PROCEDURES OF VASCULAR STROMAL FRACTION ISOLATION FROM ADIPOSE TISSUE IN OSTEOARTHRITIS TREATMENT- RESEARCH DEVELOPMENT AND CLINICAL APPLICATIONS

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INTRODUCTION

Regenerative Medicine, pursuant to the world of science has broadened its horizons over the past years towards new and promising technologies that enable surgeons to obtain cell concentrates and homogenized tissue to be used in patients forthwith.

The new procedures envision minimal manipulation of processed tissues that are not subject to GMP or Cell Factory regulations. The current guidelines concerning cells and tissue (Directive 2009/120/CE Advanced Therapy Medicinal Products) define products of Advanced Therapy as extensively manipulated cells and tissue among which cellular expansion or products intended for non-homologous use in relation to the physiological characteristics of the processed tissue or cell.

Centrifugation, homogenization, disaggregation are methods that imply minimal processing of tissue or cells and the relative biological products are not equivalent to Advanced Therapies and thus not considered as medicinal products. Such biological products, albeit exempt from regulatory restrictions, still require extreme caution in handling and adequate clinical application under strict sterile conditions, using only certified instruments and medical devices that fully ensure the safety of the patients undergoing such advanced therapies.

Cellular localization and characterization of ASCs

Adipose tissue is mainly composed of organized adipose cells into lobules characterized by highly complex material with mature adipocytes constituting more than 90% of the material and a stromal vascular fraction (SVF) including pre-adipocytes, fibroblasts, vascular smooth muscle, endothelial

cells, resident monocytes/macrophages, lymphocytes and ASCs. Numerous studies have revealed that ASC characteristics vary and are mainly dependent on the localization of the adipose tissue collection. ASCs collected in the superficial abdominal wall are significantly more resistant to apoptosis compared to ASCs collected in the upper arm, the medial thigh, the trochanter and in the superficial deep abdominal deposits. The density of the reserve stem cell population will vary within the adipose tissue and may alter according to the localization as well as to type and species (i.e. human or mouse species). In white adipose tissue, ASCs are more highly represented in subcutaneous deposits compared to abdominal fat and are most abundant in the arm and the adipose tissue of the groin with greater plasticity, besides, in the latter.

Similarly, studies have reported that ASCs are present in brown adipose tissue deposits distributed within the deposits of white adipose tissue and are subject to skeletal muscle differentiation.

To date, despite numerous attempts to establish clear consensus guidelines, none have been reached regarding ASC phenotype definition and distinction between cells and fibroblasts. Cells that have been freshly isolated from SVF are considered a highly heterogeneous cell population including stromal cells (CD31-, CD34+/-, CD45-, CD90+, CD105+, CD117+/- e CD146-), endothelial progenitor cells (CD31+, CD34+, CD45-, CD90+, CD105- e CD146+), vascular smooth muscle cells o pericytes (CD31+, CD34+/-, CD45-, CD90+, CD105-, CD146+ e NG2+) and hematopoietic cells (CD45+). Furthermore, freshly isolated cells from the SVF and ASCs, at an early stage, express high levels of CD117 (c-kit) compared to “aged” ASCs. The human leukocyte antigen DR (HLA-DR) is also indicated as well as stem cells that co-express markers such as CD34 with low level stroma markers including CD13, CD29 (Beta-1 integrin), CD44 CD63, CD73, CD90, CD105 e CD166. Reduced expression of CD34 at a later stage may consequently trigger ageing of the stem cell (or a variation in one of the several cytotypes) but one study reveals that CD34 expression may be preserved in twenty days of culture providing conditions of stem cell growth in suspended sphere cultureswhich display embryonic features allowing the cell to remain undifferentiated. In addition, various investigations show that ASCs CD34+ possess greater proliferative potential while ASCs CD34- possess higher plasticity.

ASCs as an adipose tissue source

Fat grafts used as fillers to correct defects were first used by Neuber in 1893 and many surgeons have since employed traditional measures of assisted aspiration to conduct the autologous fat grafting, to collect the adipocytes and re-inject the fat tissue? However, the life expectancy of the grafts remains unpredictable, most likely, due to poor revascularization and subsequent death of the undifferentiated adipocytes that were previously injected. The discovery of ASCs has yielded ample interest motivating the use of these cells as an adipocyte source which serves to fill and regenerate

the fat tissue. Predictably, ASCs have displayed potential to differentiate into mature adipocytes if exposed to an adequate culture media containing steroids (promoters of differentiation). Terminal differentiation into adipocytes may be ascertained via OilRed O staining and immunohistochemical analysis. This procedure will stain the lipid droplets red within the cytoplasm. In addition, specific gene expression of the adipocytes has exhibited ASC differentiation into adipocytes. Lipoprotein lipase (LPL) genes and the fatty acid-binding protein (FAB4, also known as aFABP) are regular markers to define fatty acid metabolism. Clinical trials are underway to improve the outcomes of fat grafting for contour deformities or for breast enlargement. Other studies are focusing on a novel strategy known as cell-assisted lipotransfer which envisions isolation and recombination of the SVF with different purified fat before proceeding to re-injection. Such procedure would yield a higher volume of re-injected fat. Another study has refined the purification method of lipo-aspirated fat by means of centrifugation permitting a more adequate engraftment of the implanted fat without recurring to compensatory defect corrections. Automated systems for fat processing and subsequent enrichment of fat grafts using ASCs have been produced and used in a clinical context in Europe and Asia, though results are pending. Other studies relative to ASCs in fat grafting concern the treatment of radiation necrosis and the design of large constructs for the reconstruction of the breast following a mastectomy as well as congenital deformities. Noteworthy is the speed of preparation, and specifically, herein, the preparation of the lipoaspirate, to ensure an optimal surgical result. Hence, an equilibrium between surgical technique and purity of the ASCs is crucial for the welfare of the patient.

Commercial systems

Numerous collection techniques, expansion and enhancement of stem cells are available starting from the so- called “enzymatic” methods to the customary “mechanical” methods. Herein, we assess a novel technology which enables the collection and concentration of lipoaspirated fat as well as separation of the adipose stem cells within a closed sterile environment. These methods may be used in a course of such clinical procedures as repair of cutaneous tissue loss or for aesthetic purposes such as skin rejuvenation.

The lipofilling technique was described in 1893 and re-proposed in the 1980s by Illouz, the father of modern liposuction. The technique was standardized in 1990 by Coleman but the regenerative potential of lipofilling was only revealed in 2001 with the discovery of ?? totipotent stem cells among the adipose cells, thus the objective of volumization developed into a hypothesis of tissue regeneration precisely for stem cells. The advance of the technique has allowed for the development of smaller and smaller cannulae and filters to best exploit the regenerative role of adipose tissue which has subsequently provided new definitions of adipose-aspirated tissue treatment and

formulation for injection. Macrofat obtained with liposuction cannulae used mainly for its volumetric effects has advanced to Microfat obtained by means of specific cannulae maintaining favourable preservation of the stem component.

The SVFs isolated from the ASCs have been employed with favourable outcomes in clinical trials and experimental studies, however, disadvantages have been reported regarding current European and international regulatory strategies and the possibility of using such commercial systems. Implementation of the regulations concerning therapies based on cells and their clinical application still remains a challenging and elusive issue. First and foremost, a universal standard operating protocol devoid of chemical reagents which includes the substitution of the enzymes is urgently required as well as a rapid assessment of the quality and efficacy of these systems. Methods and systems present advantages and drawbacks and indeed the standardization of a series of parameters are underway which include operative instructions (manual or automatic), handling procedures, costs (expensive equipment and high costs of medical supplies).

The following systems are currently available:- 1)The Rigenera System, a disposable device also known as Rigeneracons, a mechanical disruptor of biological tissues, i.e. the dermis, connective tissue, bone tissue, dental pulp, that is able to create micro-grafts in an autologous, homologous and minimally invasive manner; 2) The Lipogems System is a processing cylinder containing stainless steel spheres and two filter sieves. The mechanism activates the steel spheres by means of vibrational agitation and enables tissue emulsion with the continuous flow of physiological solution reducing the clusters which maintain the vascular-stromal niches intact, simultaneously eliminating residues of oil and blood elements; 3) The HY Tissue SVF system is composed of processing bags through which the lipoaspirated fat is filtered into the first filtration bags. The residue is placed into the relevant syringes which is subsequently centrifuged. The liquid residue is then passed into the second filter bag which thus enables acquisition of the SVF; 4) The nanofat system transfers the fat in a closed system which permits extraction, emulsification and acquisition of fat cells (nanofat) in a completely sterile contamination-free environment; 5) The Q-graft System contains a series of chambers to mix the lipoaspirated material during incubation at 38° C and the cross-flow filtration for the concentration of the SVF cell suspension.

Current clinical applications and future perspectives

ASCs have been investigated in pre-clinical trials to treat various disorders that affect a range of tissues and organs as shown in Table 1. Most preclinical studies related to ASCs have used rat models considering their small size, low cost and accessibility. Clinical studies have been authorized in numerous countries (Table 2) according to data collection. Clinical trials are currently recruiting patients with regeneration of soft tissue, craniofacial tissue, cardiovascular tissue and patients affected

by immune disease. Evidence suggests the efficacy of ASCs in a clinical context.

TABLE 1. Preclinical animal studies using ASCs

Tissue	Defect	Animal model	References
Adipose/soft tissue	Burn	Rabbit	Piccinno et al., 2013
	Radiation trauma	Rat	Huang et al., 2013
	Skeletal muscle trauma	Mice	Lee et al., 2014
Bone	Craniofacial defect	Canine	Liu et al., 2013
Liver	Acute failure	Rat	Koellensperger et al., 2013
Inflammation	Endotoxemia	Rat	Shin et al., 2013
Skin	Wounds	Murine	Huang et al., 2012
Vascular	Hind limb ischemia	Murine	Harada et al., 2013

TABLE 2. Clinical trials studies using ASCs

Indication	Study type	Number of patients/follow-up	References
Soft tissue			
Breast reconstruction	Comparative	10/12 months	Gentile et al., 2012
Irradiation	Case report	20/31 months	Rigotti et al., 2007
Orthopedic			
Craniofacial	Case report	2/12 months	Mesimaki et al., 2009
Osteoarthritis	Phase I and II	18/6 months	Jo et al., 2014
Immune			
Pulmonary fibrosis	Phase Ib	14/12 months	Tzouvelekis et al., 2013
Crohn's disease	Phase II	43/12 months	Yong et al., 2013
Cardiovascular			
Myocardial ischemia	Study design		Qayyum et al., 2012
Hind limb ischemia	Phase I	7/2 weeks	Bura et al., 2014

Osteoarthritis and adipose tissue

Osteoarthritis (OA) is a severe chronic disorder affecting a vast number of patients representing a significant economic burden on the European health care system. The lack of long-term treatment and consequent impact on the course of the disease exacerbates the financial strain of OA. Furthermore, OA is the main cause of pain and impairment worldwide associated to a higher incidence of cardiovascular disease and obesity which suggests an increase in the biomechanical load of the joints or systemic inflammation in relation to these dysfunctions. Investigators are currently analysing novel techniques to address and improve existing therapeutic challenges (1). Alternative approaches including cell-based therapy have yielded favourable outcomes (2). Adipose-derived stromal cells represent optimal candidates considering they can be obtained in relevantly large quantities by means of liposuction. The repair mechanism of ASCs has acquired ample translational implication in a number of diseases including OA (3,4). Preclinical and clinical studies investigating ASCs have produced promising results in regulating biological tissue response and in patients affected by OA. ASCs are able to mediate via trophic and anabolic activities, anti-fibrotic and anti-apoptotic growth factors with the OA microenvironment which will reduce the inflammatory response and the risk of fibrosis (8-10).

Studies have recently focused on the entire adipose niche serving as a reservoir of a heterogeneous cell population which include the progenitor cells, pericytes, endothelial cells, fibroblasts, preadipocytes, monocytes, macrophages as well as the extracellular matrix (ECM) (11,12). Immune cells and macrophages within the adipose niche may play an important role in mediating the clinical outcomes. The first applications of the adipose niche as the stromal vascular adipose fraction were possible due to specific enzymatic treatment including collagenases (17). The enzymatic treatment

is currently arousing concern regarding the extensive manipulation of cells and is accordingly restricted to European regulations, thereby obliging researchers to investigate alternative procedures to obtain integral adipose tissue from the adipose niche (20,21). The Food and Drug Administration has specifically defined the mechanical effects of minimal manipulation (23). In addition to the European legislations, the aim of research is to address important biological issues or rather to envisage and assess novel therapeutic strategies to acquire a vascular-stromal adipose niche. Several authors have recently reported the migration of ASCs towards the synovial membrane following intra-articular injection in animal models observing the chondroprotective effects (8). The regulation of cellular homing in joint tissue through micro-grafts in an osteoarthritic environment is still to be defined.

Discussion and Conclusions

ASC intra-articular injection is considered one of the main routes of administration in a minimally invasive treatment of OA allowing a direct release of the cells into the lesion site and active bio-molecular synthesis to enhance tissue repair (28,29).

The use of the adipose niche has been brought to light with regard to its micro-architecture, its rich source of progenitor and immune cells (11,12) as well as its natural characteristics (32,33). The recruitment of progenitor cells in lesion sites from a biological perspective greatly contributes to its ability to repair tissue.

The behaviour of ASCs in various preclinical subjects affected by OA has been analysed. However, therapies regarding the use of the SVF and adipose micro-grafts are currently scarce (8,19,35-37). Similarly to the SVF, the adipose micro-grafts have demonstrated significant positivity towards CD-90 and CD-146 and a lower percentage of CD-45+ cells (38,39). Furthermore, adipose micro-grafts have shown a higher protein expression for the CD-163 markers compared to the SVF suggesting that the mechanical process is not able to reduce the subset of M2 macrophages (15,16). Besides, the physical characteristics of the SVF and the adipose micro-grafts may influence their migration. ASCs and the SVF are cellular suspensions, conversely to adipose micro-grafts, which contain a variety of cells within a collagen mesh and the structure of the adipose micro-grafts are most likely to contribute to the long-term survival of the cells in tissue hypoxia, specifically in cartilage tissue, preventing thus enzymatic degradation and consequently safeguarding a gradual release of cytokines over time (44).

Treatments have generally presented differing responses regarding repair of joint tissue. The adipose micro-grafts, similarly to ASCs, have displayed prolonged secretion potential by means of release of the granulocyte colony stimulating factor (G-CSF) and the hepatocyte growth factor (HGF) involved in cartilage repair (46). Paolella et al. have recently reported in an in-vitro preclinical study, the active role of adipose micro-grafts in mediating synovial macrophage activity via a reduction of

CCL2/MCP1 and CCL3/M1P1 (41). Moreover, Nava S et al. demonstrated that adipose micro-grafts release mediators with more sustainable anti-inflammatory properties compared to mesenchymal stromal cells under cell culture medium without serum (46).

It is generally assumed that the synovial membrane is able to mediate recovery by commutation of inflammatory signals and activation of M2 macrophages. To this purpose, Manferdini et al. demonstrated that ASCs reduced inflammation via cyclooxygenase 2 (COX2)/prostaglandin E2 (PGE2) and via inflammatory commutation of M1 macrophages with a similar phenotype to M2 (48,53) within the synovial membrane. As regards the adipose micro-grafts, numerous authors hypothesize that this treatment may exert an anti-inflammatory and reparatory effect directly on cartilage tissue due to the trophic potential of the CD-163+ found within the niche.

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