of the patients were subject to the CAL procedure immediately after the removal of pre-existing breast prostheses. (Yoshimura et al., 2010) [68]. An important aspect of these studies is that the authors reported objective measurements obtained by MRI, ultrasound, and mammography, additionally to the classic video and photos employed for pre- and post-surgical evaluation. In cases of mammary augmentation, 3D measurements performed 6 months after transplantation indicated that 40-90% of the fat transplanted by the CAL procedure survived the transplant. The final volume increase varied between 100-250 ml, below that generally observed in augmentation procedures using silicone implants. A small number of patients developed cysts ranging in size from 5-15 mm (6 patients), and 2 patients had calcifications, which could be easily distinguished from the adjacent tissue. However, the technique proved to be superior in terms of natural appearance and breast consistency (Yoshimura et al., 2010) [68].

A small number of clinical trials have assessed the efficiency of *in vitro* purified and amplified ADSCs co-transplanted with autologous fat. Preclinical studies using animal models have provided a first indication that grafting a larger number (10^6-10^7 compared to 10^5 cells) of purified ADSCs leads to superior short-term results, although differences between different cellular concentrations become minimal on the long term (Toyserkani et al., 2016) [64].

However, not all clinical trials reported superior results when CAL was compared to classical lipotransfer. Peltoniemi et al. compared classic AFT (applied to 8 patients) with CAL technique (applied to 10 patients) in the context of mammary augmentation. (Peltoniemi et al., 2013) [71]. The postoperative evaluation was performed by MRI technique over a period of 12 months. The authors concluded that although fat resorption after CAL is slightly reduced compared to post-AFT resorption (50% vs 54%), the difference is minor and statistically irrelevant (Peltoniemi et al., 2013) [71]. However, the study used a small number of patients, making it difficult to generalize the observations.

Following a comparative study of literature, Grabin et al. (2015) [72] also states that current data does not support the superiority of CAL technique compared to classical lipotransfer. The authors point out that most clinical reports use a small number of patients, reported volumetric augmentation is often done subjectively, and moreover, the statistical analysis of the data presented is often suboptimal. The authors have acknowledged that in some situations, objective evaluation by MRI is not possible if the transplanted fat volumes are too small to allow the detection of significant differences. The authors of the retrospective study therefore underlined the need to carry out randomized, blind or double-blind clinical trials, to avoid subjective reporting. In addition, it is essential that future clinical trials contain a larger number of patients and that the follow-up period is prolonged (Grabin et al., 2015) [72].

The current study's results seem to confirm the data reported so far in the literature. Lipotransfer supplemented with the SVF fraction resulted in good and very good aesthetic results, with a low complication rate.

A limitation of the current study is the absence of a control group. However, comparing the results presented with those previously reported, we could conclude that the CAL technique is a safe method that leads to stable results, superior to classical lipofilling.

V.4: Safety of CAL technique

Taking into account that studies on animal models have often highlighted a better fat graft vascularization in ADSC-supplemented adipose tissue (Toyserkani et al., 2016) [64], concerns arised whether using the CAL technique in patients who have undergone oncologic treatments can lead to the activation of latent metastases.

Gentile et al., (2012) [52] concluded that the use of CAL for breast augmentation in patients previously diagnosed with breast carcinoma did not lead to an increase in relapsing tumors.

However, Perrot et al. (2010) [73] reported a case of local recurrence of an osteosarcoma 18 month after a lipofilling surgery using the CAL technique. The 17-year-old patient did not experience any tumor recurrence for 13 years following oncological and surgical treatment for the primary tumor located in the upper arm. The authors conducted an *in vitro* and mouse study, and monitored tumor cell amplification rates as well as tumor volume increase in the presence and absence of ADSCs. The co-culturing of tumor cells with ADSCs led to increased tumor cell amplification in the first 24 hours (Perrot et al., 2010) [73], although the effect was not maintained at the same rate by prolonging the co-culturing. The correlation between local relapse and use of CAL in this case study seems reasonable. However, the authors' conclusions are not supported by solid experimental data. The authors attempted to justify the potential pro-tumorigenic effect of transplanted ADSCs as a result of local vascular amplification (Perrot et al., 2010) [73], although the study did not evaluate whether tumors co-transplanted with ADSCs are indeed better vascularized compared with those developed in the control group.

The current study did not include any patients previously diagnosed with cancer and therefore we cannot formulate an opinion on the pro- or anti-tumorigenic effects of transplanted ADSCs.

VI. CONCLUSIONS AND PERSPECTIVES

The current study aimed to evaluate the applicability of using adult adipocyte stem cells in reconstructive, aesthetic and corrective procedures.

Firstly, we showed that ADSCs can be isolated and amplified in culture for a limited time (maximum seven passages) before entering senescence. Cells isolated in culture could be differentiated into mature adipocytes by supplementing the culture medium with a cocktail containing 3-iso-butyl-1-methylxanthine, dexamethasone, insulin, indomethacin and triiodothyronine. The ability of ADSCs to differentiate into mature adipocytes *in vitro* opens possibilities for the development of new tissue engineering approaches.

In parallel, ADSCs were used in 23 clinical CAL procedures. In the current study we preferred using the SVF fraction, enriched in mature stem cells, in contrast to *in vitro* amplified ADSCs. This approach allowed us to avoid ethical problems and also shortened the duration of the surgical procedures. According to our knowledge, the current study is the first in Romania to evaluate the effectiveness of the CAL procedure in various aesthetic and reconstructive procedures. Clinical observations have made it possible to confirm that the CAL technique is well tolerated by patients and leads to satisfactory results for both the patient and the attending physician. In addition, the technique has proven to be safe and was not associated with significant complications.

Future studies are needed to evaluate objectively the effectiveness of the CAL technique compared to classical lipotransfer. In particular, future studies will need to involve a larger cohort of patients, include a control group and should use (where possible) objective methods for pre- and post-surgical evaluation, such as MRI, tomography, ultrasound.

Aspects to be pursued in future research include: the effect of the donor area and the receiving area on the percentage of surviving fat tissue; to what extent the age of the patient affects the success of the procedure; chemical factors (chemokines, growth factors) that can be added to improve the survival of the transplanted fat; the number / density of transplanted ADSCs minimally required to effectively reduce the resorption of transplanted fat. In addition, for the use of CAL in patients who have undergone oncological therapies, studies should include a large cohort of patients in both the control group and the treatment group to investigate if ADSC-supplemented transplantation may lead to an increased risk of tumor recurrence.

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Abbreviations

ADSC	Adipose derived stem cells
AFG	Autologous fat grafting
AFT	Autologous fat transfer
AT	Adipose tissue
BSA	Bovine Seric Albumin
CAL	Cell-Assisted Lipotransfer
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CREB	cAMP response element-binding protein
ECM	Extracellular matrix
FBS	Fetal Bovine Serum
FDA	U.S. Food and Drug Administration
FG	Fat grafting
hADSC	Human Adipose derived stem cells
HSC	Hematopoietic stem cells
HIF-1	Hypoxia inducible factor
IBMX	Isobutyl methyl xanthine
IGF	Insulin-like growth factor
LA	Lipoaspirate
MAPK	Mitogen Activated Protein Kinase
MSC	Mesenchymal stem cells
MSH	Melanocyte-stimulating-hormone
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate Buffer Saline
PKA	Protein Kinase A
PLA	Processed lipoaspirate
PPARγ	Peroxisome proliferator-activated receptor-γ
PRP	Platelet-rich plasma
RBP4	Retinol binding protein 4
SAT	Abdominal subcutaneous adipose tissue
SA-β-Gal	Senescence Associated-β-Galactosidase
ST	Soft tissue
STA	Soft tissue augmentation

SVF	Stromal vascular fraction
TE	Tissue Engineering
TNFα	Tumor necrosis factor alpha
tPA	Tissue plasminogen activator
WAT	White adipose tissue