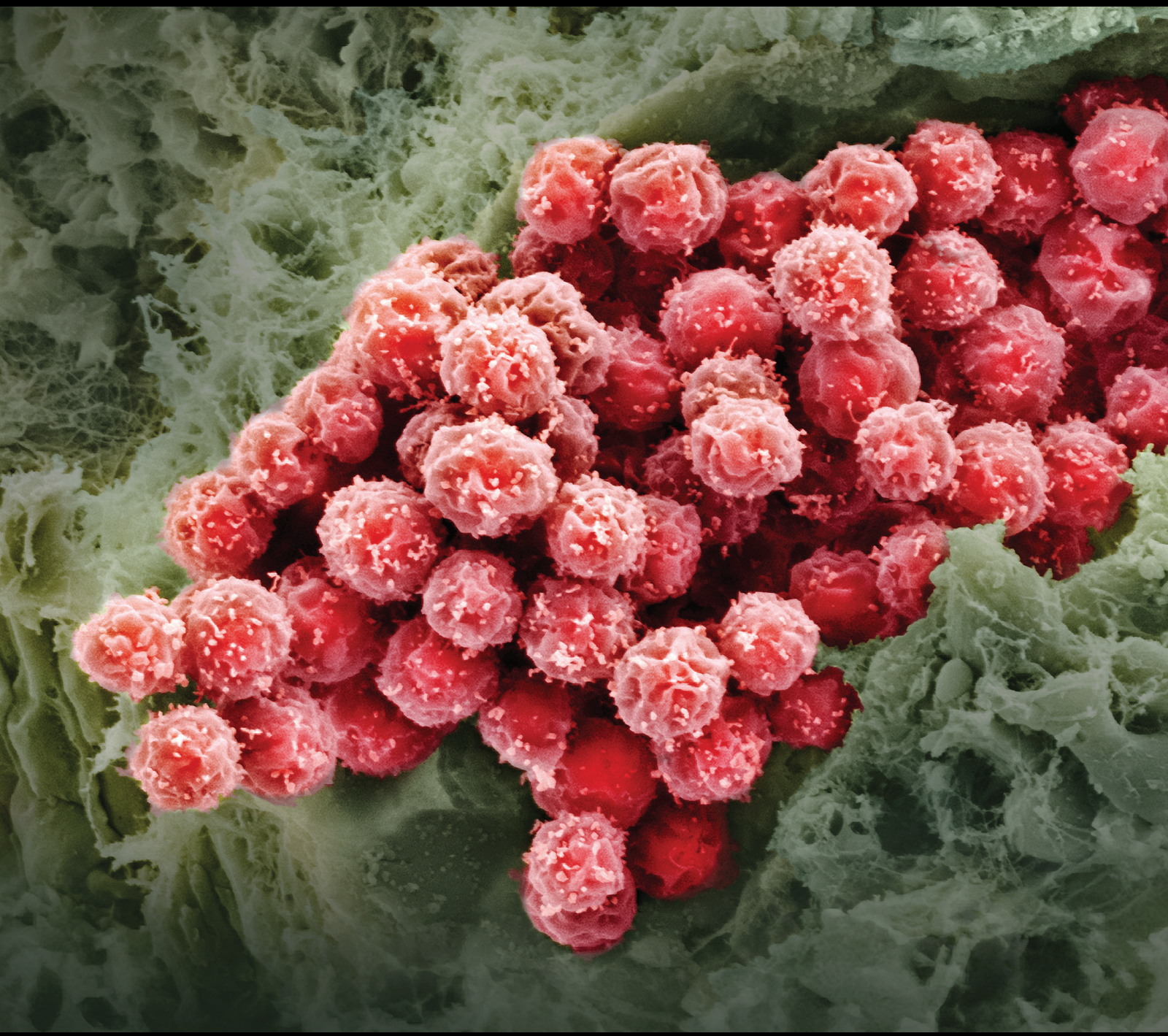
Stem Cells International

Adipose Stem Cells: From Bench to Bedside

Guest Editors: Giuseppe A. Ferraro, Hiroshi Mizuno, and Norbert Pallua



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Editorial

Adipose Stem Cells: From Bench to Bedside

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Stem cell biology plays an important role in promoting cell-based treatment. Adult mesenchymal stem cells (MSCs) are derived from various tissues including bone marrow [1], adipose tissue [2], dental pulp [3], and Wharton jelly [4]. When compared to bone marrow mesenchymal stem cells (BM-MSCs), adipose tissue represents an ideal source for multipotent progenitors in adults [5]. Adipose stem cells (ASCs) share many characteristics with BM-MSCs, including extensive proliferation and the ability to undergo multilineage differentiation [6] (like bone marrow MSCs, they can differentiate *in vitro* into adipogenic, osteogenic, chondrogenic, and myogenic cells when cultured in specific lineage-inducing culture media and into endothelial cells), and they display a noticeable plasticity both *in vitro* and *in vivo*. Moreover, the high abundance of adipose tissue within the body, its high surgical accessibility, and the demonstrated multipotency of ASCs show adipose tissue as a promising candidate for MSCs harvest and increase the interest in its use in tissue repair, regenerative medicine, and degenerative disease management [7].

ASCs have been studied widely since stem cell investigations emerged in 2001 [8]. Since then, knowledge of their characterization, immunological characteristics, and potential of multilineage differentiation has increased considerably [9–18]. Many international medical conferences have emphasized the importance of ASCs and the International Federation of Adipose Therapeutics and Science (IFATS) has been extremely active in promoting the study and discussion of ASCs [19].

Many surgical strategies for tissue loss replacement initially focused on the historical maxim "replace tissue with like-tissue" procedure. In more recent years, several allogenic and alloplastic materials have been developed and used for tissue repair [20–22]. Current research aims to reduce concerns such as foreign-body reactions, rapid degradation, and risk of immunogenicity.

The development of regenerative medicine strategies requires an appropriate cell source and scaffold, "smart" biomaterials (novel "intelligent" biomaterials with appropriate physical properties able to support *in vivo* the commitment of adipose stem cells), and a suitable microenvironment to provide the cues and signals for cell growth and tissue formation. Biomaterials are able to direct and organize the cellular events involved in the regenerative process *in situ* [23, 24]. ASCs are undifferentiated cells with the ability to self-renew and differentiate into different types of specialized cells with a regenerative potential even if not combined with biomaterials. The proliferation and differentiation of adipose stem cells can be regulated biochemically, as well as through the physical properties of microenvironments, such as the topography of the scaffolds, the "stiffness," and mechanical forces.

The potential of adipose stem cell therapies and regenerative medicine is effective and challenging, offering the possibility of tissue repair and replacement in tissue defects related to congenital diseases, trauma, and cancer [25].

This special issue has examined the importance of "adipose stem cells" focusing on the basic biology and potential

role of ASCs in the treatment and regeneration of cells, tissues, and organs.

Acknowledgments

We would like to thank Dr. *Francesco De Francesco*, researcher and plastic surgeon, for his scientific support for the realization of the special issue. It is with honour that we acknowledge the participation of international investigators that have submitted original research and review articles to contribute to the evolving research field of adipose stem cell-biomaterial interactions and the development of clinically effective tissue engineering strategies.

Giuseppe A. Ferraro
Hiroshi Mizuno
Norbert Pallua

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Review Article

Mitochondria in White, Brown, and Beige Adipocytes

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Mitochondria play a key role in energy metabolism in many tissues, including cardiac and skeletal muscle, brain, liver, and adipose tissue. Three types of adipose depots can be identified in mammals, commonly classified according to their colour appearance: the white (WAT), the brown (BAT), and the beige/brite/brown-like (bAT) adipose tissues. WAT is mainly involved in the storage and mobilization of energy and BAT is predominantly responsible for nonshivering thermogenesis. Recent data suggest that adipocyte mitochondria might play an important role in the development of obesity through defects in mitochondrial lipogenesis and lipolysis, regulation of adipocyte differentiation, apoptosis, production of oxygen radicals, efficiency of oxidative phosphorylation, and regulation of conversion of white adipocytes into brown-like adipocytes. This review summarizes the main characteristics of each adipose tissue subtype and describes morphological and functional modifications focusing on mitochondria and their activity in healthy and unhealthy adipocytes.

1. Introduction

Over the past few decades, the number of studies in the field of adipose tissue biology has increased exponentially since obesity and associated diseases are occurring at epidemic rates not only in developed countries, but also in developing countries. Obesity arises from an imbalance between energy intake and expenditure. It is associated with an increased risk of type 2 diabetes, hypertension, atherosclerosis, heart disease, stroke, cancer, infertility, and so forth [1–5]. Current clinical approaches to obesity include diet, physical activity, psychological support, drugs, and surgery treatment. Unfortunately, these treatment methods show efficiency limited only to small percentage of patients and some of them may be accompanied by serious side effects.

Studies published over the last two decades have established adipose tissue as a dynamic organ that carries out several important physiological processes. It is composed of a number of cell types: adipocytes, preadipocytes, vascular endothelial cells, pericytes, macrophages, and fibroblasts [6]. However, the dominant cells present in adipose tissue are mature adipocytes.

Two major types of adipose tissue exist in mammals, brown and white fat that have essentially antagonistic functions, brown fat expending energy and white fat storing it [7]. Brown adipocytes may occur after thermogenic stimulation at anatomical sites corresponding to WAT. This process is called the "browning" of WAT and these brown-like adipocytes that appear in WAT are called "beige" or "brite" [8, 9]. These three types of adipose cells have many specific

characteristics related to localization, cell composition (lipid droplet, mitochondria), function, pathways of homeostatic control, obesity related changes, and so forth [8, 10].

Recent data suggest that adipocyte mitochondria might play an important role in the development of obesity through defects in mitochondrial lipogenesis and lipolysis, regulation of adipocyte differentiation, apoptosis, production of oxygen radicals, efficiency of oxidative phosphorylation, and regulation of conversion of white adipocytes into brown-like adipocytes [11, 12]. Thus, therapeutic intervention into any of these mitochondrial processes could be a useful approach to reduce adiposity [13].

This review summarizes the main characteristics of each adipose tissue subtype and describes morphological and functional modifications focusing on mitochondria and their activity in healthy and unhealthy adipocytes.

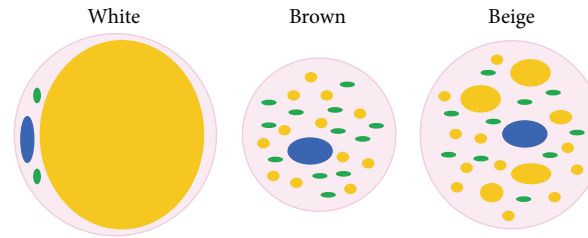

Figure 1: Main morphological characteristics of white, brown, and beige adipose tissues. White adipocyte cell is classically spherical, it is full of single lipid droplet, and it contains few mitochondria. Brown adipocyte is usually smaller than white and beige ones. It contains a large number of mitochondria and contains multiple small lipid droplets. Blue: nucleus, green: mitochondria, and yellow: lipid droplets.

2. Overview of Mitochondrial Functions

Mitochondria are the cytoplasmic organelles in human and animal cells where many distinct metabolic pathways take place [14]. Mitochondria are highly dynamic, pleomorphic organelles comprising at least six compartments: outer membrane, inner boundary membrane of significantly larger surface area, intermembrane space, cristal membranes, intracristal space, and protein rich matrix. They are found in almost all human cells except mature erythrocytes [15, 16]. Although mitochondria contain their own small mtDNA and some RNA components of mitochondrial translational apparatus, the vast majority of the mitochondrial proteins are encoded by nuclear DNA, synthesized in the cytosol, and then imported into the mitochondria posttranscriptionally [15]. Mitochondria are involved in the crucial metabolic processes including tricarboxylic acid cycle, pyruvate decarboxylation, oxidative decarboxylation of fatty acids (β-oxidation), and degradation of branched amino acids. Mitochondria also substantially contribute to biosynthetic processes taking place in the cytosol by providing key intermediates like urea cycle, fatty acids, and heme synthesis. However, the principal role of mitochondria is to synthesize more than 95% of adenosine triphosphate (ATP) for cellular utilization [14, 16]. Production of ATP requires two major steps, oxidation of highly reducing metabolites and coenzymes such as nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) and phosphorylation of adenosine diphosphate to generate ATP to support various cellular functions (OXPHOS, oxidative phosphorylation) [17]. The mitochondrial respiratory system consists of four enzymatic multiheteromeric complexes (I–IV) embedded in the inner membrane of mitochondria and two individual mobile molecules, coenzyme Q (CoQ) and cytochrome *c*, along which the electrons liberated by the oxidation of NADH and $FADH_2$ are passed and ultimately transferred to molecular oxygen. This respiratory process creates the electrochemical gradient of protons and membrane potential about 180 mV across the inner membrane that has the potential to do work. The proton flux drives the F_0F_1 ATP synthase (complex V) to phosphorylate matrix ADP by inorganic phosphate [18, 19]. On the other hand, mitochondria generate heat by a mechanism called "proton leak." Protons leak from the intermembrane space to matrix and reduce membrane potential generating heat instead of energy [17]. Mitochondria are also deeply involved in the production of reactive oxygen species (ROS) through electron carriers in the respiratory chain. Oxidative stress can induce apoptotic death and mitochondria have a central role in this process due to cytochrome *c* release in the cytoplasm and opening of the permeability transition pore [20, 21]. Mitochondria are essential for the maintenance of normal physiological function of tissue cells and mitochondrial dysfunction may cause pathological changes in the human body [14]. In addition, eukaryotic cells have the ability to initiate adaptive responses to different environmental stimuli (e.g., cell growth, death and differentiation, or modification in energy demands) by altering the number, morphology, or remodelling of mitochondria [11].

3. White versus Brown versus Beige Adipocyte Tissue

In mammals, we can find three types of adipose depots commonly classified according to their colour appearance: the white (WAT), the brown (BAT), and the beige/brite/brown-like (bAT) adipose tissues. Main characteristics of WAT, BAT, and bAT in humans are shown in Table 1 and main morphological differences are shown in Figure 1. Most mammals have WAT dispersed throughout the body in two major types of depots, subcutaneous and intra-abdominal (or visceral). Intra-abdominal fat includes retroperitoneal, omental, mesenteric, epicardial, and gonadal deposits. WAT weight generally represents as much as 20% of the body weight of normal adult human and primarily acts as a storage site for triglycerides, conserving excess calories for use in times of scarcity. White adipocytes contribute to the whole body insulation and have endocrine functions including secretion of leptin, TNF-α, adiponectin, resistin, and other compounds related to the degree of obesity and insulin sensitivity [22].

Humans have relatively large depots of BAT in infancy; only small amounts of BAT dispersed throughout the depots

Table 1: Main differences amongst the three types of adipocyte tissue.

Characteristic property	White	Brown	Beige	References
Morphology				
(i) Shape	Spherical	Ellipsoid/polygonal	Spherical	
(ii) Cell size	Variable, large (25–200 μm)	Small (15–60 μm)	Variable, smaller than white	[8, 9, 23]
(iii) Lipid droplet (LD)	Single large LD	Multiple small LD	Multiple LD with variable size	
(iv) Mitochondria	+	+++	++ (upon stimulation)	
Development	From Myf5$^-$ or Myf5$^+$ precursors	From Myf5$^+$ precursors	From Myf5$^-$ or Myf5$^+$ precursors	[23–26]
Location	Subcutaneous and visceral	Suprarenal, paravertebral, supraclavicular	Inguinal, neck (near carotid sheath and musculus longus colli), other locations?	[8, 27]
Function	Energy storage	Heat production	Adaptive thermogenesis	[9]
Uncoupling protein	Nearly undetectable	+++	++ (upon stimulation)	[28, 29]
Adipocyte-type-specific markers	*PPARγ, PLIN1, HOXC9, TCF21, TLE3, C/EBPα, Rb, Rip140*	*LHX8, ZIC1, EPSTI1, PRDM16, CIDEA, ELOVL3*	*HOXC8, HOXC9, CITED1, CD137, TMEM26, TBX1, CD40*	[13, 23, 30, 31]
Vascularization	Low	High	High upon stimulation	[32, 33]
Impact on obesity	Positive	Negative	Negative	[34]
Correlation with insulin resistance	Yes	Probably yes	Probably yes	[35–38]

CD40: CD40 molecule, TNF receptor superfamily member 5; CD137: tumour necrosis factor receptor superfamily, member 9; C/EBPα: CCAAT/enhancer binding protein (C/EBP), alpha; CIDEA: cell death-inducing DFFA-like effector; CITED1: Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain 1; ELOVL3: ELOVL fatty acid elongase 3; EPSTI1: epithelial stromal interaction 1; HOXC8: homeobox C8; HOXC9: homeobox C9; LHX8: LIM homeobox protein 8; PLIN1: perilipin-1; PPARγ: peroxisome proliferator-activated receptor gamma; PRDM16: PR domain containing 16; Rb (Rb1): retinoblastoma 1; Rip140: nuclear receptor interacting protein 1; TBX1: T-Box 1; TCF21: transcription factor 21; TLE3: transducin-like enhancer of split 3; TMEM26: transmembrane protein 26; ZIC1: zinc finger protein of the cerebellum 1.

of WAT persist in adults. Classical brown fat is primarily distributed in the interscapular space, paravertebrally, axillary, and perirenally. Recent studies have confirmed the presence of active BAT containing both classical brown and beige adipocytes in adult humans, with depots residing in the cervical, supraclavicular, mediastinal, paravertebral, and suprarenal regions [27, 39, 40].

White adipocyte cell is classically spherical and large with flattened nucleus, which is situated in the periphery. Because it is nearly completely filled with a single lipid droplet, thin ring of cytoplasm contains few mitochondria and little but recognizable smooth endoplasmic reticulum [41]. Brown adipocyte is usually smaller than the white one and its shape is elliptical with round or oval nucleus situated centrally. Cytoplasm volume is large containing multiple small lipid droplets, poor endoplasmic reticulum, and high amount of mitochondria.

Beige adipocyte has the mixed characteristics of both white and brown adipose cells. During basal state, it displays unilocular morphology as white adipocyte, but, upon cold stimulation, its appearance acquires features of intermediate morphology ultimately resulting in expression of proteins typical for BAT and transformation of stored fat into the small lipid droplets typical for brown adipocytes [8, 42, 43]. The origin and function of beige adipocytes are less clear and currently under intense discussion. It is thought that they arise from unique precursor cells [42], but there is also evidence that they stem from white adipocytes by transdifferentiation of preexisting white adipocytes. Himms-Hagen et al. treated rats with β3-adrenoceptor agonist (CL-316243); the results of their study showed that at least a subpopulation of unilocular adipocytes underwent conversion to multilocular mitochondria-rich adipocytes [44]. Interestingly, Morroni and coworkers suggested a new mechanism of reversible physiological transdifferentiation of adipocytes in the mammary gland: mouse mammary adipocytes are able to transform into secretory epithelial cells during pregnancy and revert to adipocytes after lactation [45]. Moreover, recent research has shown novel mechanism of the bAT formation. Wang et al. suggested that during cold-induced "browning" of subcutaneous fat, most "beige" adipose cells stem from *de novo* differentiated adipocytes [46]. Vargas et al. found that adipocytes differentiated with total and partial agonists of peroxisome proliferator-activated receptor gamma (PPARγ) and exposed to 31°C are able to respond to cold by a significant increase in the expression of thermogenic proteins such as uncoupling protein 1 (UCP1), peroxisome proliferator-activated receptor c coactivator 1 (PGC1α), and Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain 1 (CITED1), a marker of the beige phenotype [28]. Two potential models of mature WAT into bAT transformation are shown in Figure 2.

Interestingly, exercise has been recently considered as a physiological stimulus for brown adipose tissue activity [47]. Even vibration training changed lipid metabolism in rats and promoted brown fat-like modifications in white adipose

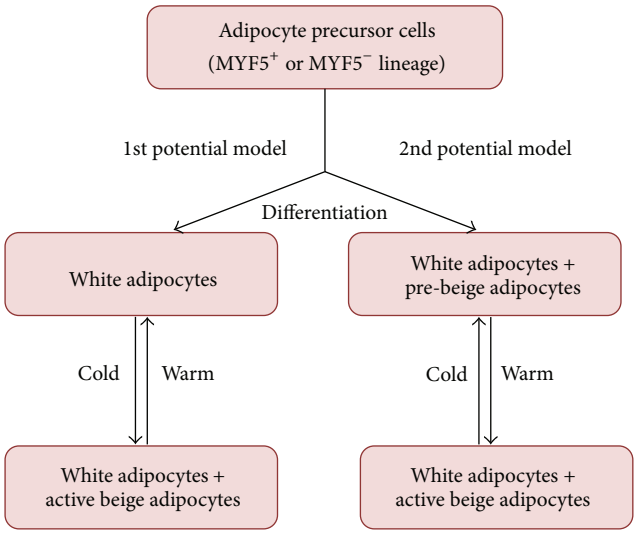

Figure 2: Two potential models of how WAT can be transformed into beige adipocytes [23, 25, 26].

tissues through triggering BAT-associated gene expression, inflammatory response, and decrease in energy reserve [48]. Understanding these biological processes and stimulation of the activity of brown and beige/brite adipocytes could help us with fight against obesity, potentially facilitate weight loss, and improve metabolic health [49].

4. Mitochondrial Activity in Adipocytes

Mitochondria play a central role in metabolism of adipose tissue, as documented by their contribution to metabolic pathways of particular importance in adipocytes, like lipolysis and lipogenesis [11]. In addition, specific function performed by brown fat is converting mitochondrial energy into heat in adaptive thermogenesis. Tissue-specific functions of mitochondria in white fat are less characterized [50], although their role in orchestrating metabolic homeostasis and weight control is now widely accepted [51].

Lipolysis in adipocytes is the hydrolysis of triglycerides from lipid droplets within the cell into glycerol and free fatty acids by hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL). The hydrolytic action of HSL is regulated by perilipin A, a lipid droplet-associated protein. Phosphorylation of perilipin A by cAMP-dependent protein kinase (PKA) facilitates the translocation of HSL into the lipid droplet [52]. In the cytoplasm, free fatty acids are presumably bound to binding proteins and subsequently moved across the inner mitochondrial membrane by diffusion or, in the case of long carbon chains, by the carnitine shuttle [39, 53]. B-oxidation, metabolic process breaking down free fatty acids into acetyl-CoA takes place in the mitochondrial matrix. Acetyl-CoA then undergoes oxidation through the tricarboxylic acid cycle and the electron transport system.

The lipogenesis *de novo* is an important pathway to convert fatty acids to triglycerides for storage in the WAT. The human liver is mainly responsible for the conversion of carbohydrates into fatty acids, but a small part of triglycerides is synthesized in adipocytes [52]. Although fatty acids and triglycerides synthesis take place in the cytosol, mitochondria provide key intermediates needed for lipogenesis, like glycerol 3-phosphate and acetyl-CoA. Key enzyme in glycerol 3-phosphate synthesis is mitochondrial pyruvate carboxylase that converts pyruvate into oxaloacetate. Pyruvate also undergoes decarboxylation to acetyl-CoA by the mitochondrial pyruvate dehydrogenase complex, which facilitates fatty acid and triglyceride synthesis [54].

As mentioned above, brown/beige adipocytes, when activated by sympathetic stimulation, dissipate chemical energy stored in the form of triglycerides by channelling fatty acids into β-oxidation. Energy of substrate oxidation is then converted into heat [49, 55, 56]. This process, termed nonshivering thermogenesis, is specific function of BAT/bAT and is particularly important during hibernation and for small animals and infants who have greater demands on thermogenesis due to a large surface-to-volume ratio [57]. The molecular substrate of this unique function is a protein containing three similar repeats of about 100 amino acids coded by nuclear genes and inserted into the inner mitochondrial membrane [58]. As the major role of the protein is proton translocation resulting in uncoupling of the electron-transporting system from ATP synthesis in the mitochondria, it was named uncoupling protein (UCP) [59].

Uncoupling proteins belong to a family of mitochondrial carrier proteins that are present in the mitochondrial inner membrane. Mammals express five UCP homologues (UCP1 also named thermogenin), UCP2, UCP3, UCP4, and UCP5, also known as brain mitochondrial carrier protein 1 (BMCP1) [60]. UCP1 is expressed almost exclusively in fully differentiated BAT cells [17], although some findings suggest that UCP1 can be detected also in other tissues including uterine smooth muscle and even WAT, where induction of uncoupling protein expression is associated with acquiring of brown fat features [61, 62]. Expression of UCP1 in WAT has been questioned by finding of brown adipocytes in white depots and white adipocytes that potentially could transdifferentiate into cells expressing markers of BAT/bAT after appropriate stimulation [56, 62]. UCP2 and UCP3 mRNAs have been detected in a number of tissues and organs, for example, thymus, stomach, testis, white and brown adipocytes, pancreatic β-cells (UCP2) and skeletal muscle, heart, and brown adipocytes (UCP3) [63–67]. The physiological function of UCP1 is to mediate a regulated proton leak and thus dissipate the proton electrochemical gradient built up by the respiratory chain in the form of heat. Maximally stimulated brown adipose tissue can produce about 300 W/kg of heat compared to 1 W/kg in all other tissues [29, 68]. The thermogenesis in BAT is induced and positively regulated by fatty acids; in fact, no heat generation can be elicited without simultaneously initiating lipolysis. Further oxidation of acetyl-CoA the end product of β-oxidation, through tricarboxylic acid cycle and the electron transport chain, provides energy dissipated as heat through the action of UCP1 [69]. In contrast to UCP1, physiological function of its homologues is still debated. Recent studies

have shown that UCPs might have an important role in pathogenesis of various disorders as type 2 diabetes, obesity, heart failure, neurodegenerative diseases, aging, or tumorigenesis [70–75].

Metabolic differences between mitochondria of WAT and BAT are associated with specific morphological characteristic of mitochondria in the brown adipocytes. These mitochondria are apparently more numerous and bigger in size and contain more cristae than mitochondria in white adipocytes. In addition, content of the heme cofactors in the mitochondrial enzyme cytochrome oxidase gives the tissue the brown macroscopic colour [16, 76]. Compared to BAT, WAT has fewer mitochondria, mostly undetectable expression of UCP1, and lower expression levels of the fatty acid β-oxidation-related enzyme, acyl CoA dehydrogenase, suggesting that the intensity of β-oxidation in WAT is lower than in BAT [53, 56, 77, 78]. As in other tissues, mitochondria represent the main source of ATP in the white fat. White fat mitochondria are well equipped for oxidative phosphorylation, with pyruvate serving as a main source of energy for ATP synthesis. Due to low activity of carnitine palmitoyltransferase 1 in the inner mitochondrial membrane, oxidation of fatty acids is relatively slow and fatty acids are directed towards esterification, unless the transferase is activated by leptin [79].

Forner et al. reported a systematic analysis of mouse mitochondrial proteomes of brown and white adipocytes documenting significant differences in the two sets of proteins, both qualitative and quantitative. Acetyl-CoA synthetase 2-like (gene *Acss1*), converting acetate to acetyl-CoA, and pyruvate dehydrogenase kinase 4 (gene *Pdk4*), inhibiting the pyruvate dehydrogenase complex thereby reducing the conversion of pyruvate to acetyl-CoA, were detected only in BAT. Conversely, MOSC domain-containing protein 1 (gene *Mosc1*), component of prodrug-converting complex, and acyl-coenzyme A synthetase ACSM5 (gene *Acsm5*), having CoA ligase activity, were detected only in WAT [50]. At transcript and proteome levels, BAT mitochondria were more similar to their counterparts in muscle cells. In contrast, WAT mitochondria not only selectively expressed proteins that support anabolic lipogenic function but also degrade xenobiotics and endogenous molecules, revealing a protective role of this tissue. These observations might help in better understanding of physiological processes in adipose tissue [50].

During adipocyte differentiation, the appropriate function of mitochondrion-specific metabolic processes is essential [11]. Adipogenic differentiation is characterized by the enhanced expression of some critical transcriptional factors, for example, *C/EBPa* and *PPARγ* [80, 81], lipid droplet accumulation, mitochondrial biogenesis [82], and a 20- to 30-fold increase in the concentration of numerous mitochondrial proteins [83]. ATP needed for the mitochondrial biogenesis, lipogenesis, and synthesis of numerous cytosolic and mitochondrial proteins is generated in the increased amounts due to the enhanced synthesis of mitochondrial DNA, subunits of respiratory complexes, cytochrome c, and enzymes of the tricarboxylic acid cycle [84]. In addition, tricarboxylic acid cycle generates citrate, which is then transported from the mitochondrion into the cytosol via the tricarboxylate carrier. Citrate is the only precursor of cytosolic acetyl-CoA, key intermediate used for fatty acid synthesis. Thus, citrate export from the mitochondria is essential during early differentiation stages of preadipocytes [85].

It has been also reported that a new adipose-specific protein, mouse ISG12b1, which is localized in the mitochondria, is predominantly overexpressed in adipocytes and dramatically induced at the terminal stage of adipogenesis. Functionally, ISG12b1 inhibits mitochondria biogenesis and adipocyte differentiation [86].

Taken together, although mitochondria in the brown fat are mainly acknowledged as important regulators of thermogenesis and those in the white fat as providers of constituents essential for lipogenesis, recent evidence suggests that mitochondria in adipose tissues might play plentiful roles in the regulation of the whole body energy homeostasis, crosstalk between adipose tissues and striated muscle, or control of insulin sensitivity and glucose metabolism [87–90].

5. Mitochondrial Dysfunction in Adipocyte

Mitochondrial dysfunction can result from a decrease in mitochondrial biogenesis, reduced mitochondrial content, and/or a decrease in the protein content and activity of oxidative proteins "per unit of mitochondria" [91]. The major tissues affected by mitochondrial dysfunction are those with a high energy demand such as heart, muscles, brain, and endocrine glands [11, 92]. However, in the past few years, many studies have targeted mitochondria in adipocytes or adipose tissues providing convincing evidence that impairment of mitochondrial functions in adipocytes could have the whole body pathological consequences [12, 51]. As mitochondria house crucial metabolic processes like fatty acid oxidation, oxidative phosphorylation, and ROS production, it is not surprising that impaired mitochondrial activity often has an association with metabolism and adipocyte differentiation [92].

5.1. Mitochondrial Dysfunctions in Metabolic Disorders. As shown in previous paragraphs, mitochondria contribute substantially to normal functions of adipose tissues. Although it is not clear yet if the mitochondrial dysfunction plays a causative or adaptive role in various metabolic disorders, further research in the field could reveal the correct timing of processes leading to obesity, insulin resistance, diabetes mellitus, or lipodystrophy. Compelling lines of evidence indicate that major factors contributing to mitochondrial defects in adipose tissues are (i) oxidative stress, (ii) insulin resistance, (iii) genetic factors, and also (iv) sedentary lifestyle without physical activity [93].

Oxidative stress is defined as a disturbance in the balance between the production of ROS and antioxidant defence [94]. Mitochondria are a major source of cellular free radicals that might damage proteins, lipids, and DNA. Defects in the transfer of electrons across the mitochondrial membrane can cause electrons to accumulate on the respiratory chain complexes, which results in an increase of the potential

for electrons to bind with free oxygen and stimulation of ROS production [95]. Furukawa et al. have shown that elevated levels of fatty acids increased oxidative stress via NADPH oxidase activation in cultured adipocytes. ROS then caused dysregulated production of various adipocytokines, including adiponectin, plasminogen activator inhibitor-1, IL-6, and monocyte chemotactic protein 1 [96]. In obese mice, fat accumulation correlated with systemic oxidative stress and treatment with NADPH oxidase inhibitor reduced ROS production in adipose tissue, attenuated the dysregulation of adipocytokines, and improved diabetes, hyperlipidaemia, and hepatic steatosis in humans and mice [96]. Wang et al. have reported that higher intracellular ROS levels elicited by mitochondrial dysfunction resulted in the impairment of adipocyte function in the maintenance of glucose homeostasis through attenuation of insulin signalling, downregulation of the glucose transporter (*GLUT4*) expression, and decrease in adiponectin secretion [97].

Insulin resistance is a key defect associated with obesity and type 2 diabetes. It is defined as "a relative impairment in the ability of insulin to exert its effects on glucose, protein, and lipid metabolism in target tissues" [98]. Decreased insulin response to glucose, dyslipidaemia, and obesity frequently progress into overt type 2 diabetes with a decline in β-cell function, sustained hyperglycaemia, and increased advanced glycation end products (AGE) formation. In turn, AGE accumulation in adipose tissue may contribute to obesity-associated insulin resistance [99]. The role of mitochondria in adipose tissues in the onset and progression of insulin resistance is still a matter of controversy. Some recent findings suggest that dysregulation of mitochondrial calcium influx and efflux could be a crucial factor contributing to decreased insulin sensitivity [100] that is associated with impaired mitochondrial biogenesis and decreased expression of mitochondrial proteins in adipose tissues [101, 102]. However, mitochondrial dysfunction is not always essential for insulin resistance as reported by Martin et al. [103]. In addition, ROS-induced mitochondrial dysfunction seems to be a valid mechanism leading to insulin resistance in skeletal muscle, but not necessarily in adipocytes [103].

Genetic factors could play an important role in the onset and progression of obesity or type 2 diabetes mellitus. Among genes with positive associations of variants with obesity or obesity-related phenotypes, there are some deeply involved in the regulation of mitochondrial activity and biogenesis in adipose tissues, like *ADRB3* (adrenergic, β3 receptor), *INS* (insulin), *PLIN* (perilipin), *PPARγ* (peroxisome proliferative activated receptor, gamma), or *UCP1-UCP13* (uncoupling proteins 1–3) [104]. In addition, impaired expression of genes related to mitochondrial functions in adipose tissues can be caused by acquired mutations of both mitochondrial and nuclear genomes. Mitochondrial DNA displays a high mutation rate due to its specific features, like limited repair, proximity to ROS production, and absence of histones [105]. Defects in the expression of mitochondria-related genes were found at the mRNA as well as the protein levels in various organs and tissues including adipose cells [106–110].

Transcriptional coactivators PGC-1α and PGC-1β seem to be of particular importance in coordination of expression of mitochondrial and nuclear genes related to mitochondrial functions in both BAT and WAT [111, 112]. PGC-1α is able to direct human WAT PPARγ toward a transcriptional program linked to energy dissipation through an increased expression of UCP1 [113]. Accordingly, decreased PGC-1α mRNA levels were reported in subcutaneous fat in morbidly obese subjects. Although it is not clear whether low PGC-1α expression is a prelude to the development of obesity or a consequence of it, upregulation of expression of thermogenic genes in white adipose tissue could offer new tool in the therapy of obesity [114].

However, it should be noted that impairment of expression of mitochondria-related genes does not necessarily lead to obesity as documented by manipulations with mitochondrial transcription factor A (TFAM), one of the major controllers of mitochondrial mass: in mice deficient in TFAM in adipocytes, activity of proteins in respiratory complexes I, III, and IV was severely compromised, which resulted in adipocyte death and inflammation in WAT and whitening of BAT [88].

Changes in human behaviour and lifestyle over the last century have resulted in a dramatic increase in the incidence of diabetes and obesity worldwide. Sedentary lifestyle, changes in work (from heavy labour to sedentary) have had an impact on human health [4, 115]. Physical activity is a major regulator of mitochondrial function in muscle cells and long-time inactivity is associated with reduced mitochondrial function and number [116].

In adipose tissues, regular physical activity and exercise training have long been known to cause increased expression and activity of mitochondrial proteins [117, 118]. In the last decade, the "beiging" of WAT associated with the expression of typical markers of BAT (like UCP1) in white adipocytes was revealed in response to exercise training [119, 120]. In rodents, even a single bout of exercise increased expression of a marker for mitochondrial biogenesis, PGC-1α mRNA in WAT [121]. This increase was presumably induced by stimulation β-adrenergic receptors at least in visceral WAT [122]. In the subcutaneous WAT, endothelial nitric oxide synthase has been proposed to regulate training-induced increases in mitochondrial biogenesis [118].

5.2. Mitochondrial Dysfunction during Adipocyte Differentiation. There is ample evidence that any damage to the mitochondrial respiratory chain results in compromised adipocyte differentiation. Inhibition of complex I by rotenone led to the significant reduction in the expression of mitochondrial malate dehydrogenase and a number of differentiation transcription factors, like PGC-1β, PPARγ, CAAT/enhancer binding protein alpha (C/EBPα), and sterol regulatory element binding protein-1c (SREBP-1c). In addition, apparent decreases in the synthesis of triglycerides and ATP were reported [84]. Antimycin A, inhibitor of complex III, and oligomycin, inhibitor of ATP synthase commonly used for developing of mitochondrial dysfunction models, also prevented preadipocyte differentiation [123, 124].

High concentrations of mitochondrial ROS generated by the respiratory chain have also detrimental influence on

adipoblast proliferation and differentiation. Genetic manipulation of mitochondrial complex III revealed that ROS generated by this complex were required to initiate primary human mesenchymal stem cells differentiation [81]. Thus, the production of ROS and mitochondrial metabolism are not simply a consequence of adipogenesis but are causal factors in promoting adipocyte differentiation [81].

6. Conclusions

Adipose tissue is an extremely plastic organ capable of massive expansion, reduction, or transformation according to appropriate stimulation. Research motivated mainly by the desire to understand adipocytes in the context of obesity and related diseases resulted not only in promising data opening new ways to fight obesity, but also in the discovery of multipotent stem cells within WAT [125]. It is now widely accepted that adipose tissue acts not only as repository for excess nutrients but also as integrator and regulator of the balance between food intake and energy output. It secretes a number of substances affecting the function of several organs in the body and also the function of adipose tissue itself [51, 126, 127]. This review summarizes the main characteristics of each adipose tissue subtype and describes morphological and functional modifications focusing on mitochondria and their activity in healthy and unhealthy adipocytes. Increasing evidence in adipocyte-related mitochondrial research demonstrates the important role of mitochondria in the onset or progression of obesity and related pathologies and offers a large spectrum of potential therapeutic targets, like differentiation and transformation of adipocytes, ROS production, substrate channelling to energy dissipation, or changes in the lifestyle.

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contribution

Miroslava Cedikova and Michaela Kripnerová contributed equally to this work.

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Research Article

Human Adipose-Derived Mesenchymal Stem Cells Cryopreservation and Thawing Decrease α4-Integrin Expression

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Aim. The effects of cryopreservation on adipose tissue-derived mesenchymal stem cells are not clearly documented, as there is a growing body of evidence about the importance of adipose-derived mesenchymal stem cells for regenerative therapies. The aim of this study was to analyze human adipose tissue-derived mesenchymal stem cells phenotypic expression (CD34, CD45, CD73, CD90, CD105, and CD49d), colony forming unit ability, viability, and differentiation potential before and after cryopreservation. *Materials and Methods*. 12 samples of the adipose tissue were collected from a healthy donor using the liposuction technique. The cell isolation was performed by enzymatic digestion and then the cells were cultured up to passage 2. Before and after cryopreservation the immunophenotype, cellular viability analysis by flow cytometer, colony forming units ability, differentiation potential into adipocytes and osteoblasts as demonstrated by Oil Red O and Alizarin Red staining, respectively. *Results*. The immunophenotypic markers expression was largely preserved, and their multipotency was maintained. However, after cryopreservation, the cells decreased α4-integrin expression (CD49d), cell viability, and number of colony forming units. *Conclusions*. These findings suggest that ADMSC transplanted after cryopreservation might compromise the retention of transplanted cells in the host tissue. Therefore, further studies are warranted to standardize protocols related to cryopreservation to attain full benefits of stem cell therapy.

1. Introduction

In the development of new therapies using stem cells, mesenchymal stem cells (MSC) from diverse origins have exhibited immense potential [1, 2]. Although the reports related to the therapeutic potential of MSC, most of them are performed mainly on hematopoietic origin, more from bone marrow (BM), that is explained by the extensive clinical experiments with stem cells, particularly in the treatment of oncohematological diseases [3, 4]. However, adipose tissue-derived mesenchymal stem cells (ADMSC) emerged as a promising candidate. As ADMSC are adult stem cells that

can be isolated easily from adipose tissue, the cells are obtained through plastic surgery that represents an ample and accessible source of adult stem cells with the ability to differentiate into adipocytes, osteocytes, chondrocytes, and cells phenotypically similar to neurons [5]. Due to ADMSC ability to differentiate into several cell types of clinical interest, they hold an immense potential for the future therapeutic use of these cells in clinics. Although many reports related to the therapeutic potential of ADMSC, most of the current investigations were performed mainly on freshly isolated cells [6]. As this cell type holds a clinical translation value, there is an increasing interest in stem cell banking of ADMSC.

In consequence of the inherent characteristics of the ADMSC: low immunogenicity, higher cellular yield, in comparison to bone marrow cells and pluripotency, the cryopreservation of these cells would be a very convenient alternative. However, the effects of the cryopreservation and the thawing proceedings could impact their therapeutic outcome and therefore should be evaluated. Once the cells are cultivated and are attached to the plastic, before the cells cryopreservation, these are subjected to a subtract desegregation stress, being able to put in risk the integrity of the cell membrane and its surface molecules for the cell adhesion. After thawing, the cryopreserved cells have also the risk of injuring the membrane and other cellular functional characteristics; both proceedings could affect the therapeutic outcomes.

In this context, the present study aims to address whether the ADMSC cryopreservation could compromise the cell integrity, the expression of adhesion molecules, and/or the differentiation potential of ADMSC and other stem cells lineages.

2. Material and Methods

2.1. Experimental Design. Adipose-derived mesenchymal stem cells were obtained from adipose tissues of 12 adult healthy donors (10 female and two male) by liposuction performed by a plastic surgeon with informed consent signature from each patient. The cell isolation was performed by enzymatic digestion with collagenase type I. Then, the cells were cultured in DMEN/F12 supplemented with 10% of calf fetal serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. Before and after cryopreservation, the following assays were performed: colony forming units, immunophenotype, and cellular viability with Annexin V and 7-AAD analysis by flow cytometer as well as ADMSC differentiation to adipocytes and osteoblasts.

2.2. Isolation of ADMSC. The adipose tissue samples were collected from a healthy donor after consent signature from each patient. All the samples were collected from specialized plastic clinics, using the liposuction technique. After the samples were collected, they were cultured before 24 hours, using the following protocol: 50 mL of each sample was extensively washed with phosphate buffered saline (PBS) (Sigma, St. Louis, MO, USA) containing 300 units/mL of penicillin and 300 μg/mL of streptomycin (Sigma, St. Louis, MO, USA) (PBS/PS). After washing the samples and debris removal, these were placed in 50 mL tubes with 0.075% of type I A collagenase (Sigma, St. Louis, MO, USA), prepared in PBS/PS. The cells were homogenized and incubated with agitation at 37°C for 30 minutes. After the incubation, the type I collagenase activity was ceased by adding the same volume of Eagle modified from Dulbecco/F12 (DMEM/F12) (LGC Biotechnology, Brazil), containing 10% of fetal calf serum (FCS) (GIBCO B Life Technologies, Inc., Rockville, USA, RL) as determined to be standard culture medium (SCM). Then, the samples were centrifuged at 400 g for 10 minutes. The supernatant was discarded and the pellet was resuspended in 10 mL of PBS/PS; then, another centrifugation was made at 400 g for 10 minutes. The supernatant was discarded and the pellet was suspended in 5 mL of culture medium. The cell suspension was filtered in cell strainer of 100 μM (Becton Dickinson, USA). The cell count was made by a hemocytometer and the utilization of Trypan Blue (Sigma, St. Louis, MO, USA) (for the dilution of 1 : 1, 10 μL of the cell suspension was added for 10 μL of Trypan Blue). After that, 1×10^5 cells for cm^2 in 25 cm^2 and 75 cm^2 culture flasks were harvested and incubated at 37°C and 5% of CO_2 [7–9].

2.3. Cultivation and Expansion. 72 hours after, on cultivation, the medium was aspirated from the flasks and the cells were washed with preheated PBS/PS; then, 7 mL of culture medium was added in the 25 cm^2 and 12 mL in the 75 cm^2 flasks. The cells were kept incubated at 37°C and 5% CO_2. The medium was changed twice a week until the cells reached between 80% and 90% of confluence. After the desired confluence was reached, the cells were briefly washed with preheated PBS/PS and added trypsin/EDTA (0,25%) (Sigma, St. Louis, MO, USA) for disaggregation of the adherent cells. The cells were incubated for 10 minutes at 37°C. Then, the same volume of SCM was added for the neutralization of the trypsin action. The cell suspension was transferred to sterile centrifuge tube and centrifuged at 400 g for 10 minutes. The supernatant was discarded and the cells were resuspended in approximately 5 mL of SCM. The cell count was realized with a hemocytometer chamber, using Trypan Blue (1 : 1 dilution) [9]. After the counting, cells were once again harvested in 75 cm^2 flasks at a concentration of 1×10^3 cells/cm^2 and incubated at 37°C and 5% of CO_2 until they reached 80% of confluence. When they reached the desired confluence, the cells were trypsinized and were available for analysis. For each sample, the culture was done at passage 2 (P2).

2.4. Cryopreservation Proceedings. After trypsinization at P2 cells as described previously, and confirming their viability by the Trypan Blue exclusion method, a fraction of the cells were submitted to the cryopreservation proceedings. The cells were counted with a hemocytometer (approximately, 1×10^6 cells/mL). The cryoprotectant medium had 80% of BFS, 10% of dimethyl sulfoxide (DMSO), (Sigma, St. Louis, MO, USA), 10% of DMEM-F12 (LGC Biotechnology, Brazil), and 100 units/mL of penicillin and 100 μg/mL of streptomycin (Sigma, St. Louis, MO, USA). 1 mL aliquots (containing 1×10^6 cells) were transferred to preidentified cryogenics tubes

Table 1: Panel of monoclonal antibodies used in this study for immunophenotypic characterization.

Marker	Clone	Reactivity	Fluorochrome	Function
CD34	581/CD34	Human	FITC	Stem cell marker (precursor), also found in hematopoietic progenitor cells, vascular endothelium, and fibroblasts of the same tissue. Probably, it works as a transduction signer and has a function in endothelium specific antigen adhesion.
CD45 (common leukocyte antigen)	HI30	Human	PE	Leukocytes marker. CD45 proteins are located in all hematopoietic cells, but not in erythrocytes.
CD49d (integrin $\alpha 4$)	9F10	Human	PE	Transmembrane glycoprotein, integrin $\alpha 4$. It makes several cell-cell interaction and cell-matrix and participated in cellular adhesion.
CD73 (ecto-5′-nucleotidase)	AD2	Human	PE	It have been suggested that this marker can mediate costimulator signals in the cells T activation and endothelium as well catalyses the dephosphorylation of adenosine monophosphate in adenosine.
CD90 (THy-1)	5E10	Human	FITC	The role of interaction in cell-cell and cell-matrix has been speculated, with related neurites growth, nerve regeneration, apoptosis, metaphase, inflammation, and fibrosis.
CD105 (endoglin)	266	Human	Purified	Responsiveness modulator of TGF-β cellular complex.

(cell type, density, and date). The tubes were cooled in a programmable freezing device (Nicool LM10; Air Liquide, Marne La Vallée, France). The cryopreservation equipment starts at room temperature and in the end of the first stage (Program 3–15 minutes) reaches the temperature −30°C; in the end of step 2 (Program 5–45 minutes), the temperature is −60°C; finally, in the last stage, step 3 (Program 9-10 minutes), the temperature is −110°C. After step 3, the cells were transferred to the liquid nitrogen to −196°C. This method prevents the crystals formation inside the cell, because the freezing occurs slowly [9–11]. Shortly after, the tubes were transferred to the liquid nitrogen where they remained stocked for approximately 20 days. All the samples were cryopreserved in triplicate.

2.5. Thawing Proceedings. 20 days after, the cryopreserved cells were thawed at 37°C and transferred in 5 mL of SCM and gently homogenized. The cell suspension was transferred for sterile centrifuge tube and centrifuged at 400 g for 10 minutes. The supernatant was discarded and the cells were resuspended in approximately 5 mL of culture medium. The cells were submitted to analysis.

2.6. Colony Forming Units (CFU) Analysis. Before and after the cryopreservation, 10 cells per cm^2 were seeded, in triplicate; in wells of 9 cm^2 with SCM, the medium was changed according to other steps of culture. After 14 days, the medium was removed and the cells were fixated with methanol for 5 minutes and then stained with 0.5% of crystal violet in methanol for more 5 minutes. The flasks were washed twice with PBS/PS and dried. The number of colonies with more than 2 mm was counted and the results represent the number of former colonies by 100 seeded cells (counted colonies/inoculated cells) × 100 [12].

2.7. Immunophenotypic Analysis. All samples were analyzed for the expression of surface markers (Table 1), using monoclonal antibodies against cluster of differentiation (CD) antigens, conjugated with fluorochromes and analyzed, before the cryopreservation and after thawing, by flow cytometer. Cells (number) were incubated with the antibodies that are described in Table 1 and corresponding isotypes were used. The primary antibodies were incubated for 10 minutes in the dark. The antibody against CD105 is a purified antibody and it is not conjugated with fluorescence; for this reason, it was necessary to incubate it with a secondary antibody for more 15 minutes in the dark room and redo the cell washing step. Further 5 μL of 7-AAD was added and incubated in the dark room for 15 minutes. After that, 400 μL of binding buffer was added and the samples were analyzed by flow cytometer (FACS Calibur; Becton Dickinson, San Diego, USA) [11, 13, 14]. Prior to each test, the equipment was calibrated using BD Calibrite (Becton Dickinson, San Diego, USA), according to the manufacturer's instructions. All antibodies were used according to the manufacturer's instructions. 20.000 events (cells) were captured; the percentage values of each marker were analyzed through specific analysis software: Cyflogic version 1.2.1.

Table 2: Surface markers expressions before cryopreservation and after thawing.

Molecular marker	CD34−		CD34+		CD45−		CD49d+		CD73+		CD90+		CD105+	
Cryopreservation	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Medium	98.88	99.3	1.12	0.78	99.79	99.8	88.67	77.8	99.57	99.5	99.55	99.5	99.4	98.3
Max	99.86	100	3.32	2.1	100	100	96.38	93.1	99.94	99.9	99.97	100	99.91	99.9
Min	96.98	97.9	0.14	0.03	99.27	99.6	80.15	41.6	98.51	98.5	98.15	97.5	97.87	93.9
SD	1.08	0.61	1.08	0.6	0.22	0.14	6.55	14.5	0.43	0.43	0.07	0.7	0.64	2.07
p value	**0.113**		**0.158**		**0.791**		**0.007***		**0.528**		**0.618**		**0.05**	

* $p < 0.05$.

2.8. Cell Viability with Annexin V and 7-AAD Analysis. The adherent cells were detached with trypsin (0.25%), centrifuged for 3 minutes at 400 g, and resuspended in 2 mL of PBS. After that, in 5 mL tubes, 100 μL of this solution was added. After that, 400 μL of PBS was added in each tube; they were homogenized and centrifuged. The supernatant was discarded and 100 μL of the binding buffer was added. Afterwards, 5 μL of Annexin V was added, 5 μL of 7-AAD was added, and the tubes were incubated in the dark room for 15 minutes. After incubation, 400 μL of binding buffer was added and homogenized; the data was collected by flow cytometer (FACS Calibur; Becton Dickinson, USA) [11].

2.9. Adipogenic Differentiation. The cells were treated with adipogenic medium consisting of DMEM-F12 medium containing 10% FBS, 0.5 m M isobutylmethylxanthine (Sigma, St. Louis, MO, USA), 1 mmol/L dexamethasone (Sigma, St. Louis, MO, USA), 10 mg/mL insulin, and 50 μM indomethacin (Sigma, St. Louis, MO, USA) and were incubated at 37°C and 5% CO_2. Control cells were treated with SCM. Medium was changed two times a week. 14 days after, the cells were fixed and stained with Oil Red Stain (Sigma, St. Louis, MO, USA) to demonstrate the fat droplets in the cells [7, 8].

2.10. Osteogenic Differentiation. The osteogenesis was induced by keeping the cells in DMEM-F12 medium with 100 units/mL of penicillin, 100 μg/mL of streptomycin, 1 nM of dexamethasone, 2 mM of β-glycerophosphate (Sigma, USA), and 50 μM of ascorbate-2-phosphate (Sigma, USA) and incubated at 37°C and 5% CO_2. Control cells were treated with SCM. The cells were kept in these conditions for 35 days and the medium was changed twice per week. The experimental and control cells were fixed glutaraldehyde at 2.5% for ninety minutes and stained with Alizarin Red Stain (Sigma, St. Louis, MO, USA) to demonstrate mineralization [7, 8].

2.11. Statistic Analysis. The obtained results in this study were expressed as mean ± standard deviation (SD). For the comparison between the molecules expression analysis before and after cryopreservation, the t-test was performed; $p < 0.05$ was considered significant. The data were organized in Excel spreadsheet and were analyzed with Statistica version 9.0 software.

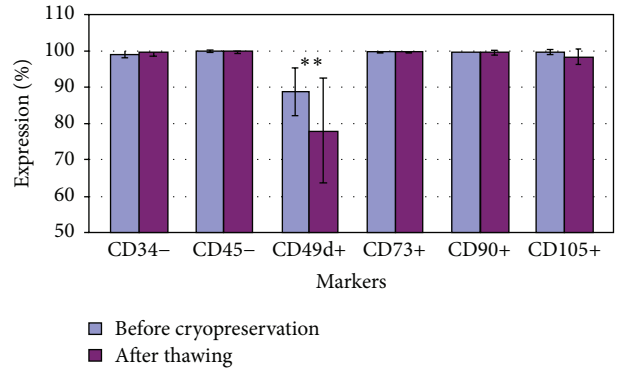


Figure 1: Surface markers expression of cells before cryopreservation and after thawing. The analysis was done by Cyflogic software 1.2.1. The t-test was done; $p < 0.05$ was considered significant. The results were $p^{**} = 0,001$.

3. Results

3.1. Phenotypic Analysis of ADMSC. Flow cytometric analysis showed a typical mesenchymal phenotype of ADMSC with expression of CD73, CD90, and CD105 whereas these cells lacked expression of CD34 and CD45. Interestingly, we observed a significant reduction of CD49d expression after thawing cryopreserved ADMSC (Table 2; Figures 1 and 2).

3.2. Annexin V 7-AAD Staining. The differences in CD49d expression before and after cryopreservation led us to look at the cell viability before and after cryopreservation. Cell viability was assessed by Annexin V 7-AAD staining; we observed a significant reduction in viability from 91.34% ± 4.54% to 74.99% ± 14.19% ($p = 0.001$) after cryopreservation, losing an average of 17.9% viable cells. Concerning labeling with Annexin V (apoptosis), values were very close to the values of cellular viability, being 91.39% ± 5.5% before cryopreservation and 76.31% ± 13.33% after thawing ($p = 0.003$) (Table 3; Figure 3). Thus, suggesting that, the majority of Annexin V stained cells were also stained with 7-AAD, which means that the amount of cells only in apoptosis was a small proportion.

3.3. Colony Formation Assay. Further, we looked at the colony formation ability of ADMSC and observed a significant decrease in the colonies formation capacity; CFUs

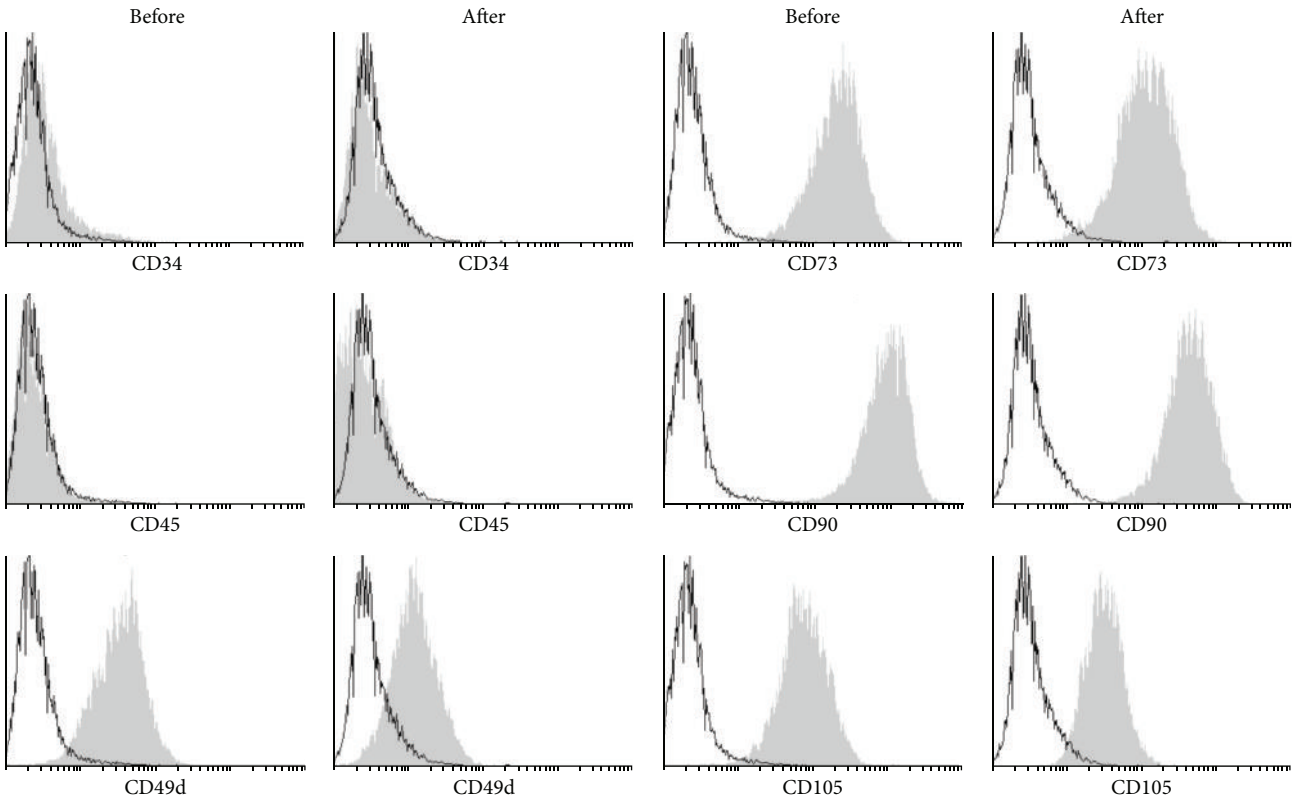


Figure 2: Histograms of ADMSC markers before and after cryopreservation. The grey color represents specific marker and the white color represents an isotype control.

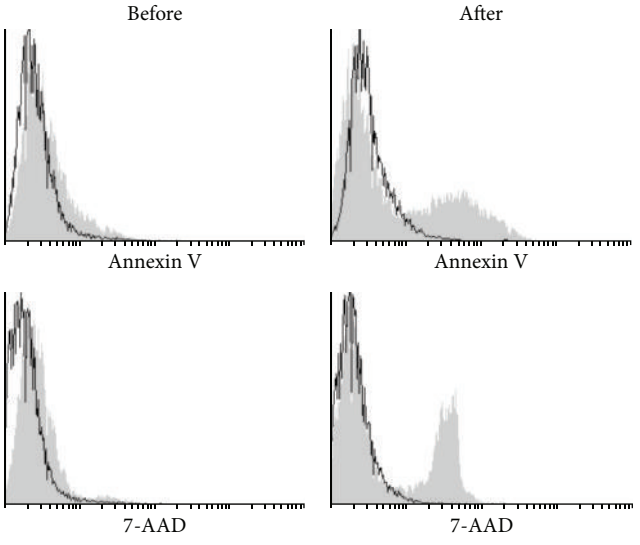


Figure 3: Histograms of Annexin V (apoptosis marker) and 7-AAD (viability marker) of the cells before and after cryopreservation. The grey color represents specific marker and the white color represents an isotype control.

before and after cryopreservation were 28.08% ± 7.06% versus 21.51% ± 6.61% ($p < 0.01$).

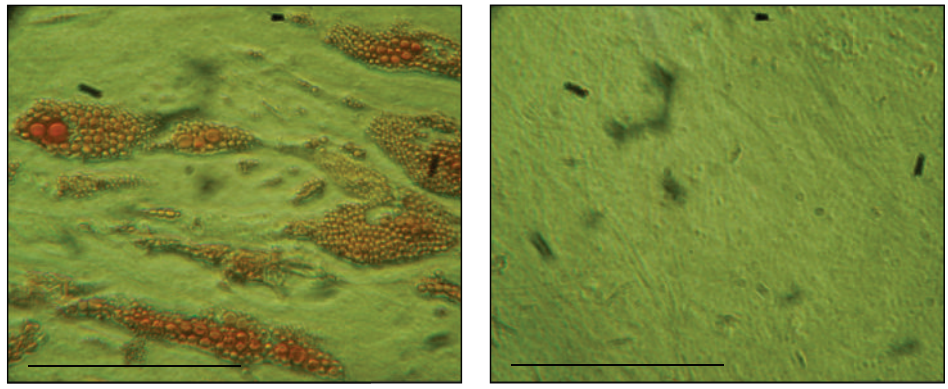
3.4. Adipogenic Potential of ADMSC. It was assessed, after cryopreservation with a lineage-specific induction medium, the cells differentiated into adipogenic as evidenced by Oil Red, whereas control cells did not take up Oil Red Staining (Figure 4).

3.5. Osteogenic Potential of ADMSC. In addition, upon treatment with a lineage-specific induction medium, the cells differentiated into osteogenic as evidenced by Alizarin Red, whereas control cells did not take up Alizarin Red staining (Figure 5).

4. Discussion

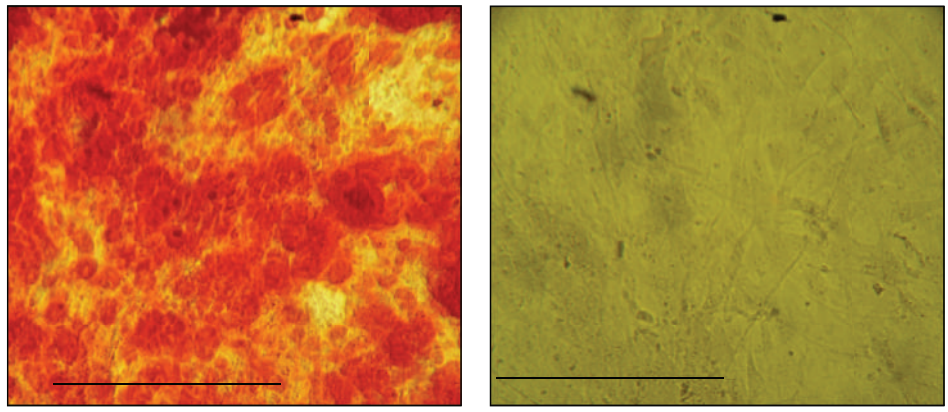
The ADMSC are cells with a huge therapeutic potential and important tool to cellular metabolism studies. Therefore, the characterization of cryopreserved cells and their maintenance after thawing cryopreserved are relevant.

About the CFU analysis, we observed a significant decrease in the colonies formation capacity; CFUs before and after cryopreservation were 28.08% ± 7.06% versus 21.51% ± 6.61% ($p = 0.01$), respectively. These results are in agreement with the results found by Goh and colleagues (2007) that cryopreservation causes decrease in adhesion efficiency of ADMSC [15]. This difference could be related to decreased expression of integrin $\alpha 4$ (CD49d) that we have observed. Mitchell et al. (2006) reported that the ability to form colonies of ADMSC is considerably greater than BMSC, even after cryopreservation. This group also reported that the more the passages, the greater the number of CFUs



(a) (b)

Figure 4: Adipose differentiated cells after 14 days in induction medium: sample after thawing of cryopreserved cells, phase contrast microscopy, 250x. (a) Presence of fat droplets (stained with Oil Red) in ADMSC cultivated with adipogenic induction medium. (b) Control does not have fat droplets, indicating the undifferentiated cells cultivated with standard medium. Scale (10 μm).



(a) (b)

Figure 5: Osteogenic differentiated cells after 35 days in osteogenic induction medium: sample after thawing of cryopreserved cells, 250x rise. (a) ADMSC stained with Alizarin Red, demonstrating the presence of calcium in extracellular matrix. (b) Control without matrix demonstration, indicating the undifferentiated cells cultivated with standard culture medium. Scale (10 μm).

and suggests that the passages select a particular cell type, possibly the stem cells [16]. The same parameters were kept before and after cryopreservation and, however, can indeed be a delay in cell growth, considering the fact that the cells were again adapting to cultivation conditions and the limited size of the colonies. Regarding cellular therapy, the increase of number of the passages could compromise the safety and that is why it may be related to increasing mutations and increasing the transformation of the cells [17]. On the other hand, about the differentiation process, most of the groups studying with ADMSC report that, after 14 days of adipogenic induction, all samples of cells have a morphology consistent with adipocyte, which was confirmed by staining of lipids with Oil Red as well as by PCR that identify genes consistent with adipocytes and which are not present in undifferentiated cells. Rodriguez and colleagues (2004) reported that after this period about 90% of the cells accumulate intracellular lipid and that there might also be no significant accumulation in untreated cells [18]. All samples, before and after thawing, that were induced to the adipogenic differentiation medium stained positive for Oil Red, showing the presence of lipids inside the cells (Figure 4(a)). In the control medium, the standard culture medium was added and there was no trace of lipids, indicating that these cells did not differentiate into adipocytes (Figure 4(b)). These results clearly show that ADMSC have after appropriate inducing an adipogenic differentiation capacity. To identify osteogenic differentiation, several groups have reported that, between 14 and 21 days after induction, we can observe the presence of a mineralized matrix, by the calcium phosphate staining with Alizarin Red [16, 19]. In the present work, it was observed only after 30 days in the induction medium. All samples, before and after thawing, that were induced to the osteogenic differentiation medium stained positive for Alizarin Red (Figure 5(a)). In the control medium, the standard culture medium was added and there was no evidence of mineralization, indicating that these cells did not differentiate into osteogenic differentiation (Figure 5(b)).

Both adipogenic and osteogenic differentiation results suggest that cells were stem cells. Other researchers reported the maintenance of the capacity of differentiation of ADMSC in both adipocytes and osteocytes after thawing [19–21]. In this study, 100% of the samples had the same induction time.

There are some controversies about the markers for MSC. This occurs because of different designs of different types of markers; however, there is a consensus that MSC are negative for CD45 (a marker of HSC) [22]. In this study, all samples were negative for CD45, which is an indication that they would be large MSC. In this study, four samples showed a small population of cells positive for CD34 (1 to 3%).

In this study, varying values for the CD49d were found (88.67 ± 6.55), consistent with the values found by Katz and colleagues (2005) (78 ± 20) [23]. Maybe, if those cells will be submitting culture again, it will be possible to recover this expression of CD49d. In this work, cultivating the cells after cryopreservation was not possible due to the low number of cells. However, thinking about translation to humans, the most likely hypothesis in a cell therapy would be the immediate transplant of the cells after thawing. Thus, as already described in the literature, increasing the number of passages also increases the probability of cell transformations such teratomes, which makes impracticable therapy [18]. In addition to varying values of expression, this surface marker showed significant decreases after thawing of cryopreserved cells (77.8 ± 14.45, $p = 0.007$). This marker represents the α4-integrin, an adhesion molecule that interacts with the β1 integrin forming a heterodimer, late activating antigen-4 (VLA-4) [13]. Lei and colleagues (2007) found low positive values for CD49d (average 12.6%), but the way of isolation of ADMSC was through the subcutaneous tissue and not through lipoaspirate; another issue is that they used DMEM with low glucose; these differences protocols can select different types of cells with similar characteristics, but not identical [24]. For Katz and coworkers (2005), some differences between groups with respect to expression of CD49d reflect adjustments of countless variations of these cells to the extracellular medium, such as density, cell cycle, culture time, and the number of passages [23]. The environment produces appropriate niches for stem cells and regulates the maintenance of these niches for specific cell lines. For this regulation, the adhesion of stem cells in the extracellular matrix is critical as it allows communication between the cells and the matrix, being a prerequisite for the maintenance of tissue [25]. Thus, these changes in expression of CD49d after cryopreservation may mean a major problem in transplantation of these cells, causing them not properly communicating with the injured tissue.

In this study, only viable and intact cells were analyzed. Gonda et al. (2008) stated that cryopreservation can cause structural and functional damage to cell proteins and reduce their viability, but immunophenotypic exchanges could hardly occur [19]. Expression changes really would not have excuses, but the loss of expression is very relevant because the group itself mentioned that cryopreservation can cause damage to the membrane protein, which is the case of CD49d, which represents an adhesion protein. Few studies are related to immunophenotypic difference after thawing of cryopreserved cells, which is an extremely important feature that should be studied [19, 26]. Cellular viabilities before cryopreservation and after thawing were analyzed by staining with the kit of viability (BD) Annexin V PE_7-AAD on 20,000 events. Apoptosis of the cells is characterized by phosphatidylserine, a component of the inner leaflet of cell membranes. When a cell enters apoptosis process, phosphatidylserine becomes exposed on the outer wall of the membrane, but the cell membrane remains intact. The cells positive for Annexin V represent cells that have this translocation of phosphatidylserine [27]. After thawing there was a significant loss of the cells integrity, being 16.5% lower than the cells before the cryopreservation (Figure 5). Maybe that cell integrity is also lost with thawing? Another cells line, which was positive for 7-AAD, indicates that the membrane integrity was compromised; consequently, there was cell death. Accordingly, these markers are used to distinguish dead cells from cells that are in the process of apoptosis. To analyze these markers, Cyflogic software 1.2.1 was used, which provides the histogram overlay of the isotype control with the results of fluorescence of each marker and quantifies the percentage of positive (not overlapping the isotype control) and negative (superimposed on the isotype control) markings. Thus, it was possible to analyze three variables: (i) 100% viable cells (negative for Annexin V-PE and negative for 7-AAD); (ii) dead cells (positive for Annexin V-PE and positive for 7-AAD); and (iii) the process of apoptosis in cells (positive for Annexin V-PE and negative for 7-AAD). Following these parameters, before cryopreservation, viability was 91.34% ± 4.54%. After thawing, the cells had a significant drop in cell viability, 74.99% ± 14.19% ($p = 0.001$), losing on average 17.9% viable cells. Concerning labeling with Annexin V (apoptosis), values were very close to the values of cellular viability, being 91.39% ± 5.5% before cryopreservation and 76.3%± 13.33% after thawing ($p = 0.003$) (Table 3). This study demonstrates that the majority of Annexin V stained cells were also stained with 7-AAD, which means that the amount of cell only in apoptosis was small.

Table 3: Representation of viability and integrity cells before cryopreservation and after thawing.

	Annexin V		7-AAD	
	Before	After	Before	After
Media	91.39	76.31	91.34	74.99
Max	96.2	95.83	79.29	49.21
Min	75.18	52.38	95.17	95.27
DP	5.85	13.33	4.54	14.19
p value	**0.003**		**0.001**	

The ADMSC viabilities of cryopreserved cells after thawing may be explained with the concentration of cells in each cryotube. Goh et al. (2007) tested four cell concentrations: 2.5×10^5, 5×10^5, 1×10^6, and 2×10^6 per mL and found a viability of 71.4%, 81.10%, 77.9%, and 69.2%, respectively. In this study, the cryopreservation of cells in 1×10^6 cells

per mL and viability found values similar to values found by Goh group (2007); however, the method used by Goh et al. (2007) was staining by Trypan Blue which is more relative to be counted manually; the method used in this study is more accurate, by flow cytometric analysis [15]. Thirumala and colleagues (2010) found viabilities, staying at 84% ± 8% when using the same cryoprotectant in their study, but the test was performed on P1 [27]. De Rose and colleagues (2009) found amazing values of cellular viability 92.5%. This high rate of viability may be related to the form of thawing these cells, which were transferred to culture medium with 10% FCS prior to complete thawing; it could be explained by the fact that the cells stayed less time in contact with DMSO in room temperature that is known for its cytotoxic effects [10]. The researchers have shown that many factors influence the intracellular dynamics when cells are frozen, affecting the viability of these cells. Among these factors can be highlighted the formation of intracellular ice, which can perforate the cell membranes, and high concentration of cells which can limit the space preventing cell growth during freezing [15]. A standard protocol was used for various cell types, but some cells have specific characteristics, which may require special care. For this reason, it would be ideal to develop a specific protocol for ADMSC, to improve the viability rate and other characteristics [11, 19, 28].

Some authors used as long-term storage method freezing in a freezer at −80°C rather than liquid nitrogen (−196°C), but this method has revealed lower levels of viability while retaining the functional characteristics. Other variables could influence the viability, like the speed of freezing, because if it will be faster, there are greater probabilities of intracellular ice formation and consequent membrane damage [19, 26] and the choice of serum free media, which may not benefit from a large cell viability. However, considering therapeutic applications, even with this, low viability could be indicated by the reduced risk of contaminating [26].

5. Conclusions

In this study, the standard protocols of the human adipose-derived mesenchymal stem cells cryopreservation and thawing proceedings are demonstrating the decrease: α4-integrin expression (CD49d), cell viability, and colony forming units after thawing. These findings can compromise the integration of cells in the extracellular matrix of the host tissue. Further protocols should be established to improve and ensure the cell graft.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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Review Article

Advances in Adipose-Derived Stem Cells Isolation, Characterization, and Application in Regenerative Tissue Engineering

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Obesity is a complex, multifactorial disease that has been extensively researched in recent times. Obesity is characterized by excess deposition of adipose tissue in response to surplus energy. Despite the negative connotations of adipose tissue (AT), it serves as a critical endocrine organ. Adipose tissue is a source of several adipokines and cytokines which have been deemed important for both normal metabolic function and disease formation. The discoveries of metabolically active brown AT in adult humans and adipose tissue derived stem cells (ADSC) have been key findings in the past decade with potential therapeutic implications. ADSCs represent an enticing pool of multipotent adult stem cells because of their noncontroversial nature, relative abundance, ease of isolation, and expandability. A decade and a half since the discovery of ADSCs, the scientific community is still working to uncover their therapeutic potential in a wide range of diseases. In this review, we provide an overview of the recent developments in the field of ADSCs and examine their potential use in transplantation and cell-based therapies for the regeneration of diseased organs and systems. We also hope to provide perspective on how to best utilize this readily available, powerful pool of stem cells in the future.

1. Introduction

Regenerative medicine has evolved tremendously with recent advances in stem cell research. The last couple of decades have shown glimpses of the exciting potential of stem cells in tissue regeneration as well as in the restoration of diseased organs and systems. Despite these advances, the availability of stem cells remains a challenge for both scientists and clinicians with an interest in regenerative medicine. In general, a stem cell is characterized by its ability to self-renew and differentiate into multiple lineages. With regard to regenerative medicine, the ideal stem cell population should be present and accessible in abundant numbers, harvestable by a relatively noninvasive procedure, able to differentiate into a variety of cell lineages, easy to transplant to an autologous or allogeneic host, and able to be manufactured in accordance with the currently accepted good manufacturing practice guidelines set by the FDA [1].

The two main types of stem cells are embryonic stem (ES) cells and adult stem cells, also known as somatic stem cells. Other types, such as induced pluripotent stem cells (iPSCs), are produced in the lab by reprogramming adult somatic cells to express ES characteristics. ES cells are obtained by extracting cells from the inner cell mass (ICM) of embryos at the blastocyst stage and subsequently expanding them in culture. ES cells' nature of origin makes them difficult to obtain and raises ethical concerns. In contrast to ES cells, adult stem cells produce only a limited set of specialized cells based on location in a particular tissue. In adults, tissue-specific stem cells are located throughout the body in several tissues. The hematopoietic stem cells found in bone marrow and umbilical cord blood can differentiate into all

the different types of blood cells. They are the easiest to isolate and have been used in therapy for decades in bone marrow transplants for diseases such as leukemia, where there is abnormal development of blood cells. Other types of tissue-specific stem cells are usually found deep within tissues and are thus more difficult to obtain and study, especially in humans. The epidermal stem cells continually renew the outer layer of skin, and the epithelial stem cells in the gut similarly continuously replace the gut lining. More recent discoveries include bronchoalveolar stem cells from the lungs of adult humans, which are thought to renew the cellular linings of the lungs [2]. One particularly new and enticing addition to the types of known stem cell sources is the pool of adipose-derived stem cells (ADSCs). ADSCs are found in the perivascular region of white adipose tissue, which include subcutaneous fat deposits [3, 4]. Because of their abundance, ease of isolation, and noncontroversial nature, ADSCs have been considered for applications in regenerative medicine.

This review focuses on isolation techniques for ADSCs, the latest developments in protocols, and therapeutic implications of ADSCs for several diseases. We will also discuss the future directions of ADSCs and promising avenues of research.

2. Historical Overview of ADSC

In 2001, a research group at UCLA identified and described a putative population of multipotent stem cells that they termed processed lipoaspirate (PLA) cells due to their derivation from processed lipoaspirate tissue obtained through cosmetic surgery [5]. These stem cells were isolated with the help of enzymatic digestion of the stromal vascular fraction (SVF) of adipose tissue. The SVF can be defined as a minimally processed population of fibroblasts, endothelial cells, smooth muscle cells, pericytes, several types of immune cells, and preadipocytes that have yet to adhere to a tissue culture substrate [6, 7]. Culturing the SVF over time is thought to eliminate many of these cell populations, resulting in an adherent population primarily composed of preadipocytes. However, as Zuk et al. suggested through histology and preliminary PCR analysis, the adherent population derived from the SVF contains a significant number of cells that display characteristics of multipotent stem cells [5]. Specifically, it was proposed that the culturing of the SVF results in a relatively homogenous population of PLA cells that are free of contaminating cell populations and capable of displaying phenotypic characteristics of adipocytes, osteoblasts, and chondrocytes [8]. Moreover, it was demonstrated that clonal cell populations derived from a single PLA cell could also be differentiated into the aforementioned cell types, thus confirming an important prerequisite along the path of identifying a stem cell.

Adipose tissue originates from the mesodermal layer of the embryo and develops partially prenatally, but mostly postnatally [9, 10]. The exact location and developmental history of the adipogenic progenitor cells that constitute stem cells are still unknown, and there is not a standardized nomenclature. In addition, stem cells isolated from different types of adipose tissue depots behave differently [11]. There are two main physiologically distinct types of adipose tissue depots: white and brown adipose tissue, with each performing a distinct biological function. Brown adipose tissue (BAT) is best known for its thermogenic properties, generating heat through the expression of a unique uncoupling protein (Ucp1), which utilizes the mitochondrial proton gradient during oxidative phosphorylation to generate thermal energy rather than energized substrates (ATP). In humans, BAT is present in newborn infants, and the amount of BAT generally decreases as humans age to a point where it is virtually absent in the mature adult. Recently, a few groups have reported the presence of metabolically active BAT in adult humans [12, 13]. White adipose tissue (WAT) is by far the most abundant of two. The principal functions of WAT are to store energy and to provide insulation throughout development. WAT is present in several parts of the body in small depots and can acquire distinct characteristics based on the location of the depot. With the rising epidemic in obesity, characterized by excessive amounts of fat deposition, WAT has received significant attention from researchers worldwide. More recently, WAT has been regarded as playing a multifaceted role as both an energy storage site and an endocrine organ. Secretion of hormones such as adiponectin, leptin, resistin, and other adipokines in addition to several classical anti- and proinflammatory cytokines allows WAT to induce systemic physiological and pathological effects.

Although recent advances have shed light on the functional and physiological differences of different adipose tissue depots, the stem cell content of individual depots is still unexplored. In particular, the stem cell population in WAT may hold greater differentiation potential and multipotency [14]. Furthermore, within the broad category of WAT, it is well established that there are differences between the subcutaneous and omental white adipose depots in human subjects, particularly with respect to preadipocyte and endothelial cell numbers [15]. Because of its volume, visceral adipose tissue may be favored over other tissue types as a source of multipotent cells.

3. Isolation Procedure and Characterization

In 1960s, Rodbell et al. developed a method to isolate cells from adipose tissue [8, 16–18]. They minced rat fat pads, washing them several times to get rid of the contaminating blood and immune cells, and incubated washed tissue fragments with collagenase enzyme to aid in digestion. Following centrifugation, the digested samples would separate into a floating population of mature adipocytes and a pelleted stromal vascular fraction (SVF). The SVF consisted of a heterogeneous cell population, including circulating blood cells, fibroblasts, pericytes, and endothelial cells as well as "preadipocytes" or adipocyte progenitors [8, 18, 19]. After washing with PBS, the SVF population from this stage may be cultured with high FBS (20%) containing DMEM media, with media changes occurring every 12–24 hours to alleviate the effects of collagenase and cell stress (Figure 1).

Adipose tissue is a heterogeneous mixture of several types of cells ranging from immune cells and blood cells to cells of adipogenic origin. To isolate the adipose-derived stem

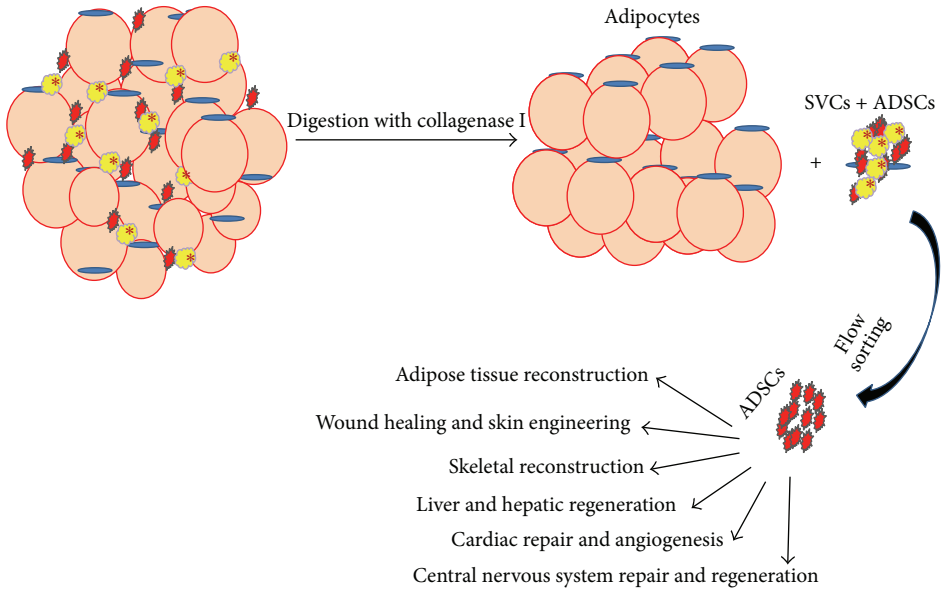

Figure 1: Schematic diagram for ADSCs isolation from adipose tissue and several uses of ADSCs in tissue regenerative medicine.

cell (ADSC) population, the cells within the SVF can be categorized using a number of cell type specific markers. The separation of adipocyte precursor populations from nonadipogenic cells using a single cell surface marker is almost impossible [20]. Like most cells, ADSCs are characterized by expression of selective cell surface markers. With the help of multicolor flow cytometry, these putative progenitor cells can be differentiated from nonadipogenic cells such as endothelial and blood cells [21].

An exact phenotypic characterization of ADSCs is still in development, as there is still an unclear distinction between ADSCs, fibroblasts, and several immune cells based on several published protocols. However, adipocyte precursors can be identified by the lack of expression of lineage cell surface markers such as CD45 (blood cell specific) and CD31 (endothelial specific) and characterized by expression of stem cell specific markers including CD29, CD34, and platelet-derived growth factor (Pdgf) receptor-α, Sca-1, and CD24 [22, 23]. ADSCs within the SVF putatively express $CD31^-$, $CD34^{+/-}$, $CD45^-$, $CD90^+$, $CD105^-$, and $CD146^-$; endothelial progenitor populations express $CD31^+$, $CD34^+$, $CD45^+$, $CD90^+$, $CD105^-$, and $CD146^+$; vascular smooth muscle cells (pericytes) express $CD31^-$, $CD34^{+/-}$, $CD45^-$, $CD90^+$, $CD105^-$, and $CD146^+$; and hematopoietic cells express $CD45^+$ in uncultured conditions [24, 25]. ADSCs share many of the same cell surface markers as pericytes and bone marrow-mesenchymal stem cells (MSCs). In addition to the previously mentioned markers, pericyte markers expressed by ADSCs include smooth muscle β-actin, pdgfr-β, and neuroglial proteoglycan 2 [26]. The MSC markers expressed by ADSCs include CD13, CD29, CD44, CD58, and CD166. Expression of several of these markers varies based on the method of isolation, time of incubation, and stage of growth when in culture. Furthermore, Ong et al. reported differences in surface marker expressions from subcutaneous and visceral adipose tissue depots. ADSCs from subcutaneous adipose expressed higher levels of CD10, whereas visceral adipose-derived ADSCs showed increased expression of CD200 [27]. Because of the overlapping of many cell surface markers, there must be meticulous gate setting while sorting cells via flow cytometry. Further analysis of gene or protein expression with the aforementioned markers can provide additional confirmation.

4. Clinical Applications of ADSCs

4.1. Adipose Tissue Reconstruction. The use of ADSCs for reconstruction of soft tissue defects has been extensively documented in the literature. Fat injections (FIs), where aspirated fat is reinjected in its entirety without any processing to separate cell populations, have been performed for reconstructive purposes [28]. The most common clinical implication of FIs today is breast soft tissue reconstruction. In the breast, FIs are performed for both cosmetic purposes as in breast augmentation [29, 30] and reconstructive purposes for women with congenital abnormalities or breast cancers in which tissue had to be removed due to metastasis [30–33]. Some studies suggest that supplementation with ADSCs may enhance bulk transplantation procedures such as fat injections. In one triple-blind trial, the survival of fat grafts enriched with autologous adipose-derived stem cells (ADSCs) was compared to the survival of nonenriched fat grafts. Compared with the control grafts, the ADSC-enriched fat grafts had significantly higher residual volumes. These findings support a potential usage of the ADSC stem cell pool in clinical settings. In particular, ADSC graft enrichment could render minimally invasive lipofilling a reliable alternative to major tissue augmentation, such as breast surgery [34]. While adipose tissue within FI contains ADSCs, FIs rely mostly on bulk volume from the transplanted adipose

tissue rather than ADSCs to achieve clinical results. As with all transplantation procedures, it is important to consider potential changes in the biochemical environment of the host prior to transplantation. One interesting finding is that the metabolic state of the donor could have an impact on ADSC behavior and expression profile. For instance, ADSCs from obese donors induce significantly greater inflammatory response in the form of higher Th17 promotion and monocyte activation when compared to ADSCs from lean donors. This proinflammatory environment in turn inhibits adipogenesis and the adipocyte insulin response [35]. The demonstration of the ADSCs-Th17-monocyte cell axis suggests a novel proinflammatory process taking place in adipose tissue during obesity [35].

4.2. Wound Healing and Skin Engineering. The potential of ADSCs to self-renew and regenerate tissue has great implications in wound healing and skin restoration. Some of the earliest uses of ADSCs in wound healing were in the treatment of chronic fistulae in Crohn's disease with the successful healing of rectovaginal fistula [36–38]. Reportedly, ADSCs were harvested, cultured for up to a month, and then injected directly into the fistula sites with a 75% successful healing rate. The mechanism by which ADSCs aid in wound healing is unclear, but possibilities include direct differentiation of ADSCs within the epithelium, support for angiogenesis in the local tissue, and other paracrine effects from the release of cytokines and growth factors to the region.

Recently, Kuo et al. investigated whether ADSCs can accelerate diabetic wound healing. In a streptozotocin-induced rodent model of diabetes, rats were subcutaneously injected in the diabetic wound margin twice with non-diabetic ADSCs. The ADSC-treated group demonstrated significantly reduced wound healing time compared to the control, and furthermore there was an observed reduction in the proinflammatory characteristics of the ADSC-treated group. There was also an increase in angiogenesis following injection, suggesting involvement of ADSCs in the angiogenesis pathway [39]. In addition to direct transplantation, differentiation of ADSCs to epithelial cells may be another useful application of ADSCs in wound healing. Indeed, there exist varying protocols to differentiate ADSCs to cells with epithelial characteristics using a combination of conditioned media, growth factors, contact with an extracellular matrix, and sometimes specific chemical factors such as retinoic acid. However, the use of ADSCs in skin engineering is still in the experimental stages of development and so far has been predominantly limited to in vitro investigations. Nevertheless, the relative ease of access, high cell yield, and putative anti-inflammatory effects of ADSCs make them attractive targets for skin engineering, in which a large amount of tissue must be supplied for reconstruction [40].

4.3. Skeletal Reconstruction. While ADSCs have demonstrated the capability to differentiate into bone and cartilage in the laboratory, the clinical usage of ADSCs for skeletal muscle/bone reconstruction has been limited [5, 41]. The reasons for a lack of translation to the clinical setting may include inefficient differentiation protocols and a limited ability to differentiate into elastic or hyaline cartilage types. One of the earlier instances where ADSCs were used for skeletal reconstruction was attempted bony cartilage healing by Cowan et al. in 2004. ADSCs were seeded into poly lactic-co-glycolic acid (PLGA) scaffolds and were able to repair critically sized cranial defects in mice [42] and in a 7-year-old girl [43]. More recently, a study involved treatment of four patients possessing large cranial defects with a combination of ADSCs and b-tricalcium phosphate granules. Computed tomography (CT) scanning subsequently revealed improved ossification in all of the cases [44]. The efficacy of ADSCs in bone or cartilage based restoration efforts may be improved if ADSCs are used in combination with traditional techniques such as grafts, glues, scaffolds, and supplementation with differentiation factors [45–47].

Because ADSCs are functionally similar to bone marrow derived mesenchymal stem cells, they are capable of directed differentiation to cartilage, muscle, and bone. As mentioned previously, while it seems that ADSCs can successfully generate forms of restored fibrocartilage, the formation of elastic or hyaline cartilage (necessary for joint reconstruction) has not been successfully demonstrated [48]. For other forms of cartilage, however, a recent study suggests that larger doses of growth factors and shear stressing can be beneficial to chondrogenesis during ADSC transplantation [49, 50]. In addition to cartilage, ADSCs can undergo differentiation to form muscle cells. There are a number of protocols for the myogenic differentiation of ADSCs, most of which focus on upregulating expression of the transcription factor MyoD. One recent finding may point to the importance of cell markers in determining myogenic potential among ADSCs. Specifically, Desiderio et al. reported that $NG2^+$ADSCs loaded on XHA scaffolds can be fabricated into human skeletal muscle tissue in vivo without a prior myogenic differentiation step in vitro. While $NG2^+$ ADSCs were able to differentiate into muscles, $NG2^-$ ADSCs merely formed human adipose tissue [51]. For osteogenic differentiation, culture and growth conditions seem to be particularly important. McCullen et al. showed that elevating extracellular calcium from a concentration of 1.8 to 8 mM accelerates human ADSC osteogenic differentiation and cell-mediated calcium accretion, even in the absence of any other soluble osteogenic factors in the culture medium [52]. Recently the same group reported that elevated calcium induced osteogenesis and inhibited chondrogenesis in human ADSCs. Based on these findings, stacked polylactic acid nanofibrous scaffolds containing either 0% or 20% tricalcium phosphate nanoparticles were electrospun and tested for site-specific chondrogenesis and osteogenesis [53]. Approaches such as changing the ionic concentrations and culture conditions hold great promise for osteochondral tissue engineering using an ADSC cell source in combination with a scaffold. In general, optimizing the physical and chemical microenvironment of ADSCs during transplantation may hold the key to making clinical progress in connective tissue regeneration.

4.4. Liver and Hepatic Regeneration. Evidence suggests that ADSCs can differentiate into cells with hepatocyte-like characteristics under appropriate culture conditions and that

transplantation of ADSCs can improve hepatic function [54]. While the precise mechanisms of these functional changes are still not clear, these results suggest that ADSCs may be useful for the regeneration of the liver. Injection of human ADSCs into the tail vein of mice has been shown to lead to multiorgan engraftment of the cells [55]. Two months after injection, the mice demonstrated the presence of human-derived cells in the bronchus of the lungs, the gastrointestinal tract, and the liver. Interestingly, the liver held a significant proportion (6.9%) of ADSC occupancy whereas no human cells were found in other tissues, such as skeletal and cardiac muscle, kidneys, or skin. These findings suggest a unique interaction of ADSCs with hepatocyte populations and could suggest a potential role of ADSCs in hepatic regeneration. Recently, Tang et al. showed that basic fibroblast growth factor treatment enhances the therapeutic effect of ADSCs, and secretion of hepatocyte growth factor from ADSCs plays a critical role in amelioration of liver injury and regression of fibrosis [56].

4.5. Cardiac Repair and Angiogenesis. Cardiac muscle is considered to be a tissue with very limited capacity for self-renewal and repair. In particular, there is an inability to naturally and adequately replace damaged myocardium in cardiovascular complications such as chronic cardiac disease and myocardial infarction (MI) [57]. Given the prevalence of heart diseases and their associated complications, alternative cell-based therapies enlisting the aid of ADSCs and other forms of MSCs are being tested for regenerative potential. In 2004, a research group led by Planat-Bénard identified a very small subpopulation (0.02–0.07%) of cardiomyocyte-like cells in murine SVF which exhibited contractile behavior at day 24 following primary culture [58]. These cells were positive for several cardiac-specific genes, including GATA-4 and Nkx2.5. Later, in 2007, Song and colleagues from the United States showed a similarly small (0.005–0.07%) subpopulation of spontaneously beating cells in human SVF [59]. Coadministration of control-released β-FGF along with ADSCs led to angiogenesis and vessel maturation in a murine ischemic hind limb model. This was partly assisted by secretion of hepatocyte growth factor, vascular endothelial growth factor, and transforming growth factor β1 [60].

De Francesco et al. reported different cell populations expressing mesenchymal stem cell markers, such as CD34, CD90, CD29, CD44, CD105, and CD117, and endothelial-progenitor-cell markers, including CD34, CD90, CD44, and CD54, present in SVF. Notably, the $CD34^+/CD90^+$ expressing cells formed sphere clusters and demonstrated high proliferative capacity when placed in nonadherent growth conditions. When cultured in an adipogenic medium, $CD34^+/CD90^+$ cells quickly differentiated into adipocytes and also into endothelial cells ($CD31^+/VEGF^+/Flk\text{-}1^+$). When placed in methylcellulose, these cells were further capable of forming capillary-like structures producing a high level of VEGF [61, 62].

The clinical usage of ADSCs in the treatment of chronic cardiac disease or acute MI in humans is still quite distant. Elucidating cellular markers associated with cells possessing particularly high affinity to differentiate into cardiomyocytes may be a promising avenue of research. ADSCs are known for their strong proliferative capacity and express high levels of hyperpolarization-activated cyclic nucleotide-gated (HCN) genes, which code for ion-gated HCN channels. These channels are essential for generating rhythmic activity within clusters of cardiac cells. Friis and colleagues in Denmark have published a safety and feasibility study looking at intramyocardial injection of cultured BMSCs in patients with stable CAD [63]. They treated 31 patients and concluded that the treatment was safe and resulted in significant improvements in left ventricular function and exercise tolerance. Overall, there is a growing body of evidence in laboratory and animal models that ADSCs could improve cardiac function via cell differentiation and paracrine pathways [57, 64]; we anticipate exploration with human trials in the coming years.

4.6. Central Nervous System Repair and Regeneration. There is a paucity of literature showing the potential of ADSCs to differentiate into neuronal lineage cells; however, there have been a few documented instances where ADSCs can acquire neuron-like characteristics under appropriate growth conditions. If efficient neurogenesis is possible, ADSCs could potentially provide a huge boon to the current clinically viable options to regenerate nerves and neural tissue. Endogenous neural stem cells (in the central nervous system) and Schwann cells (in the peripheral nervous system) are difficult to harvest and accordingly have limited applicability [65]. Morphological changes towards a neuron-like appearance have been reported for ADSCs when treated with individual or multiple growth factors such as BNDF, EGF, and βFGF. A variety of specific chemical stimuli have also demonstrated abilities to induce such morphology. For instance, ADSCs can be transdifferentiated into neuron-like cells using selegiline, as evidenced by an increase in the number of nestin and NF-68 immunoreactive cells following treatment [66]; however, chemically induced morphological changes may not represent actual cellular differentiation, but rather cell shrinkage. Thus, it is important to characterize the extent of neuronal differentiation of ADSCs using known neuronal markers such as vimentin, nestin, GFAP, and MAP-2, in addition to morphology. Other stimuli or conditioning media may be used to further enhance the acquisition of neuronal characteristics by ADSCs. For instance, one group recently showed that human ADSCs could be differentiated in rat brain-conditioned media towards neuron-like cells with a similar efficacy as bone marrow stem cells (hBMSCs); however, because of their higher proliferation rate and greater availability, the authors suggest that ADSCs may be a more viable option for future cell-based neuroregeneration efforts [67].

5. Miscellaneous Use

It has been demonstrated that an intravitreal injection of ADSCs can stabilize the retinal microvasculature, enabling repair and regeneration of damaged capillary beds in vivo. Interestingly, one report suggests that diabetic ADSCs are functionally impaired compared to healthy ADSCs and supports the utility of an allogeneic injection of ADSCs

versus autologous or conditioned media for approaching the treatment of diabetic retinopathy [68]. Urinary bladder regeneration is another area where ADSCs could play an important role. ADSCs have been successfully used on a PLGA scaffold to reconstruct half of a bladder wall in a nude mouse model [69] in addition to two human patients [70]. This may be very encouraging news for diseases such as urinary stress incontinence and could lead to new cell-based therapy incorporating ADSCs. However, in contrast to these findings, another group showed no difference between fat and saline injections in the treatment of female urinary incontinence at 3 months' follow-up and suggested that the fat may not be able to survive at the injection site [70]. Thus, more work is needed to determine the applicability of ADSCs in this area.

Human and rodent ADSCs can be stimulated to differentiate towards the osteogenic lineage [71, 72]. In vivo, ADSCs differentiate into bone when implanted ectopically into rodents. Rat-isolated ADSCs seeded in polyglycolic acid will begin bone formation when implanted subcutaneously [73, 74]. Similarly, human ADSCs in HA-TCP scaffolds differentiate to osteocytes in immunodeficient mice [75, 76]. Several preliminary reports have also shown the promising results of ADSC treatment in Crohn's disease [77]; however, many of these putative therapeutic applications of ADSCs need to be validated with further research.

6. Concerns and Safety Issues with Adipose-Derived Stem Cells

The use of stem cells to treat different maladies has gained popularity over the past decade. ADSCs have demonstrated tremendous potential because of their availability, plasticity, and versatility with regard to regeneration of different tissues. However, despite some clinical trials in humans and existing data from animal experiments, the various risks and safety concerns associated with ADSC transplant have not been fully elucidated. Since adipose tissue serves important auxiliary endocrine functions, transplantation of ADSCs may exert unintended paracrine and endocrine effects on peripheral tissues. For instance, the secretion of metabolically active hormones such as leptin, cytokines such as TNF-α and IL-6, and several growth factors could disrupt peripheral biochemical pathways [78]. Recently Eljaafari et al. reported that obese- but not lean-derived ADSCs induce a proinflammatory response mediated through Th17 promotion and monocyte activation, which leads to inhibition of adipogenesis and adipocyte insulin response [35].

One important consideration with every stem cell pool is the potential for tumorigenicity upon transplantation. The aforementioned production of IL-6 by ADSCs is concerning, since IL-6 is known to be a crucial cytokine in the induction and progression of tumors. Other proinflammatory and chemokine factors such as IL-8, MCP-1, RANTES, TGF-β, and VEGF belong to the expression profile of ADSCs while also being markers associated with tumor growth and metastasis; however, the relationship between ADSCs and cancer cells is not fully understood, as evidenced by mixed reports in the literature. In one animal model, ADSCs were shown to aid in chronic inflammation and assisted the transformation of gastritis to gastric cancer [79]. By contrast, another study showed that ADSC implantation actually inhibited breast cancer development in mice [80]. Additionally, ADSCs ostensibly secrete many factors related to the epithelial-mesenchymal transition (EMT), an important step in tumor metastasis; however, a discord still exists between basic science and clinical findings [81]. The applicability of these animal studies to humans undergoing clinical treatment remains doubtful. It is entirely possible that the interaction between ADSCs and tumor cells may be entirely characteristically different in humans, especially considering the differences in adipose tissue depot composition and location between humans and rodents. Thus, further basic studies on the interaction between hADSCs and various cancers will need to be performed to elucidate any potential hazards such as de novo tumorigenesis or the reinforcement of existing tumors. A series of well-designed and controlled clinical trials will be necessary.

7. Future Directions of Adipose-Derived Stem Cell Therapies

The potential of adipose tissue to be a prolific source of multipotent adult stem cells has garnered a great deal of attention in the field of regenerative medicine. With the increasing number of overweight and obese individuals, isolation of ADSCs from lipoaspirate samples may prove to be a clinically feasible option. Given the relative abundance of ADSCs, their ease of harvest and culture, and their high yield relative to other stem cell pools such as bone marrow, it is likely that the research and clinical usage of ADSCs will continue to grow. While some initial reports show positive clinical outcomes, well-designed and controlled studies as well as long-term posttreatment follow-ups will be paramount to ensure the safety and efficacy of these procedures for patients.

Stem cells have proven to be a useful tool in cell-based therapies for a wide collection of diseases. For ADSCs, the development of detailed and efficient differentiation protocols for various cell types, optimization of in vivo delivery methods, and mitigation of immune response in allogeneic transplantations are some of the challenges that need to be overcome. Many of these challenges have been considered and investigated of late, but additional work is necessary in order to bridge the gap between findings in basic science and the clinical treatment of diseases with stem cell-based regenerative medicine.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Development of Synthetic and Natural Materials for Tissue Engineering Applications Using Adipose Stem Cells

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Adipose stem cells have prominent implications in tissue regeneration due to their abundance and relative ease of harvest from adipose tissue and their abilities to differentiate into mature cells of various tissue lineages and secrete various growth cytokines. Development of tissue engineering techniques in combination with various carrier scaffolds and adipose stem cells offers great potential in overcoming the existing limitations constraining classical approaches used in plastic and reconstructive surgery. However, as most tissue engineering techniques are new and highly experimental, there are still many practical challenges that must be overcome before laboratory research can lead to large-scale clinical applications. Tissue engineering is currently a growing field of medical research; in this review, we will discuss the progress in research on biomaterials and scaffolds for tissue engineering applications using adipose stem cells.

1. Introduction

Adipose stem cells (ASCs) have the potential to differentiate into various cell phenotypes if there is a specific inducing microenvironment using suitable inductive substances [1–3]. Meanwhile, their abundance and relative ease of harvest, along with their autogenous immune-privileged status, have also made them an attractive candidate for tissue engineering and regenerative therapies [4, 5].

Tissue engineering enables the regeneration or repair of tissues and organs through combinations of stem cells, biomaterial scaffolds, and regulatory growth factors [6, 7]. ASC-based tissue engineering strategies depend primarily on the quality of the ASC fraction used. Although a large number of studies have been conducted to assess the differentiation potential of ASCs in different carriers, there are still some unclear aspects regarding the basic knowledge of ASC biology and the clinical applications. These include the following: (i) What is the best definition for ASCs, and what is the true nature of the fractions of ASCs used by the various investigators? (ii) Does heterogeneity exist between freshly isolated ASCs and ASCs expanded in several culture passages? (iii) What are the best procedures for harvesting adipose tissue, preparing the stromal vascular fraction (SVF), and isolating ASCs? (iv) What are the best procedures for cell banking and cellular cryopreservation? The past few years have seen exciting progress in tissue engineering and regenerative medicine using various biomaterials and scaffold [8–11]. Natural and synthetic materials have been developed to provide a carrier scaffold that is ideally supposed to mimic the extracellular matrix (ECM) properties of an *in vivo* microenvironment to induce tissue formation [12, 13]. Nowadays, the development of efficient biomaterials and scaffolds is still in high demand for the production of clinically useable volumes of new tissues to replace lost or malfunctioning body parts and to achieve uncomplicated wound healing.

It is crucial that scaffolding materials can positively interact with surrounding tissue to not only fill the defect, but also facilitate the natural regeneration of stem cells. Significant efforts have been made to develop such scaffolds for tissue engineering applications [14–16]. For example, electrospinning, lithography, microfabrication, and self-assembly techniques have been widely explored for the fabrication of engineered scaffolds appropriate for specific tissue applications. Considering the usage of these engineered scaffolds in the body, they should have the following characteristics:

(i) possession of appropriate surface properties to promote the adhesion, proliferation, and differentiation of stem cells; (ii) low toxicity and immunogenicity; (iii) high porosity; and (iv) degradability that is adequate for specific tissues, with an interconnected pore network for cell growth and flow transport of nutrients and metabolic waste. The objective of this review is to recapitulate the progress in the fields of biomaterial and scaffold development and various procedures for ASC selection for tissue engineering applications and to review several clinical cases for the advancement of ASCs-based tissue engineering strategies.

2. Basic Knowledge on the Biology of ASCs in Regenerative Medicine

In the past decade, a number of cell characterization studies have described the underlying biology of ASCs [17–33]. Preclinical studies on the use of ASCs both *in vitro* and *in vivo* have been performed, and the efficacy of ASCs has been determined in several clinical trials [34–40]. Compared with bone marrow or umbilical cord stem cells, ASCs have a similar self-renewal ability *in vitro*, and the ability of ASCs to differentiate in other mesodermal and ectodermal lineages has been demonstrated on several occasions [1, 41–45]. Moreover, ASCs release multiple growth factors, such as the two key factors vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF). Other factors include VEGF-B, VEGF-C, fibroblast growth factor- (FGF-) 2, angiopoietin- (Ang-) 1, Ang-2, SPARC/osteonectin, platelet-derived growth factor- (PDGF-) b, transforming growth factor (TGF), and stromal cell-derived factor-1 (SDF-1) [46]. However, the specific population of ASCs with the greatest therapeutic potential remains unclear. Since the initial reports in the late 1960s [47], many researchers have established that stromal stem cells similar to those identified in bone marrow can be isolated from adipose tissue that is either resected as intact tissue or aspirated using tumescent liposuction [17, 48, 49]. In general, the obtained adipose tissue is digested with one of the following: collagenase, dispase, trypsin, or related enzymes. A consensus exists regarding temperature (37°C), digestion duration times (range, 30 min to >1 h), and ratios of tissue weight to volume; however, protease concentrations are far more variable. Following the neutralization of the enzymes and differential centrifugation, the released elements, which are separated from the mature adipocytes, are defined as the SVF. The SVF consists of a heterogeneous mesenchymal population of cells that includes not only adipose stromal and hematopoietic stem and progenitor cells but also endothelial cells, erythrocytes, fibroblasts, lymphocytes, monocyte/macrophages, and pericytes [17, 50, 51]. For the phenotypic characterization of the SVF, the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT) proposed a stromal cell population, excluding hematopoietic and endothelial cells, based on the following combination: CD45−CD235a−CD31−CD34+ and additional markers used to identify SVF are CD13 (APN), CD73 (L-VAP-2), CD90 (Thy-1), and CD105 (Endoglin). Based on the existing literature, this population combination represents at least 20% of the cells in the SVF [52–56], and the percentage of CD34+ cells mainly depends on the method used to harvest the adipose tissue, the degree of vascular hemorrhage, and the subsequent digestion and isolation techniques. In addition to the enzyme digestion, methods for isolating SVF cells using mechanical, nonenzymatic techniques have been developed recently, and some have been applied in clinical practice [57–59].

When SVF pellets are seeded into culture, a subset of elongated cells begins to adhere to the bottom of the plastic tissue culture plate. After a combination of washing steps and culture expansion with media to remove most of the hematopoietic cell population from the SVF cells, these cells are purified as an adherent cell population termed ASCs. ASCs are less heterogeneous than SVF cells and have the ability to undergo self-renewal and the capacity to undergo multilineage differentiation and generate multiple terminally differentiated cells when cultured in specific lineage-inducing culture media. One main difference between SVF cells and ASC suspensions is the high percentage of CD45+ cells in the SVF cell population (30–70%) and the low or undetectable percentage in ASC population (2–30%). ASCs generally express CD34+ during the early phase of culture (within 8–12 population doublings after culture of the SVF), but then its expression decreases with continued cell division [51, 55]. A joint statement by the IFATS and ISCT recommended that the surface antigens used to characterize ASCs should include CD73, CD90, and CD34 without CD45 and CD31. In addition, CD13 has also been proposed as an alternative or supplement to CD105 [17]. To date, most experimental research groups have isolated ASCs by tissue digestion, centrifugation, and the capacity of ASCs to adhere to cell culture plastic surfaces [9, 43, 60]. However, the adherent cell population also contains other cell types that are not multipotential [61–63]. In order to overcome the problem of "contamination," a number of alternative methods have been proposed, including magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS). FACS is a typical cell enrichment method that utilizes complementary fluorochrome conjugated antibodies to label cells of interest. However, the sorted cells obtained from FACS can be utilized for diagnostic and experimental purposes but not for therapeutics due to problems with safety and efficacy [64, 65]. MACS is an antibody-aided technique based on immunomagnetic beads coated with specific antibodies against stem cell surface molecules, and it is technically accessible and affordable. From the view of clinical application, MACS with biodegradable magnetic beads wins over FACS on the grounds of safety, and it is the only method approved for use in clinical settings [61, 66–68].

Clinical research on adult stromal cell populations has accelerated, and multiple clinical investigations are underway to examine the use of ASCs and SVF cells for tissue engineering and regenerative medical applications [22, 69, 70]. To achieve the large numbers of ASCs required for clinical applications, either the cells need to be expanded in culture or ASCs must be pooled from multiple donors. Therefore, the development of stem cell banks is necessary. These banks must assure the quality and safety of these cell products,

Table 1: Synthesis of synthetic materials for tissue engineering applications using ASCs.

Materials	Properties	Principal uses	References
TiO_2 nanofiber	High degree of crystallinity and surface wettability	Bone tissue engineering	[85]
Poly(3-hydroxybutyrate-co-hydroxyvalerate) (PHBV) scaffold	Good integration with the surrounding tissue, stiff character, and degradability	Skin tissue engineering	[10]
Copolymer PEGylated fibrin (P-fibrin) gels	Stable urethane (carbamate) linkage, degradability	Cardiovascular and skin tissue engineering	[91]
Poly(glycerol sebacate) (PGS)/poly(L-lactic acid) (PLLA) blend scaffolds	Favorable porous microstructures, good hydrophilicity, appropriate mechanical properties for soft tissue applications, and degradability	Adipose tissue engineering	[93]
Poly(lactic-coglycolicacid)/multiwalled carbon nanotubes/silk fibroin (PLGA/MWCNTs/SF) nanofibrous scaffolds	Nonwoven structures and random fiber distribution with smooth and beadless fibers morphology	Nerve tissue engineering	[95]
[Poly-D,L-lactic acid/polyethylene glycol/poly-D,L-lactic acid (PDLLA-PEG)]/hyaluronic acid (HA) matrix	Designed architectures, high mechanical strength and biodegradability, biocompatibility, and water solubility	Cartilage tissue engineering	[99]

especially when the stored ASCs are intended for clinical use in cell therapy and regenerative medicine. Cryopreservation may be an ideal option for this and is currently the only method to preserve ASCs while maintaining their functional properties and genetic characteristics in the long term [71–73]. Slow freezing and vitrification are the currently available methods for the cryopreservation of stem cells in laboratories and clinics [74–78]. Vitrification only works well with the cryopreservation of human cells in small volumes, such as oocytes, but it is ill-suited for large volumes of ASCs [79, 80]. Moreover, vitrification techniques require higher cryoprotectant agent concentrations, which induces toxicity and osmotic stress in cells and tissues. Slow freezing is an established technique pioneered in the early 1970s and involves cryopreserving biological samples at controlled freezing rates to avoid intracellular ice formation and minimize structural damage to the cell membrane and cytoskeleton [81, 82]. Cryoprotectant agents are used at relatively low concentrations [21] in slow freezing, which has become the standard method for cell and tissue cryopreservation. However, formation of ice crystals, extreme hyperosmolarity, and dehydration are still reported when cells undergo the slow freezing process. Dimethyl sulfoxide (DMSO) is the most widely used cryopreservant for cells, but it is known to be toxic at room temperature. Trehalose is a nontoxic disaccharide of glucose that may stabilize and preserve cells and cellular structures during the freezing procedure. A cryopreservation method using trehalose as a cryoprotective agent is recommended for the long-term preservation of ASCs compared to simple cryopreservation or to cryopreservation using DMSO alone. Other cryoprotective agents such as polyvinylpyrrolidone and methylcellulose have been developed to replace DMSO; however, they are less efficient than DMSO in terms of maintaining ASC viability [75].

Adipose-derived stem cells are a promising cell source for regenerative medicine. It is important to understand the basic knowledge and biology behind stem cells, and further research is needed to guarantee the safety of ASCs and the effectiveness of tissue engineering using ASCs.

3. Advancement in Synthetic Materials for Tissue Engineering Applications Using ASCs

In the provision of an appropriate microenvironment for cellular components to interact with, the extracellular matrix (ECM) is an important component of normal tissue that must be considered. Various synthetic materials have been developed to provide carrier scaffolds that mimic ECM properties for tissue regeneration and reconstruction in combination with ASCs (Table 1). The advantages of synthetic materials and scaffolds rely on the technical possibility that chemical and physical properties (e.g., porosity, surface characteristics, and degradation products nature) can be specifically optimized for a particular application [83, 84]. Ideally, a polymeric material used for tissue engineering should be able to regulate cell proliferation without the loss of pluripotency and to direct differentiation into a specific cell lineage when desired. Tan et al. described the influence of TiO_2 nanofibrous surface structures, which were produced in situ onto Ti-6Al-4V substrate via a thermal oxidation process, on the regulation of proliferation and preservation of stemness of ASCs. The results show that ASCs exhibit better adhesion and significantly enhanced proliferation on TiO_2 nanofibrous surfaces than on flat control surfaces, thus presenting a promising potential for the application of TiO_2 nanofibrous surfaces in the field of bone tissue engineering and regenerative therapies [85]. Although much has been done to develop tissue-engineered skin substitutes in the past decade, poor visualization,

hypertrophic scarring, and keloid formation are still possible negative outcomes for current skin graft strategies [86–88]. In an effort to overcome these limitations, Zonari et al. proposed the combination of poly(3-hydroxybutyrate-co-hydroxyvalerate) (PHBV) structures with ASCs to induce skin regeneration in a full-thickness model. In this work, PHBV scaffolds demonstrated good integration with the surrounding tissue, allowing exudation and infiltration by inflammatory cells, which may contribute to rapid degradation over time. Furthermore, PHBV scaffolds offered a moist environment combined with a stiff character that withstands contraction and simultaneously stimulates the secretion of various growth factors by seeded ASCs; these factors enhance vascularization and ECM deposition with reduced scarring. Ultimately, this study revealed the great advantages of PHBV loaded with ASCs to improve wound healing and skin regeneration with reduced scarring in skin tissue engineering [10]. The advancement of tissue engineering as a regenerative therapy relies on rapid vascularization of tissue constructs, and engineered three-dimensional biomaterials are known to affect the angiogenic capacity of seeded stem cells [89–91]. Copolymer PEGylated fibrin (P-fibrin) gels were introduced by Chung et al. as an ASC-carrying scaffold for encouraging local angiogenesis in an *in vitro* culture model without added soluble factors. In P-fibrin gels, ASCs elicited higher von Willebrand factor expression than the two commonly used hydrogels (i.e., collagen and fibrin). After seven days of cultivation, vascular endothelial growth factor (VEGF) was secreted more in fibrin and P-fibrin gels than in collagen; several other angiogenic and immunomodulatory cytokines were similarly enhanced. Moreover, P-fibrin matrices were uniquely able to drive a vessel-like phenotype in ASCs and induce formation of well-organized vascular networks relative to other gels. Thus, it can be speculated that the research on ASCs' regenerative potential in a carrier scaffold can be expanded to include cardiovascular and skin tissue engineering applications based on the observed angiogenic properties of ASCs in P-fibrin [91]. Seeding cells on mechanically appropriate scaffolds and applying specific mechanical stimulation to these cells have been found to be beneficial in terms of proliferation and differentiation [92, 93]. Frydrych et al. reported a large and flexible 3D porous poly(glycerol sebacate) (PGS)/poly(L-lactic acid) (PLLA) blend scaffold with mechanical properties comparable to adipose tissue that was fabricated via a freeze-drying and a subsequent curing process. *In vitro* cell test results provided clear evidence that PGS/PLLA scaffolds are suitable for the culture of ASCs, as they are characterized by deep cell penetration and ECM growth. This work demonstrates that the PGS/PLLA scaffolds provided favorable porous microstructures, good hydrophilic characteristics, and appropriate mechanical properties for soft tissue applications [93]. Neural tissue possesses a very limited capacity to regenerate new functional neurons after nerve injuries, and tissue-engineered neural tissues using stem cells may serve as a promising alternative for neural regeneration. However, such stem cells would need to proliferate and differentiate into the desired phenotype with the aid of adequate chemical, mechanical, or biological stimuli regeneration [94–96]. Catalpol is a natural active ingredient extracted from a traditional Chinese medicine. Guo et al. evaluated the effects of a catalpol-loaded scaffold on guiding the neuronal differentiation of hASCs. In their study, the process for catalpol loading into the electrospun poly(lactic-coglycolic acid)/multiwalled carbon nanotubes/silk fibroin (PLGA/MWCNTs/SF) nanofibrous scaffolds was successfully established. As a result of adding catalpol, the diameters of the nanofibers decreased and the porosity increased. Moreover, the mechanical properties of the composite scaffolds were improved, and more neuronal-like cells were found on scaffolds with catalpol [95]. The poor self-healing ability of cartilage necessitates the development of methods for cartilage regeneration. Fabrication of scaffolds with live stem cell incorporation and subsequent differentiation presents a promising route [97, 98]. Sun et al. [99] reported the use of a visible-light-based PSL (VL-PSL) system to encapsulate hASCs into a biodegradable polymer [poly-D,L-lactic acid/polyethylene glycol/poly-D,L-lactic acid (PDLLA-PEG)]/hyaluronic acid (HA) matrix to produce live cell constructs with customized architectures. In the chondrogenic medium-treated group (TGF-β3 group), hASCs showed high viability (84%) and expressed the chondrogenic genes Sox9, collagen type II, and aggrecan at 11, 232, and 2.29×10^5 fold increases, respectively, compared to levels at day 0 in nonchondrogenic medium. After 28 days, the mechanical strength of the TGF-β3 group remained high at 240 kPa. Thus, PSL and PDLLA-PEG/HA-based fabrication method using ASCs is a promising approach for producing mechanically competent engineered cartilage.

Thus, synthetic materials provide greater control over the mechanical and biochemical properties of the carrier scaffolds and represent a promising tool in tissue engineering and regeneration medicine [100].

4. Development of Natural Materials for Tissue Engineering Applications Using ASCs

In accordance with the plastic surgery rule of "replace with alike," natural materials have recently been recognized as an attractive choice for tissue engineering applications. Natural materials chosen for tissue engineering scaffolds are either compounds of the native ECM or polymers extracted from other biological systems [12, 101]. Evidence indicates that natural materials can behave similar to the ECM and possess biocompatibility, biodegradability, and inherent biological functions that could make them suitable for a range of tissue engineering applications [102–105]. Over the past several years, a wide range of natural materials has become available for tissue engineering strategies (Table 2). Among them, decellularized extracellular matrix has received increasing attention [106–110]. During tissue decellularization, cells are discharged from tissues, but the native ultrastructure and composition of the ECM is highly preserved, which is expected to be able to direct the differentiation fate of the seeded stem cells [111]. The combined use of decellularized human adipose tissue extracellular matrix (hDAM) and human adipose-derived stem cells (hASCs) as an adipose tissue engineering strategy was first introduced by Wang et al. [12]. In this study, engineered fat grafts (hDAM combined

Table 2: Synthesis of natural materials for tissue engineering applications using ASCs.

Materials	Properties	Principal uses	References
Decellularized human adipose tissue extracellular matrix (hDAM)	Maintains the major adipose tissue ECM components and 3D structure and includes collagen, sulfated glycosaminoglycan, and and vascular endothelial growth factor but lacks major histocompatibility complex antigen I	Adipose tissue engineering	[12]
Acellular cartilage matrices (ACMs)	Ideal 3D structure and physicochemical properties and good biocompatibility	Cartilage tissue engineering	[112]
Liver decellularized extracellular matrix (DCM)	Preserves macroscopic 3D architecture and the native composition, and ultrastructure remains a viscous liquid at low temperatures (at or under room temperature) and becomes gelation at 37°C	Liver tissue engineering	[113]
Paper-based bioactive scaffold	Microfibrous porous 3D architecture and biocompatible, cost-effective, mechanical robustness and water resistance	Bone tissue engineering	[117]
Hyaluronic acid scaffold	Biocompatibility, nonimmunogenicity, high hygroscopicity, and capacity to degrade into safe products	Muscle tissue engineering	[121]
Collagen	Nontoxic, biocompatible, and bioabsorbable, and it is FDA approved for use in humans	Adipose regeneration and adipose tissue engineering	[122, 124, 125]
Matrigel	Natural polymer and biocompatible	Adipose tissue engineering	[128]
Chitosan	Biodegradable, biocompatible, and an excellent hemostatic and analgesic agent with antioxidant properties	Skin reconstruction and skin tissue engineering	[128, 129]

with hASCs) were implanted subcutaneously in nude rats. The results showed that hASCs seeded in hDAM contributed to adipose tissue formation; the implanted engineered fat grafts maintained their volume for eight weeks. Hence, this study provides a platform and novel scaffold design for adipose tissue engineering of hDAM-hASC constructs. Current cartilage tissue engineering technology has developed quickly and efforts have focused on the creation of a suitable chondrocyte scaffold. Acellular cartilaginous matrix (ACM), which is obtained from fresh cartilage using a series of acellular manipulations, is a recently developed natural matrix material. Wang et al. reported that the repair of articular cartilage defects had been achieved with ASCs and acellular cartilaginous matrix in rabbits [112]. In the tissue-engineered cartilage group (ACM combined with ASCs) after 12 weeks, articular cartilage defects were filled with chondrocyte-like tissue with a smooth surface and were rich in glucan and type II collagen, similar to normal articular cartilage. Although the development of cartilage tissue engineering is still in its infancy, the acellular cartilaginous matrix obtained in this study offers tremendous potential in cartilage regeneration medicine. Recently, a decellularized liver 3D matrix scaffold has been proved to be able to facilitate the activity and function of the hepatic cells and stem cells [113–115]. Zhang and Dong [113] compared the hepatogenic differentiation-inducing effect of decellularized liver 3D matrix scaffold and several extracellular matrices, including collagen, fibronectin, and Matrigel in combination with mouse adipose-derived mesenchymal stem cells *in vitro*. The results clearly demonstrated that decellularized liver ECM gel, either on its own or in the presence of grow factors, could significantly enhance hepatic differentiation from ASCs compared with other matrix scaffolds; this demonstrates the feasibility of liver DCM as a bioscaffold for liver regenerative medicine and tissue engineering. Paper, which is produced from natural sources, can be supplied in large quantities with fair properties of biocompatibility and cost-effectiveness [116]. Hence, paper may have the potential in establishing tissue engineering scaffolds for therapeutic application of stem cells. Park et al. reported the feasibility of a paper-based bioactive scaffold for hASCs application to repair bone tissue defects for the first time [117]. In this study, paper scaffolds were prepared from three types of commercial paper materials: weighing paper (WP), chromatography paper (CP), and wiping tissue (WT), after which a polymer-coating method called initiated chemical vapor deposition (iCVD) was employed to coat the paper scaffold to achieve favorable biochemical surface properties (e.g., adhesiveness and water resistance), without damaging the scaffolds. The results showed that osteogenic differentiation of hASCs was induced on the paper scaffolds under osteogenesis-inducing conditions *in vivo*, indicating that paper material possesses great potential as a bioactive, functional, and cost-effective natural scaffold for adipose stem cell-mediated bone tissue engineering. Insoluble (derivatized or crosslinked) forms of HA have been extensively investigated for tissue engineering purposes due to HA's role in the extracellular matrix as well as its biocompatibility, nonimmunogenicity, high hygroscopicity, and capacity to degrade into safe products [118–121]. Desiderio et al. evaluated the differentiation potential of constructs made from a new crosslinked HA (XHA) scaffold on which NG2+ ASCs were loaded. Thirty days after engraftment in mice, NG2+ ASCs underwent a complete myogenic differentiation and fabricated human skeletal muscle tissue, indicating a significant step in muscle regeneration without the need for a prior *in vitro* muscle differentiation step [121].

In summary, the application of natural materials in the field of regeneration medicine is currently progressing. The advantages of natural materials are biocompatibility and mechanical and biological properties consistent with *in vivo* features, making them perfect candidates for tissue engineering field. Apart from the neoteric materials mentioned above, other commonly used natural materials in tissue engineering include collagen [122–125], hyaluronan [126], Matrigel [127, 128], and chitosan [129].

5. Clinical Applications of Different Carrier Scaffolds in Combination with ASCs in Tissue Engineering Strategies

The major role of regenerative medicine in this century is based on cell therapy, in which ASCs hold a key position [130–135]. Recently, a number of *in vitro* and a few *in vivo* studies using ASCs in combination with carrier scaffolds can be found through searches and on clinical trial websites. However, the use of cultured stem cells in clinical settings is strictly controlled by governmental regulations around the world, which largely restrict the application of ASCs in regenerative medicine. Meanwhile, plastic surgeons in Korea and Japan have played a leading role in pioneering the use of ASCs in tissue engineering more than any other Western nations due to less stringent government regulations.

It is well known that diabetic ulcers and chronic radiation ulcers are notorious for their recurrence, or failure to heal due to patient debilitation or poor local blood supply conditions. Several conventional reconstructive surgeries have been introduced for patients with chronic nonhealing cutaneous lesions [136, 137]. Presently, Kim and Jeong reported a less invasive method using adipose stem cell-based therapy and a collagen sponge scaffold, which was covered with an artificial dermis (Terudermis®) to deal with a chronic diabetic ulcer on the knee area. Two weeks after application, vascular tissue ingrowth was seen in the lesion area and thus a skin graft could be placed on the newly engineered vascular bed [5]. Complex fistulas are difficult to manage. Currently limited surgical procedures often result in high recurrence rates, whereas extensive surgical procedures may cause fecal incontinence. One recent improvement in treating complex fistulas may be the use of ASCs in combination with a fibrin glue scaffold described by Garcia-Olmo et al. [138]. The fibrin glue used in this study contained human fibrinogen, bovine aprotinin, and human thrombin, and the ASCs were isolated from lipoaspirated fat tissue. Eight weeks after the final treatment, fistula healing was observed in 17 (71 percent) of 24 patients who received fibrin glue plus ASCs, in comparison to 4 (16 percent) of 25 patients who received fibrin glue alone. The proportion of patients with

healing strongly indicates that the combination of the fibrin glue scaffold and ASCs is an effective and safe treatment for complex perianal fistulas. Autogenous bone graft has been considered to be the gold standard for reconstructive bone surgery. However, harvesting bone for grafting is associated with significant donor site morbidity that requires additional operative and anesthetic time [139]. An alternative approach is bone tissue engineering, through which in situ bone formation by using combinations of biomaterials, bioactive molecules, and stem cells can be achieved [140, 141]. Sandor et al. reported a case that used an ASC tissue-engineered construct to treat a large anterior mandibular defect. In this report, expanded ASCs were seeded on β-tricalcium phosphate (β-TCP) granular scaffolds consisting of recombinant human bone morphogenetic protein-2; the constructs were implanted into a U-shaped titanium mesh that spanned the parasymphyseal defect. Ten months after reconstruction, dental implants were integrated into the grafted site successfully with a dental implant-supported overdenture. The patient has been followed for three years since the tissue-engineered constructs were placed; he has been pleased with the aesthetic outcome of the procedure and continues to be satisfied with the function of his dental implants.

Although the applications of scaffolding materials together with ASCs technologies are a rapidly developing field of regeneration medicine, they are highly experimental so far. Thus, there still remains a significant need to develop efficient carrier materials that may bridge the gap and lead towards clinical applications in tissue engineering.

6. Conclusions and Future Perspectives

In the past several years, evidence has demonstrated that the ECM not only offers structural support for cells but also profoundly influences the major cellular programs of growth, differentiation, and apoptosis [142, 143]. An ideal scaffold structure must accomplish the roles of the extracellular matrix for the seeded cells, which will be used to form a tissue-engineered construct and to promote the repair/regeneration of damaged tissue [144, 145]. Hence, the design of carrier materials that can regulate cell behaviors such as proliferation and differentiation is the main purpose for the fabrication of tissue engineering scaffolds. Moreover, we should begin to understand that biomaterials and scaffolds used in tissue engineering strategies are dynamic, mobile, and multifunctional regulators of cellular behavior, and not just mere carriers for stem cells or storehouses for cytokines. Indeed, as the field of tissue engineering is still in its infancy, possible biomechanics and biomechanical effects created by different types of scaffolds on seeded ASCs should be further elucidated so that carrier scaffolds with special properties can be created. The creation of such scaffolds would help us to optimize cellular activities including changes in morphology, proliferation, and differentiation.

In general, carrier materials for the successful generation and maintenance of engineered tissue constructs should have several necessary properties including biocompatibility, degradability, low toxicity, and immunogenicity. However, most carrier scaffolds possess only some of these desirable properties. If a scaffold could embody all of these properties successfully, it would provide an ideal platform for tissue regeneration.

In conclusion, ASCs have a prominent and strong role in tissue engineering and regenerative medicine due to their high cell yield in adipose tissue, their ability to differentiate into multiple lineages and secrete various cytokines, and their immunomodulatory effects. The field of ASCs-based tissue engineering therapy is still young. Ongoing and future development of carrier scaffolds together with reasonable promotion of stem cell research and clinical studies will no doubt gradually bring ASC-based tissue engineering technology down from the ivory tower and make it clinically accessible on a larger scale, thereby benefiting more patients.

Conflict of Interests

The authors confirm that there is no known conflict of interests associated with this paper.

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Review Article

Adipose-Derived Stem Cells for Tissue Engineering and Regenerative Medicine Applications

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Adipose-derived stem cells (ASCs) are a mesenchymal stem cell source with properties of self-renewal and multipotential differentiation. Compared to bone marrow-derived stem cells (BMSCs), ASCs can be derived from more sources and are harvested more easily. Three-dimensional (3D) tissue engineering scaffolds are better able to mimic the *in vivo* cellular microenvironment, which benefits the localization, attachment, proliferation, and differentiation of ASCs. Therefore, tissue-engineered ASCs are recognized as an attractive substitute for tissue and organ transplantation. In this paper, we review the characteristics of ASCs, as well as the biomaterials and tissue engineering methods used to proliferate and differentiate ASCs in a 3D environment. Clinical applications of tissue-engineered ASCs are also discussed to reveal the potential and feasibility of using tissue-engineered ASCs in regenerative medicine.

1. Introduction

Millions of people worldwide suffer from diseases, and the majority could be helped or cured through tissue or organ transplantation. However, deficiencies in tissues and organs are a huge challenge for medicine [1] that has resulted in the emergence of regenerative medicine, which is an interdisciplinary field involving biology, medicine, and engineering [2]. Regenerative medicine aims to repair, replace, maintain, or enhance tissue and organ functions and offers therapeutic solutions for many diseases [2, 3]. In recent years, the rapid development of biology, biomaterials, and tissue engineering has promoted the development of regenerative medicine. The traditional ways of culturing cells in a two-dimensional (2D) environment fail to allow interactions between cells and the extracellular matrix (ECM) [4]. As a result, three-dimensional (3D) biomaterial scaffolds combined with reliable sources of stem cells and biomolecules have become popular [5].

Adipose-derived stem cells (ASCs) are a mesenchymal stem cell source with self-renewal property and multipotential differentiation. ASCs can become adipocytes [6], osteoblasts [7], chondrocytes [8], myocytes [9], neurocytes [10], and other cell types [11]. ASCs also have the potential to treat various diseases, such as graft-versus-host disease [12], autoimmune-induced diseases [13, 14], multiple sclerosis [15], diabetes mellitus [16], and tracheomediastinal fistulas [17]. Compared to other types of stem cells, ASCs have two main advantages. On the one hand, ASCs can be easily accessible from subcutaneous liposuction in large numbers [18]. On the other hand, ASCs have no ethical and political issues compared to embryonic stem cells because they can be derived from autologous fat [19]. These two characteristics make ASCs become a more acceptable solution for tissue and organ transplantation in regenerative medicine and clinical studies [20, 21].

ASCs have been traditionally cultured in conventional 2D condition, which are inappropriate to mimic cell-cell and cell-environment interactions *in vivo* [22, 23]. Tissue-engineered

3D scaffolds have tremendous capacity to closely mimic *in vivo* cellular environments [24, 25]. These 3D scaffolds are generated using biofabrication methods by combining biomaterials, molecular growth factors, and extracellular matrices together to provide a 3D microenvironment for cell proliferation and differentiation, which further regulates the growth of tissues or organs [26]. In 3D scaffolds, the differentiation lineage of ASCs can be controlled by the mechanical, chemical, and other cues from microenvironment [27]. In addition to controlling differentiation, 3D scaffolds can also enhance the cell viability during proliferation [28]. Considering the benefits above, more and more attention has been paid to study ASCs within 3D scaffolds *in vitro*.

The most essential components for preparing and regulating 3D scaffolds are biomaterials and fabrication methods. Till now, many biomaterials have been utilized to grow ASCs in 3D scaffolds. ASCs localize, attach, and proliferate during *in vitro* 3D encapsulation. The ideal biofabricated scaffolds offer ASCs proper environments to facilitate their proliferation and maintain their differentiation potentials. Many key attributes of biomaterials must be considered as it closely mimics *in vivo* 3D environments: first, biomaterials should be biocompatible and do not cause a long-term immune reaction [29]; second, the biomaterials are desired to have highly porous structures with interconnected architecture to imitate the native tissue niche [30]; third, the biomaterials should have adjustable mechanical properties to regulate the cellular microenvironment. Maintaining biochemical, biomechanical, and biological properties during proliferation is also important to withstand the external environment impact [29]. With the development of biomaterials and biofabrication, many methodologies have been employed to fabricate 3D scaffolds for cell culturing, including bioprinting [31], patterning [32], self-assembling [31], and organ-on-a-chip [33]. Most of listed methodologies have been utilized to encapsulate the ASCs inside the scaffolds with the desired structure, which stimulates the differentiation of ASCs into a specific cell type for clinical application.

Current studies and clinical trails indicate that ASCs in 3D scaffolds can be a potential alternative for wound healing [34], cardiovascular grafts [35], orthopedic tissue repair [36], and plastic tissue reconstruction after surgery [37]. The success of aforementioned applications proves the great potential of ASCs to be served as a cell-based therapy for regenerative medicine. Although tissue-engineered ASCs are recognized as an attractive substitute for regenerative medicine, there are remaining problems to be solved, including the mechanisms of the interactions among ASCs, the serum-free culturing methodology, and the long-term safety. Therefore, many studies have focused on basic and animal experiments and a few clinical trials have been performed.

In this review, we discuss the characteristics of ASCs and the biomaterials and tissue engineering methods applied to regulate ASCs in 3D scaffolds. In Section 2, we discuss the characteristics of ASCs, including their background and methods to harvest and isolate ASCs. In Section 3, the biomaterials and biofabrication methods used for ASCs are discussed. In Section 4, we report current clinical cases using tissue-engineered ASCs as therapies. Finally, a brief prospective of ASCs in tissue engineering is introduced, and a short conclusion is presented.

2. Characteristics of ASCs

2.1. Background of ASCs. In general, stem cells can be divided into four categories based on their origin: embryonic [38], fetal [39], adult stem cells, and induced pluripotent stem cells (iPSCs) [40]. The characteristics of different stem cells are summarized in Table 1. Human embryonic stem cells (ESCs) are a type of stem cells derived from the inner cell mass of developing blastocysts [38] and widely used in tissue engineering and regenerative medicine because of their high capacity for differentiating. ESCs are pluripotent, can be grown into adult postnatal cells, and have a greater potential for regenerative medicine compared to adult stem cells. However, ethical problems and insufficient sources limit the applications of ESCs for clinical use [38]. Human fetal stem cells, such as amniotic fluid stem cells and umbilical stem cells, are a type of stem cells originated antenatal fetal tissue as well as postnatal fetal appendixes [39]. They are broadly multipotent and have less ethical issues compared to ESCs [41]. However, the limited sources of human fetal stem cells still restricted their applications. Induced pluripotent stem cells (iPSCs), since being discovered by Takahashi et al. in 2007 [42], have made a breakthrough in regenerative medicine. iPSCs have no ethical issues and are rich in sources. However, target cells are difficult to induce through current methodologies and technologies. Therefore, because of high availability of sources, easy accessibility, and relatively low ethical issues, the adult stem cells become an attractive and promising solution for current research and medical use of regenerative medicine.

ASCs are a mesenchymal stem cell source that can easily be isolated from adipose tissue. Similar to other stem cells, ASCs can self-renew and differentiate into other cell types in the body. They were first described by Zuk et al. in 2001 as a population of cells derived from human adipose tissue with the capability of multilineage differentiation [43]. This study opened a new window for regenerative medicine using adipose tissues. Adipose tissue belongs to the mesodermal layer in embryonic period [44] and is comprised of adipocytes and a stromal vascular fraction (SVF), which is a set of heterogeneous cells, including preadipocytes, fibroblasts, vascular smooth muscle cells, endothelial cells, macrophages, lymphocytes, and ASCs [45, 46]. Differentiation of ASCs was initially considered to be limited to mesodermal tissue only. However, recent studies have extended the use of ASCs to ectodermal and mesodermal tissues and organs [21]. More recent studies have revealed that ASCs have a variety of differentiation pathways, including adipogenesis, osteogenesis, chondrogenesis, and other lineages [11].

Bone marrow-derived stem cells (BMSCs) are the most commonly used adult stem cells. However, ASCs have many advantages over BMSCs. Firstly, the extraction procedure of BMSCs is painful, and the yield rate of cells is quite low [11], while ASCs have abundant sources that are localized in subcutaneous adipose tissue throughout the body. Besides, it is easy to obtain ASCs using the minimally invasive

Table 1: Characteristics of different stem cells.

Category	Origin	Advantages	Disadvantages	References
Human embryonic stems cells (hESCs)	Human blastocysts	Pluripotent; nonimmunogenic	Insufficient sources; ethical and religious debates	[38]
Human fetal stem cells (hFSCs)	Fetal tissue (i.e., fetal blood from umbilical cord and amniotic fluid) Fetal appendixes (i.e., fetal membranes and placenta)	Broadly multipotent; nonimmunogenic Less ethical and religious debates	Insufficient sources	[39, 41]
Adult stem cells	Adult tissues, such as bone marrow, adipose tissue, and skin	Multipotent; little ethical and religious debates; abundant sources; easily accessible	Relatively difficult to expand *in vitro*; limited use in clinical practice	[18, 19]
Induced pluripotent stem cells (iPSCs)	Somatic cells	Pluripotent like ESCs; no ethical and religious debates; abundant sources	Difficult to induce; abundant sources	[40, 42]

liposuction [47], and the percentage of obtained cells is relatively higher than other stem cell sources [47]. In addition, ASCs can be transplanted to autologous or allogeneic body safely with less implant migration and foreign body reaction [29]. Thus, ASCs become the most attractive stem cell source for tissue engineering and regenerative medicine.

2.2. Harvesting of ASCs. ASCs are harvested from human subcutaneous adipose tissue. Current techniques to harvest ASCs include Coleman's technique [48], liposuction [49, 50], and direct excision [48, 49]. Among them, the most popular technique is Coleman's technique. Liposuction includes conventional liposuction (tumescent) and ultrasound-assisted liposuction [49]. Iyyanki et al. showed that the yield of ASCs harvested from the abdomen through direct excision or Coleman's technique with centrifugation was higher than that from liposuction and Coleman's technique without centrifugation [48]. They also indicated that adipose tissue harvested from the abdomen contained a larger number of SVF cells than that harvested from the flank or axilla. However, Schreml et al. reported no significant difference in the number of ASCs or adipogenic differentiation potential between direct resection and liposuction [50]. In contrast, the percentage of viable cells was dramatically higher through liposuction than through direct resection [50].

Adipose tissue is widely distributed in the human body and the location influences the stem cell yield [48]. In practice, adipose tissue is usually harvested from the abdomen or the hip/thigh region [51]. Patient age is another important factor influencing ASC yield. Wu et al. showed that all age groups have similar ASCs and osteogenic paracrine activities [52]. In contrast, ASCs from infants have higher angiogenic and osteogenic capabilities than those from adults and elderly people. Another study supported this idea that proliferative activity, colony-differential potential, and population doubling are significantly different in ASCs harvested from young patients (>20 years old) and from older patients (50–70 years old) [53]. Due to the advancement of technologies, ASCs can now be acquired in large quantities using minimally invasive techniques. However, the best harvesting method to yield the largest numbers of ASCs with optimal biological function remains unclear.

2.3. Isolation of ASCs. Adipose tissue contains various cell types. The most widely utilized method to isolate ASCs from other cells relies on collagenase digestion, followed by centrifugation. A recent study suggested that incubating adipose tissue with 0.25% trypsin for 60 min is more cost-effective and efficient than tissue digestion with collagenase [54]. In that study, nine protocols, including collagenase, red blood cell lysis buffer solution, various trypsin concentrations, and centrifugation, were compared based on isolation rate, cell viability, expansion rate, immunophenotype, and the differentiation into adipogenic and osteogenic lineages. The results showed that trypsin-digested ASCs had similar proliferation capacity to those treated with collagenase and better osteogenic differentiation result. Thus, trypsin-based protocol is attractive for isolating ASCs considering cost and yield.

All of aforementioned methods separate primary adipocytes floating at the top from SVF accumulated at the bottom after centrifugation. SVF is a heterogeneous cell population comprised of various cells and ASCs [18]. ASCs are distinguished from other cells by morphology and immunophenotype. ASCs have fibroblast-like morphology and lack lipid droplets in the cytoplasm [29]. In addition, ASCs strongly express CD13, CD29, CD49d, CD73, CD90, CD133, MHC I, and MHC II, but they do not express CD106, which is commonly expressed on BMSCs [11].

Isolating ASCs is an important step for tissue engineering applications. Therefore, a cost-effective isolation method is essential for further applications. Comparing different protocols and determining the best one can contribute significantly to the development of adipose tissue engineering.

2.4. ASC's Culture and Preservation. Isolated ASCs are commonly proliferated in conventional culturing condition before 3D encapsulation. A typical culturing condition is Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 1% antibiotics at 37°C and 5% CO_2 in monolayer dishes [55]. However, when exposed to human body, animal-derived FBS may cause the risk of graft rejection or infection. Therefore, for clinical application, xeno-free culture media without animal-derived reagents should be developed. Lindroos et al. adopted serum-free and xeno-free media (using allogeneic human serum as a replacement) to

culture ASCs and these media maintained the proliferation and differentiation of ASCs [56]. Platelet-rich plasma (PRP) has been proposed as a promising alternative for ASCs culture [57]. Atashi et al. studied the efficiency of autologous nonactivated PRP (nPRP) or thrombin-activated PRP (tPRP) (1–60%) on ASC proliferation compared to conventional 10% FBS. The results indicated that nPRP showed dose-dependent performance and its influence on ASCs was higher than FBS or tPRP without changing cell properties [57]. The results were consistent with Liao et al. [58] who proved that PRP could improve ASC proliferation while inhibiting adipogenic differentiation of ASCs in adipogenic media. Combining with biofabricated 3D scaffolds, serum-free and xeno-free media have great potential to provide the less risky and cytoactive condition for the off-the-shelf therapies.

Preservation of ASCs is also essential for tissue engineering and regenerative medicine. ASCs can be preserved in the conventional cryopreservation media including 90% FBS and 10% dimethyl sulfoxide (DMSO) [59]. For the off-the-shelf therapies in clinical practice, preservation methods are required to store large quantity of ASCs and maintain their properties in the long term. Recently, De Rosa et al. employed low DMSO cryopreservation methods to reduce toxicity of DMSO in room temperature. In this study, threalose was used as a replacement of DMSO. A solution combing 4% DMSO, 6% threalose, and 90% FBS resulted in maintaining the stemness and differentiation property of ASCs [59]. Miyamoto et al. also found that ASCs kept in CELLBANKER 2 and DMEM/Ham's F-12 medium with 10% DMSO, 0.1 mol/L maltose, and 1% sericin performed better in terms of proliferation and differentiation capability comparing with standard protocols [60].

3. ASC's Growth and Differentiation on Biofabricated 3D Scaffolds

Biomaterials have been proved to affect the proliferation and differentiation of stem cells (SCs) by controlling chemical compositions and physical properties (e.g., mechanical properties and microstructural patterns) [66]. On the one hand, the chemical composition significantly affects the differentiation potential. For instance, collagen, which is abundant *in vivo* environments, can interact with SCs via integrin binding, while alginate, which is a seaweed-derived anionic polysaccharide and does not exist in native ECMs, cannot directly interact with stem cells [67]. Therefore, collagen can support more differentiation lineages of SCs compared to alginate. SCs can be differentiated to many different lineages (e.g., skin, bone, cartilage, tendon, ligament, lung, and nerve), when encapsulated in collagen [68]. On the other hand, physical properties also play an essential role in regulating SC's differentiation. For example, when encapsulated in polycaprolactone (PCL), SCs tended to differentiate to mesenchymal lineages (e.g., bone, tendon, and cartilage) since the stiffness of PCL mimicked *in vivo* mechanical property of mesenchymal tissues [67]. In addition, it has shown that the precise control of scaffold microstructural patterns using nanobiotechnology can affect the differentiation of SCs [69]. SCs could differentiate to osteoblast rather than adipocyte when the size of pattern seeding SCs was increased. Taken together, chemical and physical effects of the SC microenvironments created by biomaterials can significantly affect SC differentiation.

3.1. Tissue-Engineered 3D Scaffolds for ASC Culture. Fabricating proper scaffolds that facilitate ASC proliferation is important for the tissue engineering applications. The characteristics of scaffolds can be adjusted by encapsulating nanostructures, using different types of biomaterials, or modifying the mechanical or electrical properties of biomaterials through coating.

Silica nanoparticles (NPs) have been used to increase proliferation of ASCs by activating extracellular signal-related kinase (ERK) 1/2 [70]. The ERK 1/2 signaling pathway is a mitogen-activated protein kinase (MAPK) pathway related to biopolymer-induced proliferation of stem cells. ASCs were seeded into a 96-well plate with DMEM and 1% FBS containing silica NPs and silica microparticles (MPs). It was reported that the ASC proliferation rate increased significantly in the presence of the silica NPs, while no obvious change in proliferation was observed with silica MPs, indicating that silica composites promoted proliferation of ASCs and that the MAPK pathway is a possible mechanism regulating proliferation. Another study showed that the proliferation rate of ASCs cultured in media containing 2 or 4 μM silicon was significantly greater than that in control medium. The improved mechanical strength of the medium with silicon may contribute to this result [71]. Therefore, scaffolds containing NPs for tissue engineering may enhance ASC growth and single component silica-derived NPs could be useful for the scaffolds in stem cell therapy [70].

Natural polymers also benefit the proliferation of ASCs. Hyaluronic acid (HA) is an effective natural biomaterial to increase ASC proliferation. HA scaffolds formed porous structure, which was commonly regarded as a preferable culturing model for cell proliferation. Besides, ASCs tend to form small dispersed aggregates in HA scaffolds, and the small-sized cell aggregates facilitated metabolic exchange. The above factors may account for the enhanced proliferation of ASCs [72]. Other nature-derived scaffolds, such as collagen-HA scaffold [73], and type I collagen scaffold [74], have also been reported to facilitate the proliferation of ASCs.

Highly conductive scaffold could also upregulate the proliferation of ASCs in synthetic polymers. The proliferation of ASCs in polypyrrole-coated polylactide (PLA-PPy) and PLA scaffolds has been investigated [61]. PPy was an electrically conductive material, while PLA was electrically insulated. In this study, PLA was coated with PPy to regulate its conductivity properties under different electrical stimulation. Figure 1(a) presents the scanning electron microscope images of the microstructures of PLA and PPy-coated PLA. Electrical stimulation may serve as a potential factor to stimulate ASCs proliferation as well as differentiation like most molecular factors do. Figure 1(b) shows the representative fluorescent images of the ASCs cultured in both scaffolds after 14 days. It is clear that the number of ASCs and the attachment rate of ASCs in PLA-PPy scaffold are significantly higher

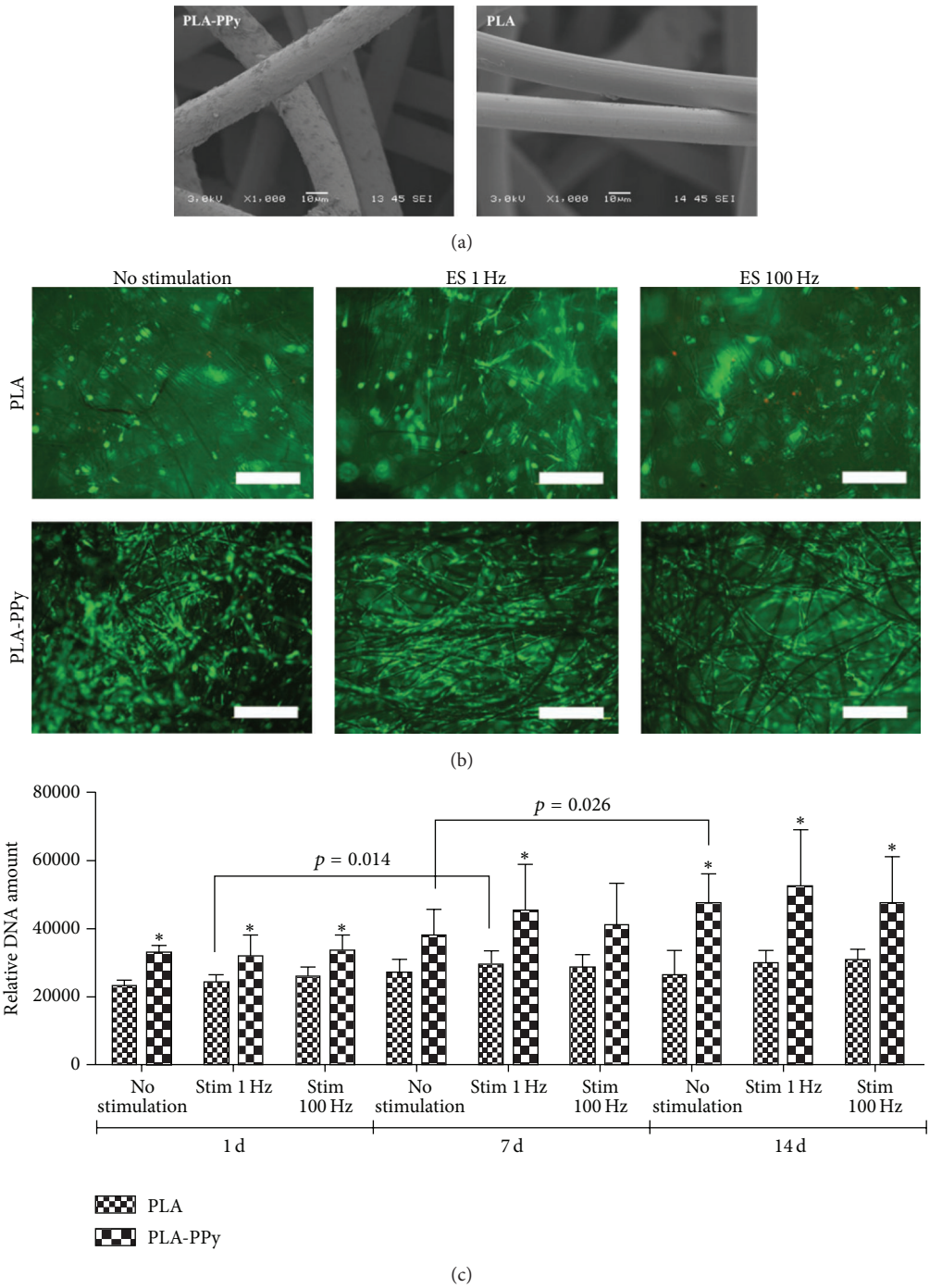


Figure 1: PPy-coated PLA scaffolds are able to enhance ASC proliferation through electrical stimulation. (a) Scanning electron microscopy image of PLA-PPy scaffold (left) without sputtered gold layer and PLA scaffold (right) with 20 nm gold coating. (PLA scaffold could not be imaged without coating due to the heavy electrostatic.) (b) Representative images of the viable (green) and dead (red) ASCs seeded in PLA and PLA-PPy scaffolds at day 14 (scale bar: 500 μm). (c) Relative DNA content of ASCs cultured for 1, 7, and 14 days in PLA and PLA-PPy scaffolds (in the form of mean ± standard deviation, $n = 3$, $^*p < 0.05$) (adopted from [61]).

than PLA scaffold, revealing that the proliferation rate of ASCs was higher in PLA-PPy scaffolds (no stimulation, 1 Hz stimulation, or 100 Hz stimulation) than that in PLA scaffolds. The DNA content analysis results are also consistent with the fluorescent images. The amount of DNA increased significantly when using PLA-PPy scaffolds (Figure 1(c)). Thus, it can be concluded that conductive scaffold benefits the proliferation of ASCs. However, judging from Figures 1(b) and 1(c), the proliferation rate under different electrical stimulation did not differ significantly. This may be due to

the fact that PPy is an electronic conductor both in air and in medium and can regulate suitable stimulation current to cells regardless of external electrically stimulation [61]. However, further study is required to quantitatively analyze the effects of electrical stimulation.

Many other biomaterials have been employed for culturing ASCs in 3D environments, such as chitosan [75], silk [76], alginate [77, 78], and natural and synthetic calcium phosphate [7]. Various scaffolds with different tissue properties including geometry, porosity, stiffness, surface characteristics, and composition have been tested. External stimulus is another factor to influence ASC proliferation. Jeong et al. found that low-dose ultraviolet B (UVB) radiation did not affect ASC proliferation, while high-dose UVB reduced the proliferation of ASCs [79]. In addition, many researches showed that magnetism could enhance ASC differentiation, while its ability to enhance ASC proliferation was not confirmed [80, 81].

3.2. Differentiation of ASCs in 3D Scaffolds. ASCs have great differentiation potential. In this section, different tissue engineering methods are presented for inducing various types of differentiation.

3.2.1. Adipogenesis. Adipogenesis is the original ASC differentiation pathway. ASCs are capable of differentiating into adipocytes on particular scaffolds when combined with appropriate cues. Both alginate and alginate/gelatin microspheres seeded with ASCs facilitate adipogenesis, whereas an alginate/gelatin matrix supports the adipogenesis pathway better than that of alginate alone [78]. PDM combined XLHA scaffolds also benefit adipogenesis, and mature adipocytes occur more frequently in the central region of these scaffolds [82]. ASCs seeded in micromolded resections of agarose hydrogel form tissue spheroids after 3 weeks of culture, and the cells were multipotent for the adipogenic lineage [83]. ASCs cocultured with human umbilical vein endothelial cells (HUVEC) in collagen/alginate microspheres could be used as a biomimetic physiological model. Abundant lipid accumulation and morphological changes representing the adipogenic differentiation were detected after 17 days of coculturing [84].

Bioprinting methods also help to maintain the differentiation potential of ASCs. ASCs have been encapsulated inside a 3D scaffold using laser-assisted bioprinting [62] (Figure 2(a)). In this study, alginate was employed as the scaffolding materials. Alginate is a natural hydrogel and has been widely used for tissue engineering applications. It has been also reported that ASCs could be differentiated to other cell types in alginate scaffolds [77, 78, 85]. During printing, a laser pulse was applied to the energy absorbing layer (red layer), generating a high-pressure bubble and propelled the suspended cells below that area. The small falling unit formed a droplet that was collected in the bottom substrate, and the prepolymers were cross-linked with a cross-linking agent. ASCs encapsulated scaffolds and ASCs cells in 2D were cultured in 24-well plates for 21 days in DMEM containing 10% FBS, 1 μM dexamethasone, 10 μg mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 0.2 mM indomethacin. The expression of adipogenic markers, including lipase (LPL), adipocyte fatty acid-binding protein (aP2), and peroxisome proliferator-activated receptor (PPAR-γ2) as well as lipid accumulation in the cytoplasm, was evaluated at days 3, 7, and 21 to investigate the degree of cell differentiation. Gene expression was measured by the real-time reverse transcriptase-polymerase chain reaction (RT-PCR). As shown in Figure 2(b), LPL, aP2, and PPAR-γ2 expression levels in the printed and nonprinted groups were similar after 3, 7, and 21 days. However, the expression levels of LPL, aP2, and PPAR-γ2 were promoted compared to those in unstimulated ASCs, indicating that bioprinted 3D scaffold may upregulate the differentiation of ASCs. The Oil Red-O staining result indicated similar lipid accumulation in the cytoplasm of the bioprinted and nonprinted groups (Figure 2(c)). The quantitative assessment of lipid accumulation using radiation absorbance also revealed that lipid quantities were equal in the two groups after 7 and 21 days. This study verified that laser-assisted bioprinting has no significant negative effect on ASC proliferation and differentiation. Therefore, laser-assisted bioprinting is a promising manufacturing method for ASCs encapsulated 3D scaffolds for tissue engineering applications.

3.2.2. Osteogenesis. ASCs can differentiate into osteocytes as well. It has been found that PLA-PPy scaffolds can provide higher alkaline phosphatase (ALP) activity levels, which benefit the early osteogenic differentiation of ASCs [61]. In another study, a silicon dioxide gel was fabricated to serve as the medium for ASC culturing. Expression of the osteogenic genes osteocalcin and osteopontin reached the highest in medium containing 2 μM silicon ions. The Alizarin Red-S staining of ACSs indicated that, with 2 μM silicon ions in the medium, there are maximum number of differentiated cells [71]. Mihaila et al. reported that combining ASCs with bioactive silicate nanoplatelets (sNPs) promotes osteogenic differentiation [63]. In their study, SSEA-4$^+$ ASCs, which is a subset of ASCs with higher differentiation potentials, was utilized. As shown in Figure 3(a), SSEA-4$^+$ ASCs were harvested from human subcutaneous abdominal tissue and isolated and selected from SVF. SSEA-4$^+$ ASCs were cultured in basal medium for 1 day and then sNPs were added and incubated in either basal or osteogenic media. Samples were collected on days 7, 14, 21, and 28 to analyze expressed ALP, which appeared in early osteogenic differentiation and considered as the marker of osteogenic differentiation. The qualitative level of ALP was assessed by staining the fixed samples with nitro-blue tetrazolium/indolyl phosphate. The quantitative level of ALP was determined by an adapted end-point colorimetric procedure based on the p-nitrophenol assay. As shown in Figure 3(b), the dark purple color became more intense with the increasing of NPs concentrations and peaked on day 14. The osteo medium presented a darker purple color than that of the basal medium, which was consistent with the quantitative results shown in Figures 3(c) and 3(d). Taken together, these results indicate that the sNPs significantly promoted ALP activity in SSEA-4$^+$ ASCs ($p < 0.05$), compared to the ASCs cultured in the same condition and upregulated osteogenic differentiation in both basal and osteogenic media.

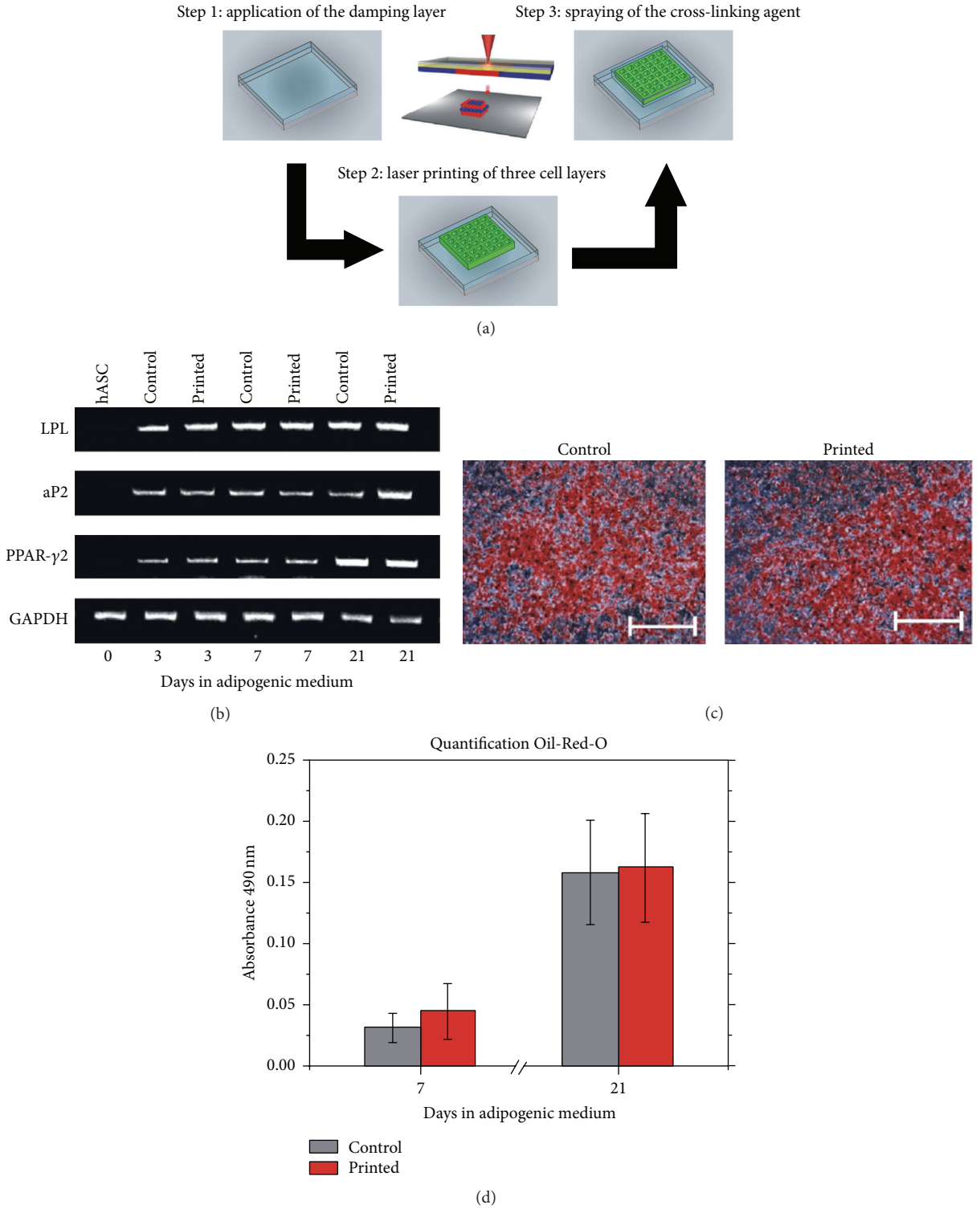
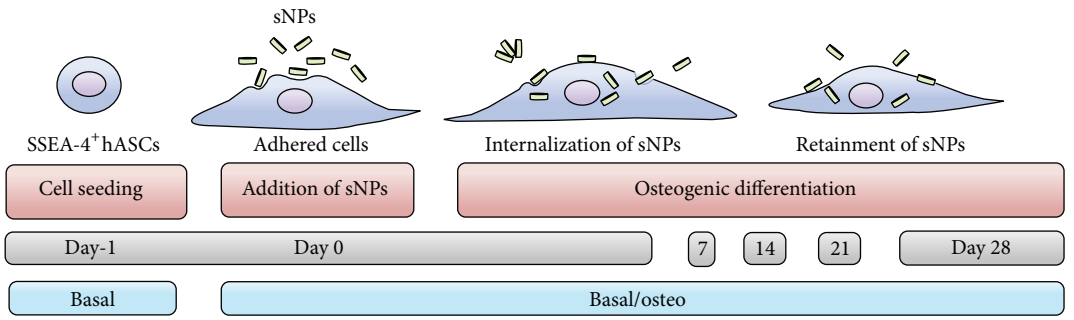
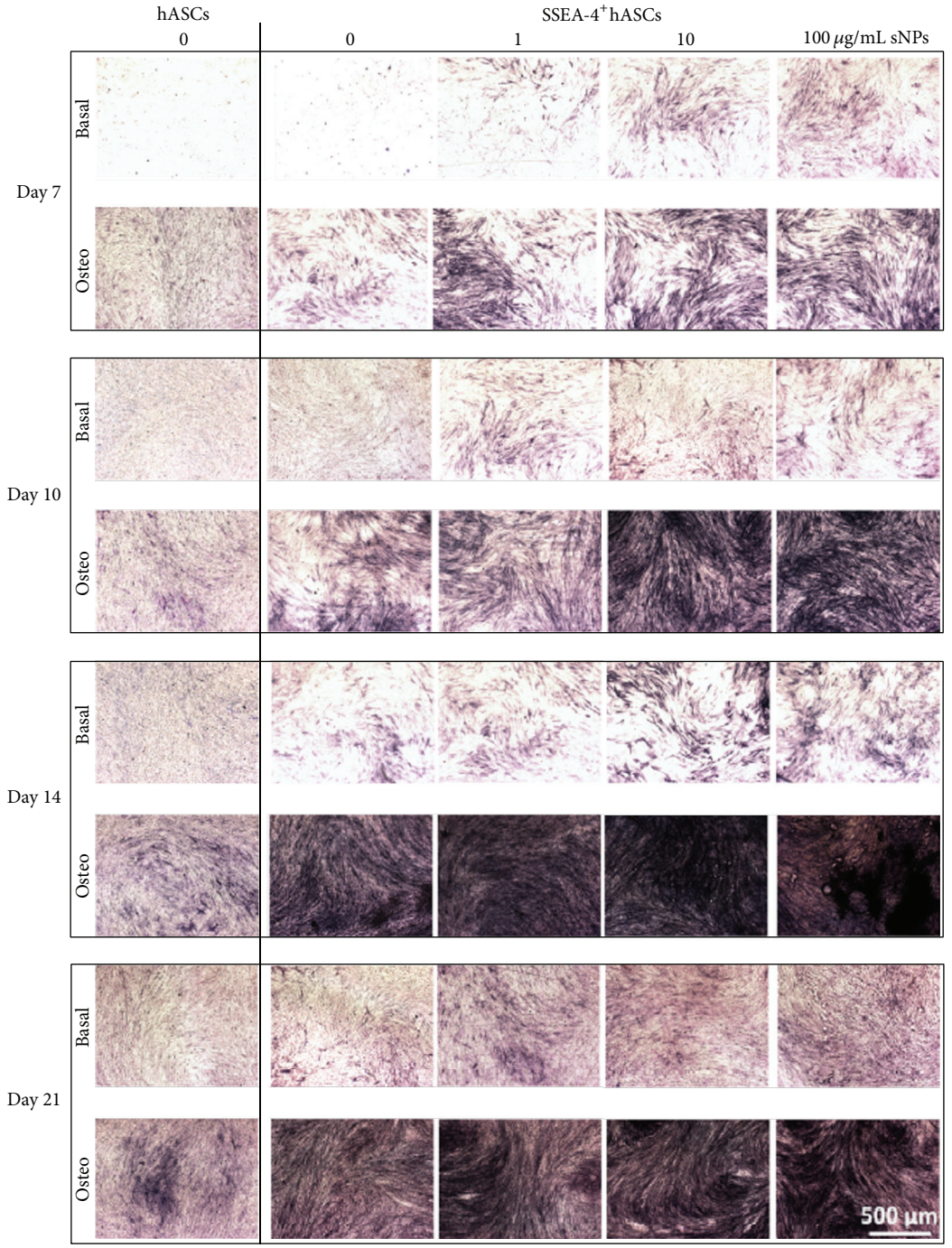


Figure 2: Laser-assisted bioprinting helps in the proliferation and differentiation of ASCs. (a) Three-dimensional scaffolds were fabricated via laser-assisted bioprinting. During printing, the laser pulse was applied to melt a certain area in the energy absorbing layer (red layer). The suspended biomaterial prepolymers below that area fell due to the loss of support from the absorbing layer. The small units formed droplets that were collected by the bottom substrate. The prepolymers were cross-linked using a cross-linking agent. (b) Lipase (LPL), adipocyte fatty acid-binding protein (aP2), and peroxisome proliferator-activated receptor (PPAR-γ2) expression were compared on printed and nonprinted control ASCs compared to unstimulated ASCs after 3, 7, and 21 days of incubation. (c) Oil Red-O staining indicated similar lipid accumulation in the cytoplasm of the printed and nonprinted groups (scale bar: 500 μm). (d) Equal lipid quantities were detected in both groups ($\alpha = 0.05$) (adopted from [62]).



(a)



(b)

Figure 3: Continued.

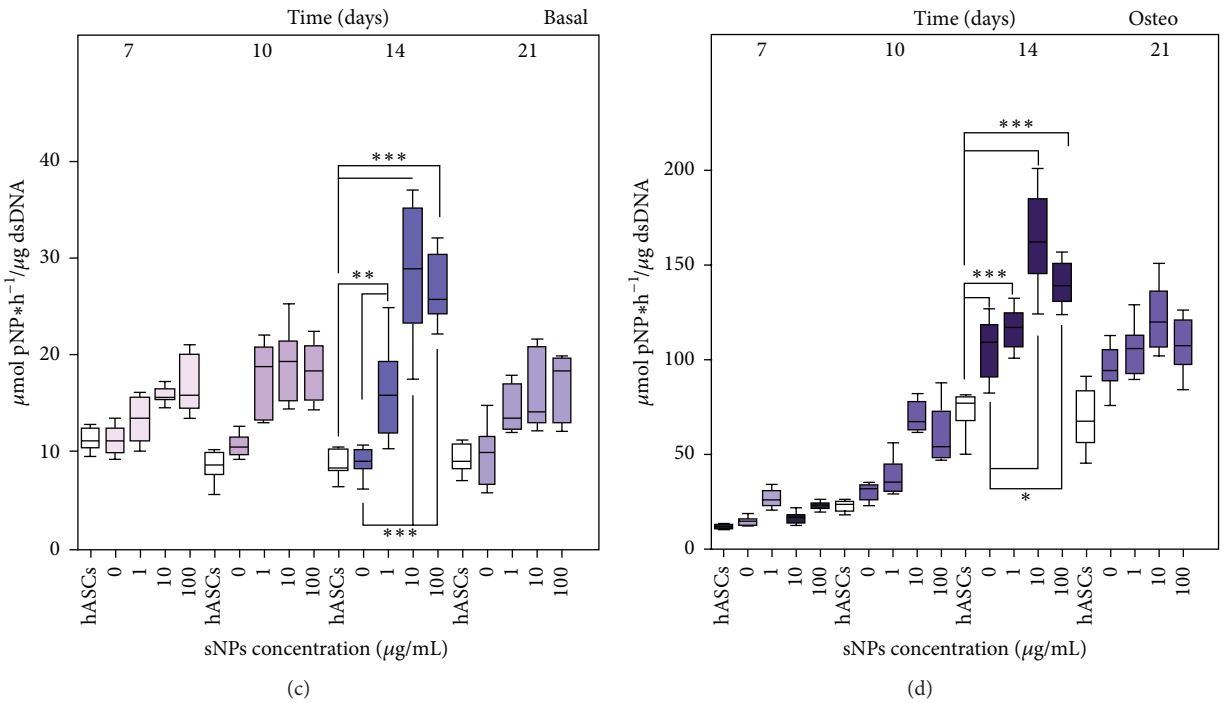


Figure 3: Culturing ASCs with bioactive silicate nanoplatelets promotes the osteogenic differentiation of ASCs. (a) Experimental procedures used to induce osteogenic differentiation. SSEA-4$^+$ adipose-derived stem cells (ASCs). SSEA-4$^+$ ASCs were seeded in basal medium for 1 day, and silicate nanoplatelets (sNPs) were added to the basal medium. The samples were incubated either in basal medium or under osteogenic differentiating conditions. Samples were collected on days 7, 14, 21, and 28 to analyze differentiation. (b) Qualitative analysis of alkaline phosphatase (ALP) in SSEA-4$^+$ ASCs on days 7, 10, 14, and 21 cultured in basal and osteo media, compared to ASCs. The dark purple color increased with sNP concentration level and osteo medium presented a darker purple color. (c) Quantitative analysis of ALP in SSEA-4$^+$ ASCs in basal medium with sNPs. The presence of sNPs enhanced ALP activity on day 14, compared to that in ASCs. (d) Quantitative analysis of ALP in SSEA-4$^+$ASCs in osteo medium with sNPs. The presence of sNPs enhanced ALP activity on day 14, compared to that in ASCs (adopted from [63]). $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Many other studies also revealed that the method regulates osteogenesis of ASCs. External stimulation as in the form of magnetic actuation was reported to influence osteogenic differentiation of ASCs [80]. In the study, Lima et al. combined magnetic nanoparticles with ASCs and cultured cells in osteogenic medium up to 28 days with or without external magnetic stimulation. The results in days 14 and 21 revealed that the alizarin red staining against calcium deposits was more intense and widespread. In addition, polyglycolic acid (PGA) mesh scaffolds could induce osteogenesis but pretreatment with osteo-induction factors did not further increase osteogenesis [86]. The mechanical properties of hydrogels could also affect osteogenesis process. ASCs had been cultured and differentiated in poly- (ethylene glycol-) diacrylate (PEGDA) of different molecular weights and concentrations. Mineralization and osteocalcin gene expression were examined as indicators of osteogenesis. The results showed that osteogenesis of ASCs increased with matrix stiffness, indicating that a stiff matrix mimicking the native microenvironment of bone is beneficial for osteogenesis [87].

3.2.3. Chondrogenesis. Chondrogenesis is another common pathway of multipotent mesenchymal cells. HA scaffolds also have the capacity to induce chondrogenesis in ASCs [72]. In this study, researchers prepared poly ethylene glycol diglycidyl ether- (PEGDG-) cross-linked porous 3D HA scaffolds and investigated their feasibility for differentiating ASCs into chondrocytes using cell sulfated glycosaminoglycan content. The results showed that chondrogenic differentiation of ASCs in the scaffolds was higher than that in micromass culture. In another study, ASCs were cultured in alginate microspheres, and upregulation of cartilage specific genes, including transforming growth factor-β, collagen type-X, and cartilage oligomeric matrix protein, was observed [77]. In another study, plasmid DNA (pDNA) containing SOX trio genes was incorporated into PLGA scaffolds with ASCs [88]. Increases in COL2A1 gene expression and protein were seen in SOX trio pDNA-incorporated scaffolds compared to that in the control group, indicating the upregulated effects of pDNA to chondrogenesis. Electromagnetic field also promotes the chondrogenic differentiation of ASCs [81]. Chen et al. found that electromagnetic field could improve chondrogenic differentiation while not affecting cell viability.

3.2.4. Other Lineages. ASCs can follow other differentiation pathways in biofabricated scaffolds mimicking specific *in vivo* environment. For example, ASCs can become smooth muscle cells and endothelial cells [35]. In this study, a customized

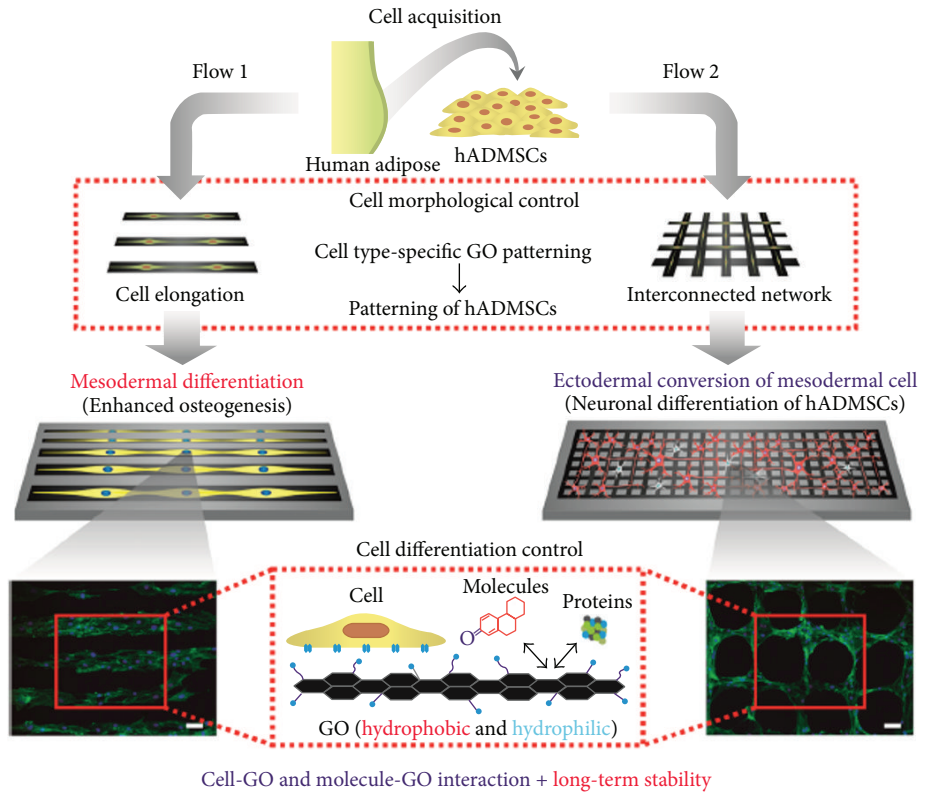


Figure 4: Graphene pattern controlled ASCs differentiation. The schematic illustrates the steps taken to modulate the morphology of ASCs using graphene oxide patterns, which further guide the differentiation of ASCs. Fluorescent images are F-actin stained ASCs. (Scale bar = 100 μm.) Flow 1: the line pattern enhances the osteogenesis of ASCs. Flow 2: the mesh pattern enhances the neurogenesis of ASCs (adopted from [64]).

electrospun scaffolds using electrospun nano to microscale collagenous and elastic fibers were created to mimic the natural cardiovascular environment and induce ASC differentiation. After two weeks' culturing, ASCs migrated into the scaffolds, interconnected with the surrounding environment, and developed into endothelial cells and smooth muscle cells under different culture environments. Desiderio et al. showed that ASCs can also form a human skeletal muscle tissue *in vitro* through the culture with cross-linked hyaluronic acid scaffold [89]. Gao et al. revealed that using ASCs could obtain neuron-like cells through seeding ASCs in photocurable 3D chitosan and gelatin scaffolds [90]. ASCs are capable of becoming cardiomyocytes for cardiovascular tissue engineering [91]. In detail, two engineered scaffolds, aligned polycaprolactone (PCL) nanofibrous electrospun scaffolds and random PCL nanofibrous scaffolds, were used to proliferate and differentiate ASCs to cardiomyocytes. The results revealed that the aligned PCL nanofibrous scaffolds were more appropriate to induce differentiation of cardiomyocytes since it guided the growth direction of ASCs. A recent study from Kim et al. further revealed the potential to use nanostructure for manipulating and guiding ASC differentiation [64]. In this study, graphene oxide (GO) was precisely patterned to control cell morphology, which significantly affected ASC differentiation. The result shows that the line pattern of GO, which closely mimics the environment of osteoblasts, enhanced the osteogenesis of ASCs (Figure 4, Flow 1). In addition, the mesh pattern of GO upregulated neuronal differentiation of ASCs (Figure 4, Flow 2). This study demonstrated that a combinatorial method combining nanomaterials and biofabrication could accurately and effectively control the differentiation of ASCs.

We summarized representative biofabrication and tissue engineering methods employed to ASCs in Table 2. In summary, the differentiation capacity and the fate of ASCs is closely related to the characteristics of the microenvironment, including the mechanical properties of scaffolds, the existence of additional inducing factors, and the alignment of the microstructures.

3.3. Possible Mechanism to Regulate ASC's Proliferation and Differentiation. The proliferation and multiple lineage differentiation capabilities of ASCs are elaborated in many previous articles. Despite their promising application in tissue engineering and regenerative medicine, the mechanisms of ASC's behaviors remain unclear. Some possible mechanisms have been proposed to address the proliferation and differentiation properties of ASCs.

3.3.1. Growth Factors. Growth factors have been acknowledged to affect cell transition [110] and stem cell differentiation [111]. Several growth factors were reported to

Table 2: Representative biofabrication and tissue engineering methods for ASCs culturing and differentiation.

Methods	Materials	Applications	References
Porous scaffolds	Hyaluronic acid (HA)	Proliferation and chondrogenesis	[72]
Microfluidics	Alginate/gelatin	3D cell encapsulation and adipogenesis	[78]
Porous scaffolds	Chitosan	Neurogenesis	[90]
Porous scaffolds	Polycaprolactone (PCL) nanofiber	Cardiomyogenesis	[91]
Bioprinting	Alginate	Adipogenesis	[62]
Nanostructure	Laponite silicate nanoplatelets	Osteogenesis	[63]
Porous scaffolds	Chitosan film	Hepatogenesis	[75]
Porous scaffolds	Poly-lactide-co-glycolide	Chondrogenesis	[88]
Porous scaffolds	Silk fibroin	Adipogenesis	[76]
Nanostructure	Graphene oxide	Osteogenesis Neurogenesis	[64]

Table 3: The effect of growth factors to ASCs.

Types of growth factors	Function	Reference
FGF-2	Promoting ASCs proliferation, endotheliogenesis, osteogenesis, and chondrogenesis	[92–94]
VEGF	Promoting angiogenesis and osteogenesis	[95]
PDGF-BB	Promoting ASCs proliferation, angiogenesis, and migration toward the tumor-conditioned medium	[96–98]
BMP-2	Promoting osteogenesis and chondrogenesis	[99, 100]
TGF-β1	Promoting ASCs proliferation	[101]

involve ASC proliferation and differentiation. Fibroblast growth factor-2 (FGF-2) can promote the proliferation of ASCs [92]. FGF-2 can also induce endotheliogenesis [92], osteogenesis [93], and chondrogenesis [94] of ASCs. Vascular endothelial growth factor (VEGF) has been used to promote capillary formation and induce osteogenic differentiation [95]. Platelet-derived growth factor- (PDGF-) BB was able to prompt ASCs proliferation [96], angiogenesis [97], and their migration toward the tumor-conditioned medium [98]. Bone morphogenetic protein (BMP-2) was used to enhance osteogenesis [99] and chondrogenesis [100]. It is also reported that transforming growth factor- (TGF-) β1 can promote ASC proliferation [101]. The effect of the growth factors on ASCs is summarized in Table 3.

3.3.2. MicroRNAs. Recently, several studies have focused on investigating the role of microRNAs (miRNAs) in regulating proliferation and differentiation capabilities in ASCs. miRNAs are a species of small noncoding RNAs of 19–23 nucleotides in length [112]; miRNAs are known to negatively regulate gene expression by translational repression or inhibiting protein synthesis of target miRNAs [113]. Several recent studies indicated that miRNAs are involved in maintaining self-renewal, proliferation, and multipotential differentiation of ASCs (Table 4). For example, the involvement of miR-26a in osteogenic differentiation of ASCs was demonstrated by Luzi et al. [102]. The expression level of miR-26a was relatively high in late stages during osteogenic differentiation, suggesting that miR-26a was a positive regulator for the osteogenesis of ASCs. Kim et al. also reported that miR-196a was upregulated during osteogenic differentiation of ASCs [103]. The alteration of miR-138 and miR-21 expression during adipogenesis of ASCs suggested that those two miRNAs played an important role in the adipoblast differentiation [105, 106]. Another study showed that the level of miR-194 targeting Sox5 was decreased during chondrogenic differentiation of ASCs, whereas the upregulation of Sox5 inhibited chondrogenesis [108]. In addition, Chen et al. reported that miR-27b was related to tolerogenic response of ASCs [109]. In this study, they discussed the rat tolerogenic orthotopic liver transplantation (OLT) and rejection OLT models. It was found that miR-27b expression in the ASCs from tolerant recipients was elevated, compared to those of rejecting recipients. Taken together, the investigation of miRNAs' role in regulating ASC's behaviors provides us with information to better understand the self-renewal, proliferation, and differentiation capabilities of ASCs.

Table 4: miRNA expression of ASCs in culturing and differentiation.

Differentiation of ASCs	Involved miRNAs	Reference
Osteogenesis	miR-26a, miR-196a, and miR-138	[102–104]
Adipogenesis	miR-21, miR-138, and miR-27	[105–107]
Chondrogenesis	miR194	[108]
Proliferation	miR-196a	[103]
Tolerogenic response	miR-27b	[109]

3.3.3. Extracellular Signal-Related Kinase (ERK) Signaling Pathway. Activation of extracellular signal-related kinase (ERK) signaling pathway is a well-known mechanism to

Table 5: List of clinical trials using ASCs.

Condition	Number	Phase	Study design	Study type	Reference
Breast reconstruction	2	Phase 2	ASCs enriched fat graft	Interventional	NCT01771913
		Phase 4	Autologous SVFs	Interventional	NCT00616135
Lipoatrophy	1	Phase 1	SVF-enriched fat graft	Interventional	NCT01828723
Fistula	6	Phase 1/Phase 2	Allogenic ASCs	Interventional	NCT01372969; NCT00999115
		Phase 1	Autologous ASCs	Interventional	NCT00992485
		Phase 2	Autologous ASCs	Interventional	NCT01011244
		Phase 3	Autologous ASCs	Interventional	NCT00475410
		Prospective	Autologous ASCs	Observational	NCT01020825
Liver cirrhosis	1		Autologous ASCs	Interventional	NCT01062750
Cardiovascular disease	4	Phase 1	Autologous SVFs	Interventional	NCT00442806; NCT00426868
		Phase 2	Autologous ASCs	Interventional	NCT01449032; NCT01216995
Peripheral vascular diseases and cardiovascular diseases	1	Phase 1/Phase 2	Autologous SVFs	Interventional	NCT01211028
Urinary incontinence	1	Phase 2	Autologous ASCs	Interventional	NCT01799694
Localized adverse reaction to administration of drug	1	Phase 1	Allogenic ASCs	Interventional	NCT01743222
Osteoarthritis	2	Phase 1	Autologous ASCs	Interventional	NCT01585857
		Phase 1/Phase 2	Autologous ASCs	Interventional	NCT01809769
Buerger's disease	1	Phase 1/Phase 2	Autologous ASCs	Interventional	NCT01302015
Brain injury	1	Phase 1/Phase 2	Autologous ASCs	Interventional	NCT01649700
Cerebellar ataxia	1	Phase 1/Phase 2	Allogenic ASCs	Interventional	NCT01649687
Spinal cord injury	2	Phase 1	Autologous ASCs	Interventional	NCT01274975; NCT01624779
Soft tissue mass removal	1		SVFs	Interventional	NCT01399307
Diabetic foot ulcer	1	Phase 1	Allogenic ASCs	Interventional	NCT02394886
Limb ischemia	2	Phase 1/Phase 2	Autologous ASCs	Interventional	NCT01257776; NCT01663376
Overweight	1	Prospective	Bone Grafts using ASCs and different scaffolds	Observational	NCT01218945
Rheumatoid arthritis	1	Phase 1/Phase 2	Allogenic ASCs	Interventional	NCT01663116
Degenerative arthritis	1	Phase 1/Phase 2	Autologous ASCs	Interventional	NCT01300598
Sepsis	1	Phase 1	Allogenic ASCs	Interventional	NCT02328612
Romberg's disease	1	Phase 2	Autologous ASCs	Interventional	NCT01309061
Depressed scar	1	Phase 2/Phase 3	Autologous ASCs	Interventional	NCT00992147
Healthy	1	Phase 1	Allogenic ASCs	Interventional	NCT01739530

ASCs, adipose-derived stem cells; SVFs, stromal vascular fraction.

regulate ASC proliferation and differentiation. ERK belongs to the mitogen-activated protein kinase (MAPK) family which is a conserved family of serine/threonine protein kinases. External stimuli, such as hormones, molecular factors, physical stimuli, and environmental changes, act on target receptors of ASCs and this cell response activates ERK. The activated phosphorylated ERK then initiates a cascade of downstream events related to ASC's biological behaviors, especially differentiation property. The role of ERK signaling pathway in enhancing ASC proliferation capacity has been well discussed in the previous section. Besides, ERK signaling pathway is also known to involve ASC osteogenic differentiation. Liu et al. reported that the blockage phosphorylation level of ERK induced by a specific ERK inhibitor, PD98059, reduced the osteogenic differentiation of ASCs in a dose-dependent manner [114]. In addition, they also revealed that the combination of dexamethasone led to adipogenic differentiation conversely [114]. Kim et al. reported that MAPK pathway also involved TGF-β1 signaling which was believed to induce chondrogenic differentiation of ASCs. In addition, TGF-β1 signaling activated SMAD that was related to increasing chondrogenic activity of ASCs [115]. While ERK signaling pathway may be advantageous to induce the osteogenesis of ASCs, the role of ERK signaling pathway in other lineage differentiation and self-renewal capacity of ASCs remains unclear. Moreover, it has been reported that MAPK signaling pathway could control cancer development and tumorigenesis [116]. A group of researchers have confirmed the effect of MAPKs in liver cancer [117], brain tumors [118], prostate cancer [119], glioma [120], head and neck squamous carcinoma cells [121], thyroid tumor [122], and breast carcinoma [123]. Therefore, it is controversial to regulate ERK signaling pathway during ASC proliferation and differentiation.

4. Clinical Applications of Tissue-Engineered ASCs

ASCs are being vigorously studies in the laboratory now, but a few clinical trials of ASCs have been reported compared to those for BMSCs. The official clinical trial website (https://clinicaltrials.gov/, keyword: adipose derived stem cells) revealed 125 stem cell studies, excluding unknown status. Among them, 35 trials have been completed (Table 5). Two are ongoing Phase IV clinical trials. One trial is designed to determine whether ASCs are effective for females with premature ovarian failure. The other trial was designed to evaluate transplantation of autologous fat with adipose-derived regenerative cells in patients with functional and cosmetic breast deformities after segmental mastectomy or quadrantectomy (lumpectomy). However, no result or relevant study was found at the website. Although some clinical cases have used tissue-engineered ASCs as a potential therapeutic solution, clinical trials investigation of the long-term effects of ASCs are still in progress.

Great successes in the laboratory level application have suggested the promising effects of ASCs-based in a variety of clinical condition. A recent clinical case reported that tissue-engineered ASCs could be utilized to treat a large anterior mandibular defect [65]. In this case, a 55-year-old man had a third 10 cm ameloblastoma recurrence in the parasymphyseal area of the mandible (Figures 5(a) and 5(b)). The defect site was treated with a tissue-engineered construct that combined β-tricalcium phosphate (β-TCP) granules, recombinant human bone morphogenetic protein-2 (BMP-2), and autologous ASCs. Adipose tissue was harvested from the anterior abdominal wall of the patient, and the ASCs were isolated by collagenase and expanded for 21 days in DMEM with 15% autologous serum without antibiotics *in vitro*. The ASCs was confirmed through cell surface marker expression and analyzed for osteogenic differentiation potential. Before seeding the cells, scaffolds containing β-TCP granules (porosity, 60%; granule size, 1.4–2.8 mm) were fabricated for cell attachment. The β-TCP granules were incubated for 48 hours in basal medium containing 12 mg rh-BMP-2. Then, the combined cell-biomaterial scaffolds were incubated and transported to the operating theater. A medical skull model manufactured using computed tomography data was fabricated before transplantation to fit the patient-specific reconstruction plate and titanium mesh. The mandibular bone was resected, and the defect site was replaced with the reconstruction plate and titanium mesh. Then, the cell encapsulated biomaterial scaffolds were added to fill the gaps, and six dental implant fixtures were used to attach the mesh. Five of six implanted fixtures allowed osseointegration (Figure 5(c)). The patient was satisfied with the appearance and function of the implanted borne during the 3-year follow-up (Figures 5(d) and 5(e)). This case shows that tissue-engineered ASCs offer a promising replacement for large bone defects [65]. Another clinical case enrolled four patients (three women and one man) with cranial defects on the right side, and all underwent ASC-based cranioplasty [124]. ASCs were also seeded in β-TCP phosphate granules. The follow-up indicated no clinically relevant postoperative complications, and the patients were satisfied with their outcomes. A cell-assisted lipotransfer technique has been employed in other clinical cases to treat facial lipoatrophy [125], cosmetic breast augmentation [126], and breast implant complications [127].

Despite ASC's positive effects in tissue engineering and regenerative medicine, many researchers reported that ASCs exhibited carcinogenic potentials. ASC enriched fat tissue grafts can be used as autologous and allogeneic transplantation for soft tissue reconstruction followed by mastectomy to reduce cosmetic and psychological problems [128]. However, *in vitro* and animal studies reported that ASCs can interact with tumor cells and induce tumor progression [129]. Yu et al. transplanted ASCs together with tumor cells subcutaneously or intracranially into BALB/c nude mice to observe tumor outgrowth [130]. The result indicated that coculture of ASCs with H460 or U87MG cells promoted tumor cell proliferation. Coinjection of ASCs with H460 or U87MG cells increased tumor cell viability *in vivo* and induced the apoptosis of normal cells. Conditioned medium from ASCs inhibited hydrogen peroxide-induced cell death of H460 or U87MG cells. These findings suggested that ASCs with H460 or U87MG cells promoted tumor growth in the nude mice. Chandler et al. also conducted a study to investigate

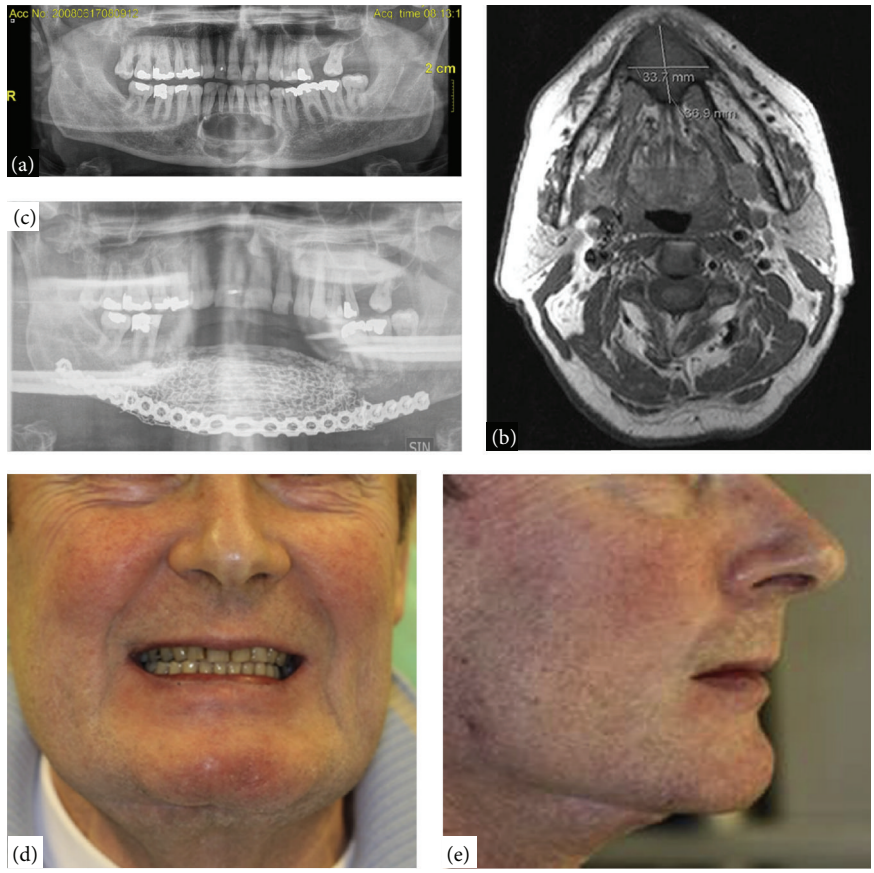


Figure 5: Tissue-engineered ASCs could be utilized to treat a large anterior mandibular defect. (a) X-ray image shows a nearly 10 cm recurrence of an ameloblastoma in the parasymphyseal area of the anterior mandible. (b) T1-weighted magnetic resonance image shows a large tumor (ameloblastoma) in the anterior mandible. (c) X-ray image shows the six implant fixtures inserted into the grafted site. (d) The reconstructed chin taken from the front. (e) The reconstructed chin taken from the lateral side (adopted from [65]).

the possible mechanism of the interaction between ASCs and tumor cells [129]. They found that tumor-secreted soluble factors from a breast tumor inhibited adipogenic differentiation but increased the proliferation, proangiogenic factor secretion, and myofibroblastic differentiation of ASCs. This changed behavior of ASCs is similar to the characteristics of breast tumors. The result was further confirmed by orthotropic mouse studies. On the contrary, Cousin et al. reported that ASCs could inhibit tumor viability and proliferation [131]. A coculture system with ASC-conditioned medium and pancreatic tumor cells was able to enhance pancreatic tumor death both *in vivo* and *in vitro*. This inhibitory effect mediated by ASCs was also found in liver, prostate, and colon cancers. Taken together, the carcinogenic potential using ASCs in transplantation remained unclear and reported results are controversial. Therefore, clinical trials using ASCs should be carefully considered by the medical history of patients, especially for the patients who has been diagnosed with any cancer previously.

5. Challenges and Future Perspectives

ASCs are good candidates for tissue repair and regeneration for plastic and reconstructive surgery because of their rich source and easy access. However, adipose tissue engineering is far from an "off-the-shelf" product. Before ASCs can be translated to clinical practice, a number of problems must be solved. First, industrialized xeno-free culture media without animal-derived reagents have not been well established. Although some labs have adopted serum-free and xeno-free media to culture ASCs. These media are not "off-the-shelf" products. To fully recognize the safety and efficiency of these media, further studies *in vivo* must be performed. Second, a well-defined preservation method maintaining ASCs properties in the long term is also of impact for ASCs' application in tissue engineering and regenerative medicine. Third, a few studies have considered the mechanisms of the interactions among ASCs, biomolecular growth factors, and biofabricated scaffolds. Understanding these mechanisms involved in ASCs proliferation and differentiation is much important for further clinical applications. Finally, as few clinical trials have been performed to investigate tissue-engineered ASCs, their long-term safety remains uncertain. At present, long-term safety issue is the biggest challenges of ASCs-based regenerative medicine. It is necessary to conduct more systematic studies to confirm that ASCs can be utilized as a standard therapeutic tool in regenerative medicine. In addition to safety issue of ASCs, the biomaterials also needed

more long-term *in vivo* experiments. Although biomaterials are biocompatible, most of them are also derived from animal sources (e.g., collagen from rat tail and gelatin from porcine skin) and have the possibility to induce an immune reaction in the long term. Moreover, with the degradation of biomaterial in the body over time, the fraction may serve as host antibodies causing severe immune reactions. In summary, although there are a number of challenges existing, ASCs are still a very promising method in regenerative medicine with a bright future because of their rich source, relatively simple process of accessing and isolating, and their multipotentials of differentiation. In the near future, adipose tissue engineering may become "off-the-shelf" products for various diseases and benefit millions of people.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Ru Dai and Zongjie Wang contribute equally to this work.

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Research Article

The Use of Human Adipose-Derived Stem Cells in the Treatment of Physiological and Pathological Vulvar Dystrophies

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"Vulvar dystrophy" is characterized by chronic alterations of vulvar trophism, occurring in both physiological (menopause) and pathological (lichen sclerosus, vulvar graft-versus-host disease) conditions. Associated symptoms are itching, burning, dyspareunia and vaginal dryness. Current treatments often do not imply a complete remission of symptoms. Adipose-Derived Stem Cells (ADSCs) injection represents a valid alternative therapy to enhance trophism and tone of dystrophic tissues. We evaluated efficacy of ADSCs-based therapy in the dystrophic areas. From February to April 2013 we enrolled 8 patients with vulvar dystrophy. A biopsy specimen was performed before and after treatment. Digital photographs were taken at baseline and during the follow-up. Pain was detected with Visual Analogue Scale and sexual function was evaluated with Female Sexual Function Index. All patients received 2 treatments in 3 months. Follow-up was at 1 week , 1 and 3 months, and 1 and 2 years. We obtained a significant vulvar trophism enhancement in all patients, who reported pain reduction and sexual function improvement. Objective exam with speculum was easy to perform after treatment. We believe ADSCs-based therapy finds its application in the treatment of vulvar dystrophies, since ADSCs could induce increased vascularization due to their angiogenic properties and tissue trophism improvement thanks to their eutrophic effect.

1. Introduction

Vulvar dystrophy is a condition which may occur in women both in physiological and in pathological conditions. Associated symptoms may include itching, burning, dyspareunia, vaginal dryness and bleeding. Vaginal dystrophy commonly affects postmenopausal women, with prevalence ranging from 10% to 50% [1, 2] and it is estimated that up to 45% of all women are symptomatic [3]. The aetiology of vulvovaginal atrophy among postmenopausal women is most commonly explained by the decrease in circulating estrogen associated with the menopausal transition, which has an adverse effect on skin collagen and elasticity [4]. Even while taking systemic estrogen, 10% to 20% of women may still have residual symptoms [5]. Symptoms related to estrogen reduction may also occur during other stages of women's lives in response to events associated with sustained decreases in estrogen such as lactation or also related to chemotherapy that induces premature ovarian failure in 14–100% of cases. These patients are at high risk of transient or permanent amenorrhea, and, for those women who continue to menstruate or who recover their cycles, there is an additional long-term risk of premature ovarian failure. In particular, breast cancer treatment increases the prevalence of vulvar dystrophy because the surgical, endocrine, and chemotherapeutic agents used in its treatment can cause or exacerbate this condition. An iatrogenic cause of vulvar dystrophy is related to allogeneic hematopoietic stem cell transplant. This procedure has found a place in the treatment of a variety of malignant and nonmalignant diseases of the bone marrow and immune system. However, it may be complicated by chronic graft-versus-host disease (GVHD), due to activation of donor immunological cells against host tissues. GVHD may occur in acute or chronic forms. The skin, mouth, eyes, liver,

and intestines are the organs most commonly involved in chronic GVHD. Genital tract involvement can occur in nearly 25% of patients with GVHD. Vulvar scarring can lead to vaginal stenosis and introital reduction and may also result in labial fusion [6]. These manifestations can lead to some complications as hematometra, hematocolpos, and abscesses. Hypoestrogenism from chemotherapy-induced premature ovarian failure and chronic GVHD can both cause vulvar or vaginal pain and irritation. Lichen sclerosus (LS) is a chronic immune-mediated inflammatory skin disorder of poorly understood aetiology [7, 8], which may be localized anywhere on the body but has a predilection for the anogenital area [9]. Although disease onset has been reported at all ages, it occurs most commonly in women in their fifth or sixth decade of life. Skin changes of the vulva initially begin with white, polygonal papules that coalesce into plaques, with skin atrophy and perifocal erythema. Additionally, subcutaneous bleeding with ecchymosis or hematosis and fissures with superficial ulceration and erosion may occur. Late progressive symptoms include thinning of the mucosa, edema and fibrosis of the dermis, shrinkage of the labia, and agglutination of the labia minora that can lead to stenosis of the introitus. Moreover, while the disorder is considered benign, some women with vulvar LS may later develop squamous cell carcinoma (SCC) of the vulva and some women have concomitant SCC when initially diagnosed with LS [10]. Vulvar dystrophy may negatively affect the entire sexual response cycle, inducing significant changes in desire, arousal, orgasm, and satisfaction at menopause and beyond. Patients may be embarrassed by the disfiguring changes that may occur and avoid sexual intimacy. Furthermore these patients may have increased risk to develop bacterial vaginosis due to vaginal pH changes and urinary tract infection or stress urinary incontinence. Main treatments to solve urogenital and sexual dysfunction due to vulvovaginal tissue alteration currently include drug therapy and lubricants, but in the majority of patients these therapies do not imply a complete remission of symptoms. Recent studies emphasized that adipose tissue is a rich source of adult stem cells, the so-called Adipose-Derived Stem Cells (ADSCs) [11–13]. We tested the hypothesis that the vulvar application of ADSCs can enhance the trophism and tone of dystrophic tissues.

2. Materials and Methods

2.1. Patients. The clinical trial protocol, conformed to the guidelines of the Declaration of Helsinki (1964), was approved by the Ethical Committee of our institution (Ref.1834/25.03.10). From February to April 2013 we enrolled 8 patients affected with vulvar dystrophy of average age of 56.5 (between 38 and 75 years old). Among them, one patient was in postmenopause, 2 women were affected with vulvar GVHD, and 5 were affected with LS. At each visit, personal, anamnestic, and objective data were collected. Our study was conducted with the patients' understanding and consent. Patients were informed of the purpose and objective of the investigation and were asked for their written informed consent for participation and publication of the data obtained during the course of the study. Inclusion criteria called for vulvar dystrophy's symptoms and signs, patients' refusal of lipofilling, and patients nonresponders to traditional therapy. Exclusion criteria were pregnancy or lactation and associated pathologies contraindicating the proposed treatment.

All patients received 2 treatments in 3 months. Follow-up was at 1 week and 1 and 3 months and at 1 and 2 years.

2.2. Surgical Techniques. The adipose tissue collection was performed through liposuction from the abdominal region or trochanteric areas, depending on the easier access to a sufficient amount of adipose tissue. The sampling was carried out under local anesthesia, using a modified Klein solution (1 liter of sodium chloride 0.9%, 20 mL of lidocaine 2%, and 1 mL of epinephrine 1 : 200,000) through a single hole of 3 mm blunt cannula connected to a 10 cc Luer-lock syringe. The harvested fat was centrifuged at 1500 rpm for 2 minutes. After centrifugation, the oil layer (upper level) and the aqueous layer (lower level) were eliminated from the syringe. The middle layer containing adipocytes and stromal vascular component was sent to the laboratory for cell cultivation within 1 hour.

2.3. Cell Isolation and Culture. The fat was transferred into a sterile tube and washed extensively with sterile phosphate-buffered saline (PBS) containing 2% PSG to remove contaminating debris and red blood cells. The stromal vascular fraction (SVF), containing the ADSCs, was then pelleted by centrifugation for 5 min at 2000 rpm [14] and then resuspended in DMEM-Ham's F-12 medium (vol/vol, 1 : 1) (DMEM/F-12, Gibco) supplemented with 20% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine and plated in a 75 cm^2 tissue culture flask coated with collagen type IV.

ADSCs were self-selected out of the SVF during subsequent tissue culture passages, on the basis of their ability to adhere to the plastic tissue cultureware, and maintained in a 5% CO_2 incubator at 37°C in a humidified atmosphere, with medium change twice a week. Cell viability was assessed by using the trypan blue exclusion assay. A homogeneous population of ADSCs was subsequently checked by determining growth kinetics and by analyzing the surface-marker expression profile.

2.4. Preparation of Cell-Enriched Hyaluronic Acid. Primary cultures of ADSCs derived from each patient were expanded in order to obtain an adequate number of 75 cm^2 flasks at 95–100% confluence. Once the appropriate number of cells was reached for each patient, cells were detached with 0.5 mM EDTA/0.05% trypsin for 5 min at 37°C and counted. Then, ADSCs were centrifuged at 1500 rpm for 10 minutes, washed twice in phosphate-buffered saline to remove serum, and resuspended in an adequate volume of synthetic stabilized low molecular weight hyaluronic acid solution (a 1.6% solution of synthetic HA, without chemical modifications and with a molecular weight of 1×10^3 KDa, very similar to the endogenous HA) at the standard concentration of 5×10^5 cells/mL, to vehicle stem cells and to facilitate subsequent infiltration. After mixing, the suspension was kept under ambient conditions for 10 to 15 minutes to allow cell adherence to the hyaluronan matrix. Homogeneous dispersion

of the cells within the gel was ensured by microscopical observation. Then, the cell-supplemented hyaluronic acid solution was loaded into an injection syringe and carried to the operating room.

2.5. ADSCs Infiltration. Usually after 3 weeks, the patient went back to the operating room for the inoculation of autologous ADSCs into the dystrophic areas. The procedure was performed in local anesthesia with sedation, with the patient in the dorsal lithotomy. The infiltration was done using two 2 mL syringes provided with a 30-gauge 1/2 needle by means of multiple passes in the subcutaneous plane of labia minora. We employed 2 mL of hyaluronic acid for each patient, keeping a constant rate of 5×10^5 cells for each mL of hyaluronic acid. All patients were dismissed the following day.

2.6. Clinical Evaluation. The evaluated parameters were tissue trophism, pain, and sexual satisfaction. Tissue trophism was evaluated by photographic documentation and biopsy performed before and after treatment. Photographs were taken in occasion of follow-up utilizing the same parameters (same camera Canon EOS 500D, same shooting distance of 50 cm, and same anatomical landmarks). Pain was estimated at T0 and weekly according to the Visual Analogue Scale (VAS). A range from 1 (no pain) to 10 (maximal sensation of pain) was considered. Sexual function was assessed by the Female Sexual Function Index (FSFI), performed before and after treatment. It consists of 19 questions on the sexual activity performed in the last four weeks. It enables assessment of six sexual functioning domains: desire, arousal, lubrication, orgasm, satisfaction, and discomfort/pain [15]. Subdomains are scored considering the values of each question and its respective conversion factors, and total FSFI Score was calculated as the sum of the six results, ranging from 2 to 36. Better levels of sexual function were indicated by highest scores.

3. Results

All patients were followed postoperatively at least for 2 years, with periodical medical assessments at 1 week, 1 and 3 months, and 1 and 2 years.

Just one month after the first ADSCs infiltration, improvement of vulvar trophism was clinically observed, enhancing progressively over time. Pain reduction was already documented at one month after the first treatment; dramatic pain reduction was obtained after 1 year. Sexual function improved significantly at one month after the second treatment. Overall results in terms of pain reduction, vulvar trophism, and sexual function improvement were well-maintained at 2 years' follow-up. In particular, photographic documentation at 2 years' follow-up revealed an improvement of vulvar trophism, showing a pink vulvar skin color, a restoration of elasticity, and the disappearing of erythematous areas. We observed that speculum exam was easier to perform and painless as indicated from the patients. A loss of vaginal rugae, vaginal pallor, and petechiae was evident in the patient affected in menopause. Histological exam in menopause patient demonstrated improvement of the elastic network.

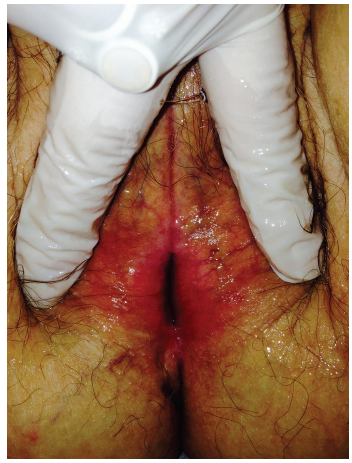

Figure 1: Before ADSCs.

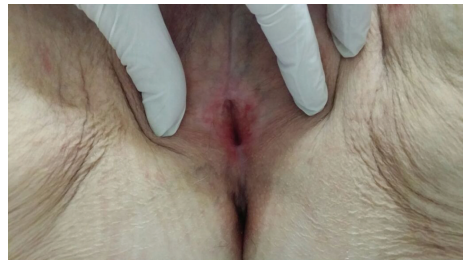

Figure 2: After ADSCs.

In patients with LS, showing extensive erythema of the labia minora extending onto the interlabial sulci (which also show lichenification) and the vaginal introitus before treatment (Figure 1), we observed that erythema was dramatically reduced and disappeared at level of the interlabial sulci and the vaginal introitus after ADSCs infiltration (Figure 2). In patients affected with GVHD, histological examination documented apoptotic cells in the basal layer, epithelial detachment from the thinning mucosa, collagen hyalinosis, and monocytic infiltrate in the submucosa. After treatment with ADSCs, a regression of the morphological alterations of epithelial cells and full attenuation of inflammatory signs in the connectival tissue were observed. Biopsy specimens of patients affected with LS displayed epithelial acanthosis, appendageal hyperkeratosis and dermal modifications with hyalinization, lymphocytic infiltrate with a longitudinal band in the medium dermis, wide ectatic capillaries, and lymphocyte tagging along the basement membrane. After treatment with ADSCs, the dermis sclerosis was significantly reduced, capillaries that resulted were less dilated, and inflammatory infiltrate was dramatically reduced (Figures 3 and 4). According to the VAS, a remarkable pain reduction could be observed in all patients, as shown in Table 1. Moreover, FSFI revealed that all patients obtained an improvement of sexual function after ADSCs treatment, as documented in Table 2. Our method was efficacious regardless of the aetiology of vulvar dystrophy.

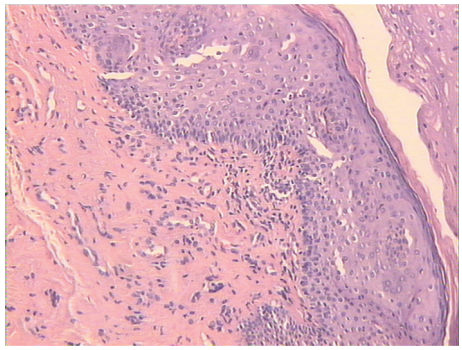

Figure 3: Hematoxylin-eosin staining ×10. Epidermis with mild parakeratotic hyperkeratosis ectatic capillaries, sclerotic dermis, and inflammatory infiltrate.

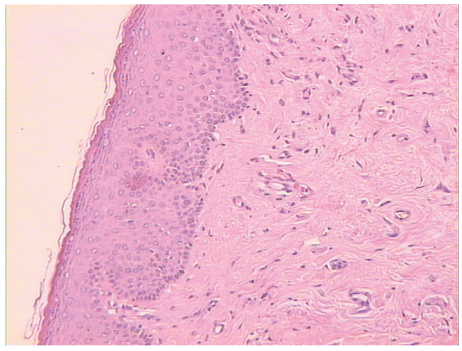

Figure 4: Hematoxylin-eosin staining ×10. Reduction of dermis sclerosis, capillaries less dilatated, inflammatory infiltrate dramatically reduced.

Table 1: Visual Analogue Scale (VAS) score before and after treatment.

Cases	Patients (N)	VAS score	
		Before ADSCs	After ADSCs
Menopause	1	7	1
GVHD	1	9	2
	1	8	1
LS	1	8	1
	1	7	1
	1	8	1
	1	9	2
	1	8	1

Table 2: Female Sexual Function Index (FSFI) before and after treatment.

Cases	Patients (N)	FSFI	
		Before ADSCs	After ADSCs
Menopause	1	10	30
GVHD	1	15	30
	1	20	30
LS	1	15	36
	1	14	35
	1	12	35
	1	10	36
	1	10	36

4. Discussion

Vulvar dystrophy negatively impacts women's lives, but women lack knowledge of the subject and are hesitant to consult healthcare professionals, who should proactively initiate discussions regarding appropriate treatment options. Main treatments to solve urogenital and sexual dysfunction due to vulvovaginal tissue dystrophy currently include drug therapies and lubricants but in the majority of patients they do not imply a complete remission of symptoms. Lubricants can be used to decrease immediate irritation during coital activity, but there is no evidence that these products have any long-term therapeutic effect. Systemic hormone replacement is indicated for women who are seeking to treat a variety of symptoms associated with the estrogen deprivation of menopause, such as hot flushes and sleep disturbance, but, in some women, it is not an effective method for relieving vulvovaginal atrophy [16]. Furthermore, estrogens cannot be used in women affected with breast cancer undergoing the hormone suppressive therapy. To date, new therapeutic approaches have been experimented. Hyaluronic acid (HA) infiltration is recently used for vaginal rejuvenation. HA is an unbranched, nonsulphated polysaccharide, and it is biocompatible, nontoxic, and biodegradable. Due to its high water-binding capacity or hygroscopicity, it induces tissue hydration and lubrication [17]. In the skin, HA is produced intracellularly at the cell membrane of fibroblasts by HA-synthases [18] and extruded directly into the extracellular matrix. Since HA is chemically and structurally identical, regardless of its origin, allergic reactions are rare and skin tests before injection are not necessary. This led to the use of HA in several tissue engineering areas, such as aesthetic surgery. Moreover, previous observations suggested that nonanimal stabilized hyaluronic acid may confer filling effects, attributable to its stimulatory effect on collagen production [19], but a major drawback of HA is the lack of a tissue regeneration, with the filling material that is only degraded and not replaced by autologous tissue. Therefore, it is almost completely reabsorbed after 1 month, and its effects are only temporary. In the last few years, autologous fat transplantation has become the first-choice treatment to restore volumes and to achieve structural modifications. This approach, using the patient's own body fat as a natural filler, takes advantage of its abundance and accessibility, thus avoiding complications associated with foreign materials [20]. Nevertheless, many studies demonstrated that the most part of adipose tissue grafts is reabsorbed through time or replaced by fibrous tissue, and they also display a low rate of survey due to partial necrosis [21]. Lipofilling benefits have already been proved in dystrophies and vulvar rejuvenating treatments. As outlined above, the adipose tissue is a rich source of ADSCs. This stem cell reservoir can be

easily obtained from a very small amount of liposuction aspirate (1–5 cc) or from adipose tissue biopsy. Furthermore, ADSCs possess the ability to differentiate into various cell types, including adipocytes, chondrocytes, osteoblasts, and myoblasts. Therefore, they may represent a promising approach to cell-based therapies [11–13]. Besides, it has been recently demonstrated that ADSCs show angiogenic properties and could also exert some immunomodulatory activities, including a suppressive response on collagen-reactive T cells and the capacity to restore immune tolerance by inhibiting the inflammatory response *in vivo* [13]. ADSCs infiltration finds its application in the treatment of physiological and pathological vulvovaginal dystrophies, since these cells could induce increased vascularization, due to their angiogenic properties, and an improvement of tissue trophism, due to their eutrophying effect. In particular, in patients affected with pathological inflammatory dystrophies, a reduction of the typical pain and burning sensation could be obtained thanks to the immunomodulatory properties of the ADSCs, which may act by reducing inflammation that represents the algogenic stimulus of these pathologies. Therefore, it is possible to obtain the appropriate number of cells to treat different pathologies, by means of conveyance with a low molecular weight hyaluronic acid gel, thus allowing an easy and nontraumatic infiltration in the affected areas. Nonanimal stabilized hyaluronic acid represents an ideal scaffold that can provide structural support and a favorable environment for growing cells, and it is a natural component of commonly used injectable soft-tissue filler [22]. Moreover, a further advantage of this procedure is the reduction of operating time and the greater compliance of patients and surgeons in comparison with the traditional invasive techniques.

5. Conclusion

ADSCs injection represents the evolution of a standardized therapy, such as conventional lipofilling, and a valid alternative method to the injection of hyaluronic acid alone for biorevitalizing purposes.

On the basis of our experience we advocate that ADSCs infiltration in dystrophic vulvar areas is a safe method, easier to be performed with respect to the traditional technique, noninvasive, and requiring short time. It could represent a valid therapeutic option when patients refuse or present conditions contraindicating an invasive procedure. In agreement with the positive results previously achieved with this technique in pathologies characterized by tissue scleroatrophy such as scleroderma [14], we obtained encouraging results in patients suffering from vulvar dystrophies, even in the long-term, in terms of cutaneous, subcutaneous, and mucous trophism improvement. Our patients experienced a restoration of the anatomy, altered by the pathology, and could improve their quality of life. ADSCs treatment leads to the reacquisition of vaginal and periurethral tonicity, thus solving problems related to the hypotrophic involutive processes affecting the genitourinary tract. After treatment our patients performed objective exam, Pap test, and other gynaecological procedures, without pain.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper and disclose no commercial interest that they may have in the subject of study and the source of any financial or material support.

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Clinical Study

Improvement of Mouth Functional Disability in Systemic Sclerosis Patients over One Year in a Trial of Fat Transplantation versus Adipose-Derived Stromal Cells

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Background. Systemic sclerosis (SSc) is a multisystem disease characterized by cutaneous and visceral fibrosis. Face and mouth changes include telangiectasia, sicca syndrome, and thinning and reduction of mouth width (microcheilia) and opening (microstomia). We applied autologous fat transplantation compared with autologous adipose-derived stromal cells (ADSCs) injection to evaluate the clinical improvement of mouth opening. *Methods*. From February to May 2013 ten consecutive SSc patients were enrolled from the outpatient clinic of Plastic Surgery Department of Sapienza University of Rome. Patients were divided into two groups as follows: 5 patients were treated with fat transplantation and 5 patients received infiltration of ADSCs produced by cell factory of our institution. To value mouth opening, we use the Italian version of Mouth Handicap in Systemic Sclerosis Scale (IvMHISS). Mouth opening was assessed in centimetres (Maximal Mouth Opening, MMO). In order to evaluate compliance and physician and patient satisfaction, we employed a Questionnaire of Satisfaction and the Visual Analogic Scale (VAS) performed before starting study and 1 year after the last treatment. *Results and Conclusion*. We noticed that both procedures obtained significant results but neither one emerged as a first-choice technique. The present clinical experimentation should be regarded as a starting point for further experimental research and clinical trials.

1. Introduction

Systemic sclerosis (SSc) or scleroderma is a multisystem disease characterized by cutaneous and visceral fibrosis. After an initial period of induration, dermis and visceral become infiltrated with collagen and become both harder and thicker. It is an autoimmune disease with a prevalence of 2-3 per 10.000 people. The ratio of women to men is 4 to 1, the majority diagnosed between the age of 30 and 50 [1].

Cutaneous manifestations of this disease are very plain and hard to conceal. Limited range of mouth opening, along with other symptoms such as dry mouth, can lead to difficulties with oral hygiene and eating. Facial involvement and oral complications are typical features of SSc, leading to aesthetic changes and impairment of the patient's self-image. The face becomes amimic, cutaneous wrinkles disappear around the mouth, vertical furrows develop, and the nose becomes sharp. Face and mouth changes also include telangiectasia, sicca syndrome, and thinning and reduction of mouth width (microcheilia) and opening (microstomia), also favoured by osteolysis of mandibular angles and by fibrosis of soft tissues.

Sclerosis of the extremities is highly disabling and results in significant dysfunction; the facial symptoms bear cosmetic disfigurement and limit expression, leading to a mask-like stiffness of the face. Currently, therapy is limited and no antifibrotic treatment has proven its efficacy. Recent studies have assessed different biological agents for the treatment of skin thickness as neutralizing antibodies, tyrosine kinase inhibitors, proteins with antifibrotic properties, or proteins

that induce immune tolerance, generally well tolerated but not showing significant efficacy [2].

Over the years, autologous fat transplantation has become the first-choice technique to hide cutaneous lesions; this approach, using the patient's own body fat as a natural filler to achieve structural modifications, takes advantage of its abundance and accessibility and avoids complications associated with foreign materials. Elective liposuction for fat transplantation is nowadays considered a safe and well-tolerated procedure [3, 4].

Recently, a stem cell population within the adipose stromal compartment has been identified, termed adipose-derived stromal cells (ADSCs). This stem cell reservoir can be easily obtained from a very small amount of liposuction aspirates (1–5 cc), since it is present in any type of white adipose tissue [5]. Moreover, ADSCs possess the ability to differentiate into various cell types, including adipocytes, chondrocytes, osteoblasts, myocytes, and neurons under specific differentiation conditions. Some studies describe the potential use of ADSCs in treating some autoimmune and inflammatory disorders, such as type I diabetes mellitus, systemic sclerosis and systemic lupus erythematosus, myasthenia gravis, and multiple sclerosis [6–8].

In this project, we applied the procedure of autologous fat transplantation compared with autologous ADSCs injection to evaluate the clinical improvement of mouth opening.

2. Materials and Methods

From February to May 2013, ten consecutive SSc patients (8 female and 2 male), fulfilling American College of Rheumatology (ACR) criteria and classified as having diffuse cutaneous scleroderma [9], were enrolled from the outpatient clinic of the Department of Plastic Surgery of Sapienza University of Rome and agreed by a written informed consent to participate in the study, which was approved by our ethics committee (Ref. 1834/25.03.10) and conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki.

These patients had advanced systemic sclerosis-related perioral thickening and mouth opening limitation. The group was homogeneous for age (age range: 20–48 years), disease state, and duration and finally for clinical characteristics.

At each visit, personal, anamnestic, and objective (clinical characteristics) data were collected and recorded, using a written form that was held securely, thus being accessible only to study investigators (Table 1).

Inclusion criteria called for signs of no active disease expressed by increasing size of lesions, appearance of new lesions, and/or clinical signs of inflammation within the last 6 months.

Exclusion criteria were as follows: pregnancy or lactation, any immunomodulating or immunosuppressive therapy within the last 4 weeks and any topical therapy within the last 2 weeks except for the use of emollients, and finally patient's refusal to participate in the study.

Patients were divided into two groups as follows: 5 patients were treated with fat transplantation "group L" (lipofilling) and 5 patients received infiltration of ADSCs produced by cell factory of our institution "group A" (ADSCs). The patients did not receive any remuneration for their inclusion or treatment in this study.

Table 1: Patients' characteristics: A: patients treated with ADSCs injection and L: patients treated with fat transplantation.

Patients	Age	Disease status	Disease duration	Localized scleroderma
1 A	23	1-year disease stabilization	3 years	Face and hands
2 A	25	9-year disease stabilization	5 years	Face
3 A	35	6-year disease stabilization	10 years	Face
4 A	38	6-year disease stabilization	10 years	Face
5 A	39	15-year disease stabilization	13 years	Face and hands
1 L	24	5-year disease stabilization	3 years	Face and hands
2 L	25	8-year disease stabilization	3 years	Face and hands
3 L	36	16-year disease stabilization	12 years	Face
4 L	39	8-year disease stabilization	15 years	Face
5 L	48	6-year disease stabilization	18 years	Face and hands

After the first treatment, all patients underwent the same procedure 3 months later. Follow-up was at 1 week, 1 month, and 1 year. During the follow-up, it was possible to compare our obtained results by using fat transplantation with ADSCs infiltration.

2.1. Disability Evaluation. We want to describe several parameters used to value disability often experienced by scleroderma patients. Various modalities can be used to measure the extent and severity of skin involvement. The modified Rodnan Skin Score (MRSS), a summation of physical examination ratings over 17 skin sites (fingers, hands, forearms, arms, face, chest abdomen, thighs, lower legs, and feet), has become the standard primary outcome measure of skin involvement during clinical trials and in practice [10].

MRSS is the current gold standard measure of skin disease, but other methods that are more objective, precise, and reproducible have been developed to assess skin involvement. These include skin biopsy, ultrasonography, electronic tonometry, cytometry, and durometry [11–16].

There are also different scales to value quality of life: the Health-Related Quality of Life (HRQoL), the Health Assessment Questionnaire (HAQ), and scleroderma HAQ (sHAQ). This latter is more specific for SSc, as it adds to HAQ 5 visual analogue scales, evaluating Raynaud's phenomenon, digital ulcers, gastrointestinal and lung symptoms, and overall disease severity [17–19].

Table 2: Italian version of Mouth Handicap in Systemic Sclerosis Scale (IvMHISS) assesses the handicap with mouth disability in SSc. It consists of 12 items (each scored 0–4): 0 Never (Mai); 1 Rarely (Raramente); 2 Occasionally (Occasionalmente); 3 Often (Spesso); 4 Always (sempre).

1	I have difficulties opening my mouth Ho difficoltà ad aprire la bocca	0	1	2	3	4
2	I have to avoid certain drinks (sparkling, alcohol, etc.) Devo evitare alcuni tipi di bevande (frizzanti, alcoliche, ecc)	0	1	2	3	4
3	I have difficulties chewing Ho difficoltà a masticare	0	1	2	3	4
4	My dentist has difficulties taking care of my teeth Il mio dentist ha difficoltà a prendersi cura dei miei denti	0	1	2	3	4
5	My dentition had become altered La mia dentatura si è alterata	0	1	2	3	4
6	My lips are retracted and/or my checks are sunken Le mie labbra sono retratte e/o le mie guance sono infossate	0	1	2	3	4
7	My mouth is dry La mia bocca è secca	0	1	2	3	4
8	I have to drink often Devo bere spesso	0	1	2	3	4
9	My meals consist of what I can eat and not what I would like to eat Devo mangiare le cose che posso e non quelle che vorrei	0	1	2	3	4
10	I have difficulties speaking clearly Ho difficoltà a parlare con chiarezza	0	1	2	3	4
11	The appearance of my face is modified L'aspetto della mia faccia si è modificato	0	1	2	3	4
12	I have trouble with the way my face looks L'aspetto della mia faccia mi crea problemi	0	1	2	3	4

Hand disability can be studied by specific instruments, such as Cochin Hand Function Scale (CHFS) and Hand Mobility in Scleroderma Scale (HAMIS) [20, 21].

In our study, we decided to examine the following parameters. Skin tightening due to subcutaneous and ligamentous collagen deposit associated with diffuse systemic sclerosis results in a mechanical inability to open the mouth. To value the mouth disability we use the Italian version of Mouth Handicap in Systemic Sclerosis Scale (IvMHISS).

MHISS assessing the handicap with mouth disability in SSc consists of 12 items (each scored 0–4) with a total score range from 0 to 48 (Table 2) [22–27].

Mouth opening was assessed in centimetres (Maximal Mouth Opening, MMO) by measuring the distance between the tips of upper and lower right incisive teeth (mean of two consecutive measurements).

The patients were asked to fill in a questionnaire in which their degree of satisfaction could be expressed by the following ratings: unsatisfied, moderately satisfied, rather satisfied, and very satisfied.

In order to assess *compliance and physician and patient satisfaction* we employed an evaluation system according to the Visual Analogic Scale (VAS) giving a score ranging from 1 up to 10, where 1 indicates no improvement and 10 indicates the maximum possible improvement.

IvMHISS, MMO, Questionnaire of Satisfaction, and VAS were performed before starting study and 1 year after the last treatment.

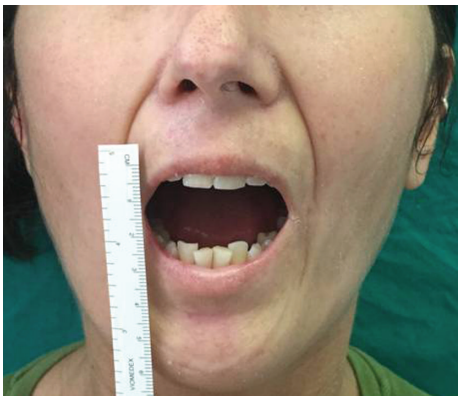

Figure 1: A 39-year-old woman with microstomia before treatment.

2.2. Case Report 1

Patient: A 39-Year-Old Woman. She first reported the onset of the disease at the age of 25. Disease was characterized by slow but progressive symmetrical skin thickening limited to the fingers (sclerodactyly) and to the face (especially microstomia). She reported that for 18 years the disease was stable (Figure 1). The patient underwent fat transplantation (Figures 2 and 3).

2.3. Case Report 2

Patient: A 38-Year-Old Woman. She reported the disease onset at 28 years of age. Disease was characterized by fast

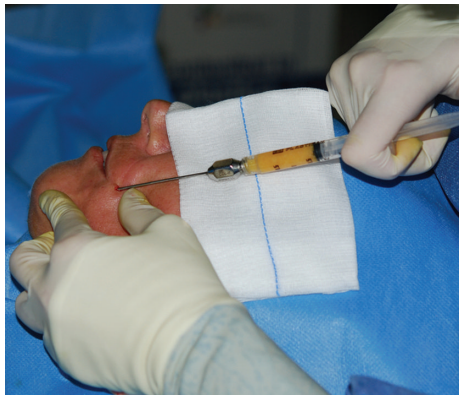

Figure 2: Autologous fat transplantation.

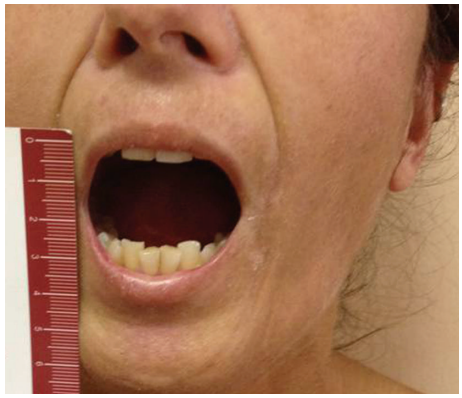

Figure 3: Follow-up at 1 year.

symmetrical skin thickening limited to either the fingers (sclerodactyly) or to the face (especially microstomia). She also reported that for 16 years the disease has not progressed (Figure 4). The patient underwent ADSCs infiltration (Figures 5 and 6).

2.4. Technique

2.4.1. Autologous Fat Transplantation Procedure. The periumbilical abdominal region represented the donor site for all patients. After the administration of local modified Klein solution, 1 liter of sodium chloride 0.9%, 20 mL of lidocaine 2%, and 1 mL of epinephrine 1 : 200,000, adipose tissue was harvested using hand-generated suction by means of a one-hole blunt 3 mm cannula attached to a 10 cc Luer-lock syringe. Such nontraumatic low-negative pressure drain method preserves adipocytes intact and viable for transfer [28].

A total amount of 40 mL of lipoaspirate was harvested from the abdomen. Afterward, it was decanted 15 minutes and only the layer containing adipocytes was used for fat injection. The fat infiltration was performed using a blunt injection cannula of 2 mm in diameter. Perioral region was injected using many radiating passages at the subcutaneous level for a total of 16 mL. The cannula was inserted in 4 symmetric

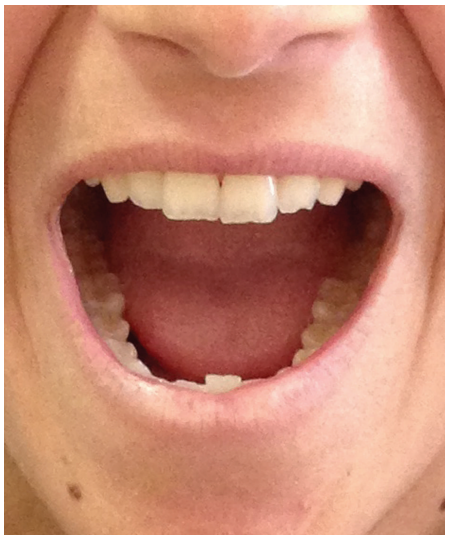

Figure 4: A 38-year-old woman with microstomia before treatment.

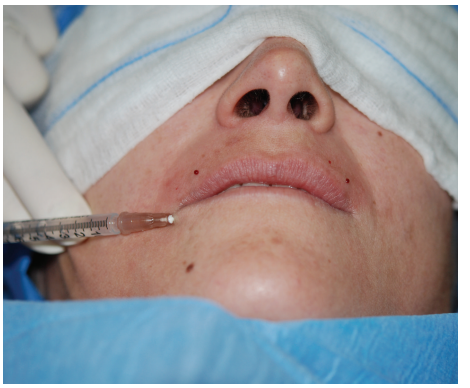

Figure 5: ADSCs injection.

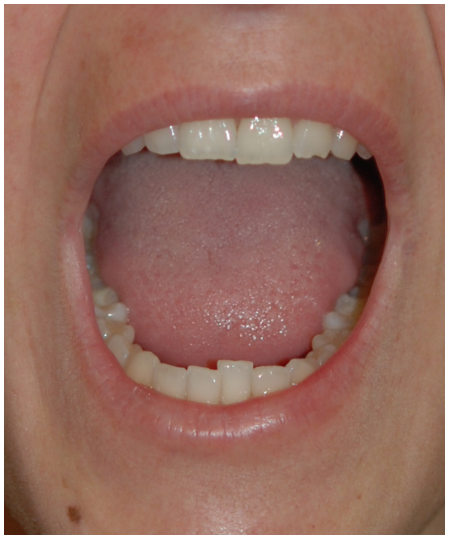

Figure 6: Follow-up at 1 year.

sites: 2 located just upon and 2 just below labial commissures. Antibiotics were given to all patients as a precautionary measure.

The selected areas for fat injection were six. Three were at the level of upper perioral region: two at the level of nasolabial fold (injected fat amount: 2 mL for each side) and one at the level of the upper lip (in which we injected 2 mL from the right side of the upper lip to the center and 2 mL from the left side of the upper lip to the center, both at the level of vermilion border). The other three selected areas were in the lower perioral region: two at the level of a line extending from the labial commissure toward mandibular border (injected fat amount: 2 mL for each side) and one at the level of the lower lip (in which we injected 2 mL from the right side of the lower lip to the center and 2 mL from the left side of the lower lip to the center).

2.4.2. ADSCs Isolation, Expansion (Standard Culture Method), and Injection. A total amount of 20 mL of lipoaspirate was harvested from the abdomen with the same technique of fat transplantation. Lipoaspirate was sent to the laboratory for cell cultivation within 1 hour and processed for ADSCs isolation.

Primary cultures of ADSCs derived from each scleroderma patient were expanded following the guidelines of current GMP. On the day of transplantation, cells were detached with 0.5 mM EDTA/0.05% trypsin for 5 min at 37°C and counted. Then, ADSCs were centrifuged at 1,500 rpm for 10 min, washed twice in PBS to remove serum, and finally resuspended in an adequate volume of synthetic stabilized HA solution (a 1.6% solution of synthetic HA, without chemical modifications and with a molecular weight of 1×10^3 KDa, very similar to the endogenous HA) at a standard concentration of 8×10^5 cells/mL. After gentle mixing, the suspension was kept under ambient conditions for 10–15 min to allow cell adherence to the hyaluronan matrix. Homogeneous dispersion of the cells within the gel was ensured by microscopical observation. Then, the cell supplemented HA solution was loaded into an injection syringe and carried to the operating room [29].

Usually after 3 weeks the patient went back to the operating room for the injection of the expanded ADSCs. This procedure did not require anaesthesia; only one patient asked for blunt sedation to calm down. The injection technique relied on preoperative topographic markings. Small aliquots of cell-enriched HA were infiltrated in the chosen areas.

The infiltration was done using 2 mL syringes provided with a 30-gauge 1/2 needle. We employed always 4 mL of hyaluronic acid for each patient, keeping a constant rate of 8×10^5 expanded ADSCs for each mL of HA.

Small aliquots of cell transferred by hyaluronic acid were infiltrated at the subcutaneous level of selected perioral regions: six areas, two in the upper lip and two in the lower lip (two lateral for each lip), plus one area for each opposite mouth corner region.

Table 3: Maximal Mouth Opening (MMO) by measuring the distance between the tips of upper and lower right incisive teeth.

Patients	Pretreatment (T0) opening mouth (cm)	Posttreatment opening mouth (cm)
1 L	3.6	4.2
2 L	3.4	3.8
3 L	2.6	3.4
4 L	3.1	3.8
5 L	3.3	4.2
1 A	3.4	4.4
2 A	2.5	3.1
3 A	3.3	3.7
4 A	2.9	3.6
5 A	3.2	4.0

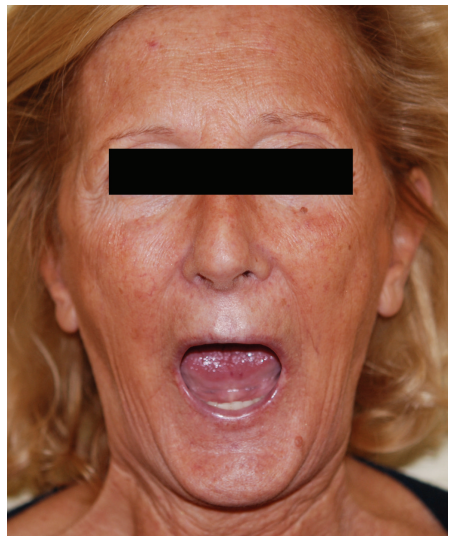

Figure 7: A 48-year-old woman before treatment.

3. Results

All patients treated presented a favourable outcome with improvement in subjective wellness of the skin in the perioral areas.

Both procedures improved the scores of IvMHISS scale at T1 versus T0. A significant score increase was shown in group L (p value 0.0234; t: 2.7940) and in group A (p value 0.0022; t: 4.4453); instead there was no statistical significant difference in improvement between groups L and A (p value 0.9619; t: 0.0485).

Maximal Mouth Opening, assessed as interincisor distance (Figures 7 and 8), was assessed by the same operator at baseline (T0) and after 1-year follow-up (T1). Patients of both groups benefited from the treatments for mouth opening (Table 3). A significant increase of mouth opening was shown in group L (p value 0.0171; t: 2.9994) and in group A (p value 0.0322; t: 2.5873); instead the difference of improvement between groups L and A was statistically insignificant (p value 0.5833; t: 0.5587).

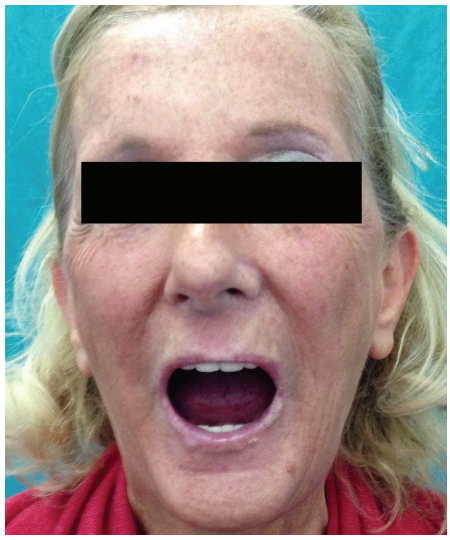

Figure 8: Aesthetic changes in the perioral region after autologous fat transplantation.

Table 4: Visual Analogic Scale (VAS). Score ranging from 1 to 10, whereby 1 indicates no improvement and 10 the maximum possible improvement.

Patients	VAS
1 L	6
2 L	5
3 L	6
4 L	7
5 L	8
1 A	7
2 A	8
3 A	8
4 A	8
5 A	8

When the patients were asked to express their overall personal opinion on the procedure they had undergone and its effectiveness, 80% (4/5) and 20% (1/5) in group L claimed to be rather satisfied and very satisfied, respectively. In group A, 20% (1/5) and 80% (4/5) claimed to be rather satisfied and very satisfied, respectively.

High values of VAS scale were obtained in both groups as shown in Table 4; instead the difference between groups L and A in terms of improvement was statistically insignificant (p value 0.0339; t: 2.5560) (i.e., we obtained improvement in groups A and L with both techniques, without any significant difference between patients treated with Lipofilling with respect to patients treated with ADSCs).

4. Discussion

SSc has an important social and emotional impact [30]. It is associated with increased functional impairment, body image distress due to skin lesions, and psychosocial comorbidity, particularly depression. Prevalence of depressive symptoms in SSc patients ranges from 36% to 65% and contributes to the worsening of any aspect of the disease [31].

Scleroderma patients report problems across multiple domains including fatigue, pain disability, sleep, interpersonal functioning, anxiety, and more generally physical and mental-health-related quality of life [30].

Fear of the disease (anxiety/panic) and depression are often not revealed by the patient because of the embarrassment of discovering an emotional illness. Scleroderma can be disfiguring and patients' psychosocial well-being is often affected more by disfigurement caused by facial changes. Low self-esteem alters social interactions and intimate relationships. The disease causes disability and may disrupt patients' ability to perform daily activities. Patients with mouth opening impairment cannot eat solid food, drink, and take care of their teeth thus highly limiting their social role in life.

Therapeutic strategies available today for chronic inflammatory diseases, such as SSc, often represent a way to obtain symptoms relief.

Au et al. [2] evaluated changes in vascular and musculoskeletal involvement in patients with interstitial lung disease comparing placebo treatment with oral cyclophosphamide (CYC). Authors demonstrated that there were no differences in dermal ulcer and musculoskeletal measures between the CYC and placebo groups at baseline and 24 months. Instead, mean oral aperture improved over time in the study participants.

Therapeutic repair encompasses the converging triad of rejuvenation, regeneration, and replacement strategies, which rely on self-healing processes, stem cell regeneration, and/or organ transplantation. Transplant medicine exploits the replacement strategy as a valuable option to recycle used parts and restore failing organ function by means of exogenous substitutes. It is, however, limited by donor shortage. Stem cell-based regeneration offers the next frontier of medical therapy through delivery of essentially unlimited pools of autologous progenitor cells to achieve structural and functional repair [32, 33].

However, translation into clinical applications requires the establishment of a regenerative medicine community of practice capable of bridging discovery with personalized treatment solutions. Indeed, this multidisciplinary specialized workforce will be capable of integrating the new science of embryology, immunology, and stem cell biology into bioinformatics and network medicine platforms, ensuring implementation of therapeutic repair strategies into individualized disease management algorithms.

Advanced cell-based therapies provide promising therapeutic possibilities to enhance repair or regeneration of damaged tissues also because their development may be greatly facilitated by the availability of an easily accessible and reproducible cell source.

In recent years, there has been growing emphasis on the use of mesenchymal stem cells (MSCs) for advanced cell therapy, due to their ability to be expanded in culture and to differentiate into multiple cell types. It is well established that MSCs secrete a broad spectrum of bioactive molecules with

immunoregulatory and/or regenerative activities. Through direct cell-cell interaction or the secretion of various factors, MSCs can exert a great effect on local tissue repair by modulating the local environment and activating endogenous progenitor cells. Taken together, these properties make MSCs promising candidates for cell therapy in various diseases.

In particular, adipose-derived stem cells (ADSCs), isolated from stromal vascular fraction, are able to differentiate into various cell lineages such as chondrocytes, osteoblasts, and adipocytes and to exert potent immunomodulatory, proangiogenic, antiapoptotic, antifibrotic, and anti-inflammatory effects important in preventing tissue degeneration. In particular, ADSCs' angiogenic and immunomodulatory properties, including a suppressive response on collagen-reactive T cells and the capacity to restore immune tolerance by inhibiting the inflammatory response in vivo, strongly suggest their use for chronic pathologies, especially for autoimmune and inflammatory disorders [34].

Whilst a set of clinical trials are demonstrating safety and efficacy of personalized cell-based treatments using ADSCs, translation to patients is faced by an obstacle: the heterogeneity in 13 treatment methods. Standardizing and automating the manufacturing and characterization of ADSCs allows better comparisons and identification of optimal treatments. Moreover, cell-based techniques are costly, as cells need to be sent to centralized cell factories for cell expansion. Cell factories often are multipurpose (supporting numerous different therapies), utilize capital-intensive clean rooms and equipment, and need highly qualified personnel to implement manual processes. Transport of biological materials to and from centralized cell factories poses additional regulatory problems.

In fact, despite the advantages of cell-based approaches, in terms of both effectiveness and therapeutic potential, the diffusion of such therapies is still limited by the high costs of manufacturing processes and qualified personnel, as well as by the stringent rules that govern the isolation and expansion of cells for therapeutic use (GMP requirements).

Because of the above discussed limitations, it was necessary to turn back to another well-established technique exploiting ADSCs' potentialities as fat transplantation.

Over the years, autologous fat transplantation or "lipofilling technique" has become the first-choice procedure to fill depressed areas, restore anatomical saliences, and correct contour deformities or volumetric defects [3, 4].

Fat is ubiquitous and easily obtainable in large quantity with a minimal invasive collection procedure, limited patient discomfort, and minimal ethical considerations: it may be injected safely and efficaciously.

After harvesting fat, it must be processed in order to limit the blood or oil within the lipoaspirates so that only pure fat will be used for injection.

There are many different ways to process fat after its collection. Many authors described sedimentation by gravity or decantation, filtering, and centrifugation [35–38].

Currently, there is no agreement among authors in terms of which is the best method for processing fat grafts. In our study, we used decantation for a personal preference; in fact, we noticed that fat processed through decantation is more fluid and so more useful for scleroderma patients who present very fibrous areas, where the access and treatment are difficult. Many experimental studies designed to compare these 3 techniques were evaluated and the debate continues as to which is the best.

5. Conclusion

In this project, based on the successful results of our previous pilot study on cutaneous manifestations of systemic sclerosis (SSc) [39], we aim to compare two methods, ADSCs infiltration and fat transplantation, to evaluate their potential in treating diseases with few or no therapeutic choices. Such personalized therapies allow each patient to represent a resource for the treatment of his/her pathology.

ADSCs infiltration is an advanced method that is not performed by many laboratories in Italy. There are several intermediate and critical substeps required for effective cell manipulation and the entire process must comply with strict regulations. It is a procedure particularly useful in cases where not even the insertion of the smallest diameter cannula is possible, such as skin fibrosis. The main disadvantage is represented by its high costs due to cell preparation in specific laboratories and because it is performed in two separate sessions, thus increasing patient discomfort.

Autologous decanted fat transplantation allows us to obtain satisfactory results in terms of tissue trophism and mouth opening improvement, taking advantage of adipose-derived stromal cells properties and exploiting the fluidity of fat obtained from fat decantation especially to treat very fibrotic areas. Compared to the ADSCs injection, its main disadvantage is represented by cannula use, which is more traumatic with respect to thin needle employment. Cannula use also requires access sites, which are sutured possibly leading to scars. Fat adsorption is unpredictable and may result in irregularities and asymmetries with respect to more precise ADSCs injection. On the other hand, fat transplantation is a more economic procedure, requiring only one surgical step and one-day hospitalization.

In our study, we compared two techniques to determine whether one prevailed in terms of results and patient satisfaction. We noticed that both procedures obtained significant results but neither one emerged as a first-choice method.

We strongly believe in fat potentialities especially in treating immune-mediated chronic diseases as scleroderma and we hope to contribute to studies aimed at standardizing fat use. The present clinical experimentation should be regarded as a starting point for further experimental research and clinical trials.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper and disclose no commercial interest that they may have in the subject of study and the source of any financial or material support.

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Clinical Study

A Prospective and Controlled Clinical Trial on Stromal Vascular Fraction Enriched Fat Grafts in Secondary Breast Reconstruction

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Background. Fat grafting is a tremendous tool in secondary breast reconstruction. Stromal vascular fraction (SVF) enriched fat grafts have been presenting promising results regarding volume maintenance. *Methods.* We developed a method that produces a superior SVF enrichment rate (2 : 1) in the operating theatre. This prospective and controlled trial analyzed quantitatively and qualitatively fat grafts with (stem cells group, SG) and without (control group, CG) SVF enrichment in secondary breast reconstruction, through MRI-based volumetry, immunophenotyping, and cell counting. Also, patient satisfaction, aesthetic outcomes, and complications were analyzed. *Results.* Volumetric persistence in the SG was 78,9% and 51,4% in the CG; however it did not reach statistical significant difference. CD90 was the only marker highly expressed in the SG and showed a positive correlation with volumetric persistence ($r = 0.651$, $p = 0.03$). Fat necrosis occurred in 4 patients in the SG and in none in the CG. Patients in the CG showed a trend to be more satisfied. Considering aesthetics, both groups presented improvements. No locoregional recurrences were observed. *Conclusions.* Results are encouraging despite the fact that SVF enrichment in a higher supplementation rate did not improve, with statistical significance, fat graft volumetric persistence. Enriched fat grafts have proven to be safe in a 3-year follow-up.

1. Introduction

One of the first descriptions of fat graft was done in 1893 [1] and only a century later did it regain credibility [2]. Coleman published new concepts and an innovative technique to obtain, process, and transfer adipose tissue, which produced consistent and long-lasting results in a variety of fat grafting applications [3–6]. In such a manner, an American survey showed that Coleman's principles were completely or partially incorporated by approximately 50% of the plastic surgeons interviewed [7]. However, many questions about the best technique to handle adipose tissue to be used still remain unanswered.

After Zuk et al. published that the adipose tissue is a rich source of mesenchymal stem cells, regenerative medicine gained an impulse [8–10]. Based on the differentiating capacity the adipose derived stromal cells (ADSCs) present, Yoshimura et al. developed the Cell Assisted Lipotransfer (CAL), the most high-tech type of fat grafting [11]. This technique transforms poor-ADSCs fat grafts into enriched ones, which, in theory, would improve graft take rate and, consequently, volume retention, by stimulating neoangiogenesis and stromal cells differentiation into new adipocytes [12–14]. Some authors have published randomized clinical trials using CAL with favorable and unfavorable results. However they employed different methods of cell obtainment, isolation, and preparation in different clinical settings [15–17]. Recently, De Francesco et al. emphasized that adipose tissue is an important living scaffold for ADSCs, which provides adequate environment for cells to survive [18]. Further, our group, in an *in vitro* model of admixed heterogeneous cell population, showed a positive correlation between the percentage of ADSCs and the increase in *in vitro* adipocyte differentiation [19].

Spear was the pioneer in the use of lipofilling to correct contour irregularities of reconstructed breasts [20] and, since then, a multitude of articles has been published regarding its versatility, safety, and complication rates in aesthetic and

reconstructive breast surgeries [6, 21–28]. Likewise, CAL was used in primary breast augmentation [29] and for correcting the sequelae of conservative breast cancer surgeries [30] and congenital deformities [31], but none of these studies was followed by quantitative evaluation. The purpose of this study was that of developing a prospective and controlled trial so as to qualitatively and quantitatively analyze the efficacy of fat grafts with and without a novel type of stromal vascular fraction enrichment as refinements in secondary breast reconstruction.

2. Patients and Methods

2.1. Ethic Statement. This prospective and controlled study was approved by the Ethics Committee of Faculdade de Medicina da Universidade de São Paulo, Brazil (code 498/11), and was registered at ClinicalTrials.gov under the number NCT01771913. This study was conducted at Carmino Caricchio Public Hospital in São Paulo, Brazil, between March 2012 and May 2015.

2.2. Patients. Patients were selected from the Breast Reconstruction Unit in order of presentation, and we started with those in the stem group (SG) followed by the control group (CG). The CG was matched by age, BMI, and radiotherapy with the SG.

The inclusion criteria in both groups were patients with primary breast reconstruction with contour irregularities and BMI between 20 and 35 kg/m^2, with sufficient fat in the abdomen. Radiotherapy, despite being a confounder factor, was not regarded as an exclusion criterion; however only patients with grades 1 and 2 in the LENT-SOMA scale [32] were included. A stratified blocked randomization was also done to evenly distribute patients with radiotherapy [33]. Patients with breast cancer active disease sequelae of breast cancer conservative treatment, smokers, and uncontrolled comorbidities were excluded.

So as to calculate the sample size, the STATISTICA software required assumptions based on volumetric persistence. In the stem group (SG), the estimated volumetric persistence was considered at 80%, while, in the control group (CG), it was considered at 40%, with a variance of 20% and an alpha error of 5%. Thus, 9 subjects were determined to be allocated in each group (STATISTICA, version 12, StatSoft, Tulsa, USA).

2.3. Suction Assisted Lipectomy, Processing, and Lipofilling Surgery. In the operating theater and standing up, patients had their breasts boundaries demarcated and split into four quadrants. Surgeries were conducted under general anesthesia, and autologous fat from abdomen [19] was harvested with a 3 mm cannula with standard low-pressure machine liposuction (−350 mmHg) [34, 35].

In the CG, fat was centrifuged in conic tubes for 2 minutes at 335 g. The intermediate layer was collected and transferred into 3 cc syringes and then grafted with a 1.4 mm blunt cannula in multiple layers mainly in the subcutaneous tissue in a crisscrossed manner.

In the SG, 600 cc of fat was obtained and centrifuged in 50 cc conic tubes for 2 minutes at 335 g. The intermediate layer collected was digested with 1/2 volume of 0.15% collagenase IA (Sigma-Aldrich, MO, USA) for 30 minutes at 37°C with constant homogenization. The aqueous layer was transferred into 50 cc tubes and collagenase was inactivated with 3 volumes of HBSS (Hank's Balanced Salt Solution, Invitrogen, CA, USA). This solution was centrifuged for 5 minutes at 750 g, and the pellets collected were transferred into a sterile bag containing the remainder volume of 300 cc fat centrifugation. The mixture of fat and stromal vascular fraction (SVF) was incubated for 15 minutes under constant homogenization allowing cell adherence to fat to occur. This process resulted in SVF enriched fat tissue at 2 : 1 enrichment ratio.

Supplemented fat grafting was conducted in the same fashion as that of CG. Samples of fat with and without SVF addition were sent to the laboratory for analysis. Considering the time for tissue processing, it took 1 minute to prepare 2.5 cc of fat in the CG while, in the same time period, 2.0 cc of fat was produced in the SG.

2.4. Cell Counting. At the laboratory, immediately after surgical procedure, fat graft samples with and without enrichment were digested in the same manner as that at the operating theater. SVF cells were counted and tested for viability using the trypan blue exclusion method in an automatic cell counter (Countess I, Invitrogen, CA, USA).

2.5. Immunophenotyping Characterization. So as to assess SVF cells immunophenotype, flow cytometric analyses were conducted in a Guava EaseCyte plus cytometer (Millipore, MA, USA) running the Guava Express Pro 8.1 software.

Freshly isolated SVF from adipose tissue samples (SG and CG) were filtered in 100 μM Nylon Net Filter (Millipore, MA, USA), so as to remove contaminant debris. The sample cells were incubated for 1 hour at 4°C with anti-human CD29-PECy5, CD31-PE, CD34-PerCP, CD45-FITC, CD73-PE, CD90-PE, and CD105-PE (BD Biosciences, NJ, USA). After incubation, labeled cells were washed with phosphate buffered saline (PBS, Invitrogen, CA, USA) and fixed with 1% p-formaldehyde (Sigma-Aldrich, MO, USA). Analyses were conducted on 5×10^3 labeled cells per sample for each antibody, and nonlabeled cell samples were used as control. Laboratory personnel were blinded to the sample analysis.

2.6. Breast Volumetry. Patients were MRI scanned without previous injection of gadolinium contrast, and, 6 to 19 months after the lipofilling surgery, they were scanned again with the purpose of determining the breast volume. A 1.5 Tesla scanner (Inthera, GE, Contagem, Brazil) was employed with 3 mm thick slices. We developed a new strategy for determining and computing the boundaries and volume of a reconstructed breast in a more precise way. Just before the MRI exam, the senior investigator marked the boundaries of each breast. With a dermographic marker, a line was drawn throughout medial, lateral, inferior, and superior breast limits. Vitamin E capsules (external markers) were applied on skin over the line, so as to allow more precise regions of interest (ROI)

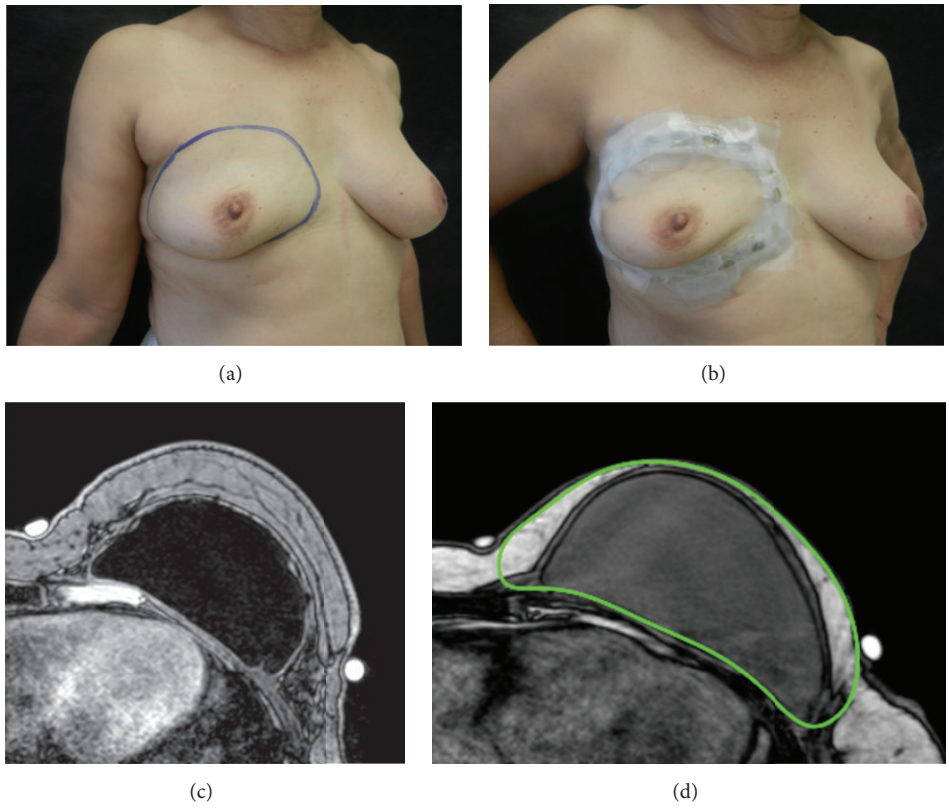


Figure 1: Method for MRI-based breast volume measurement. (a) Breast boundaries demarcated, (b) external markers applied, (c) axial sequence showing the breast medial and lateral limits, and (d) a selected region of interest (ROI) with the OsiriX software.

to be determined, and performed on axial sequences by an independent radiologist (Figure 1). OsiriX software, 32 bits, free version (Pixmeo, CA, USA) was utilized to calculate breast volume. Two calculations were done per exam and the average determined was taken as the final breast volume.

2.7. Patient Satisfaction Assessment. A patient satisfaction survey was conducted for this study. We included a modified Michigan's questionnaire [36], a visual analogue scale with 5 possibilities (very unsatisfied, unsatisfied, neither unsatisfied nor satisfied, satisfied, and very satisfied), and a score scale ranging from 1 to 10 to assess the final breast aesthetic result. Patients from both groups answered the satisfaction questionnaire at the time of the postoperative MRI scan.

2.8. Aesthetic Results Evaluation. Five plastic surgeons who were not involved in the conduction of the study and had different types of breast reconstruction expertise were invited to objectively and independently analyze improvements in breast contour. Panels containing blinded frontal pre- and postoperative photos were prepared for analysis. Surgeons were able to choose 5 different situations: strongly worse, mildly worse, no change, mild improvement, and strong improvement. For each score, a value was attributed as follows: −2, −1, 0, +1, and +2. For analysis purposes, the sum of all five scores, per patient, was taken as the final value.

2.9. Clinical Events. Patients of both groups were monitored for the occurrence of adverse events of any type, locoregional cancer recurrences, fat necrosis, oil cysts formation, skin necrosis, and infection.

2.10. Statistics. The data gathered was analyzed by means of the R Statistical Software, version 2.15.2 (R Foundation, Vienna, Austria). The data is expressed by mean (range and standard deviation), median (range), and percentages. Comparison between groups was done with Student's t-test or Mann-Whitney for age, BMI, breast volumetry, fat graft volume, time of follow-up, basal cell counting, question 6 of the self-assessment questionnaire, and surface markers expression. Wilcoxon was used to compare the number of cells in the pellets before and after enrichment with SVF cells. Fisher exact test was employed to analyze radiotherapy distribution, occurrence of fat necrosis, and questions 2, 3, 4, and 7 of the self-assessment questionnaire.

3. Results

Eleven patients were recruited for the SG and nine were recruited for the CG. However, one patient withdrew her informed consent, and the CG finished with 8 participants. Patient demographics are shown in Table 1.

The method developed by the authors (2 : 1 enrichment rate) and used to boost the fat grafts in the SG produced an

Table 1: Patient demographics.

	Age	BMI before	BMI after	Types of tumor	Type of reconstruction	Time from mastectomy to fat graft	Graft follow-up	Time between MRIs	RTX
					Stem group				
1	55	24,2	24,0	LCI	LD + IMPL	3 y 8 m	4 y 1 m	12 m	Yes
2	47	25,4	25,8	Mucinous	LD + IMPL	3 y 9 m	3 y 9 m	11 m	Yes
3	49	28,0	27,6	DCI	EXP + IMPL	3 y 2 m	3 y 2 m	15 m	No
4	48	27,2	27,6	DCI	LD + IMPL	3 y 7 m	3 y 2 m	15 m	Yes
5	51	28,7	28,7	DCI	EXP + IMPL	2 y 6 m	3 y 1 m	13 m	No
6	41	27,5	29,1	DCIS	TRAM	4 y 10 m	3 y 1 m	16 m	Yes
7	54	24,0	25,1	DCI	LD + IMPL	3 y	2 y 10 m	13 m	Yes
8	44	23,5	23,3	DCI	LD + IMPL	4 y 1 m	2 y 9 m	16 m	Yes
9	56	23,9	23,9	DCI	TRAM	4 y	2 y 9 m	19 m	Yes
10	58	25,6	25,6	Nontumor	No reconstruction	20 y	2 y 6 m	13 m	No
11	43	30,9	29,7	LCI	Seq explantation	4 y 1 m	1 y 8 m	17 m	Yes
Average						**5 y 2 m**	**36 m**	**14,5 m**	**72,7%**
					Control group				
1	51	29,2	29,2	DCI	EXP + IMPL	3 y 2 m	2 y 6 m	16 m	No
2	40	20,8	20,4	Medullar	EXP + IMPL	3 y 1 m	1 y 11 m	19 m	No
3	56	32,4	32,6	DCI	Seq explantation	7 y 3 m	1 y 9 m	19 m	Yes
4	69	25,9	28,1	DCIS	LD + IMPL	16 y 6 m	1 y 3 m	13 m	Yes
5	38	24,1	26,1	DCI	LD + IMPL	2 y 5 m	1 y 1 m	11 m	Yes
6	36	24,1	23,2	DCI	LD + IMPL	8 y 3 m	1 y	11 m	Yes
7	59	25,6	25,2	LCI	EXP + IMPL	5 y 8 m	8 m	7 m	No
8	49	24,9	24,3	DCIS	Seq explantation	2 y 8 m	8 m	7 m	Yes
Average						**6 y 1 m**	**16 m**	**12,9 m**	**62,5%**
p values	0.977	0.765	0.861	nm	nm	nm	nm	0.414	>0.999

BMI, body mass index; LD, latissimus dorsi; Impl, implant; Seq, sequelae; DCI, ductal carcinoma invasive; DCIS, ductal carcinoma in situ; LCI, lobular carcinoma invasive; Exp, expander; m, months; RTX, radiotherapy; nm, not measured; y, years.

Table 2: Basal and after-enrichment cell counting in the SG.

	Basal cell counting	Cell counting after enrichment*	Cellularity shift
n	10	11	10
Average	524.760,0	1.108.818,2	679.940,0
Median	175.000,0	400.000,0	390.000,0
Minimum–maximum	21.600–2.500.000	42.000–6.400.000	20.400–3.900.000
SD	791.542,2	1.829.290,4	1.147.997,9

*Wilcoxon $p = 0.005$.

enrichment of 2.6-fold the number of basal cells ($p = 0.005$) (Table 2). Expressions of cell surface markers done in the fresh SVF are shown in Table 3 and a wide variability in their expression was observed among all patients. Taking both groups together, CD45 was the least expressed, while CD29 and CD90 were the most expressed. However, the mesenchymal cell marker CD90, highly expressed in the SG, was the only marker that reached a statistically significant difference among all ($p = 0.026$). There seemed to be a positive correlation between CD31, CD73, CD90, and CD105 expressions and volumetric persistence; however CD90 was the only marker that showed significance ($r = 0.651$ and $p = 0.03$) (Figure 2).

Volumetric persistence in the SG was higher (78.8%, SD = 74.9) than that in the CG (51.4%, SD = 18.4); however, it did not reach a statistically significant difference ($p = 0.31$). Fat necrosis was present in four patients in the SG and in no patients in the CG ($p = 0.103$) (Table 4). Fat necrosis was surgically removed and the pathological findings confirmed this diagnosis for 3 patients. One patient was observed and the ultrasound follow-up showed no need for intervention.

Table 3: Surface markers expression in both groups (SG and CG).

	CD29 (%)	CD31 (%)	CD34 (%)	CD45 (%)	CD73 (%)	CD90 (%)	CD105 (%)
				Stem group			
n	7	7	5	7	7	7	7
Average	70,09	20,56	43,92	8,59	40,36	79,01	35,81
Median	80,54	0,74	18,64	1,6	39,78	81	35,52
Minimum	21,78	0	11	0	0	58,86	0
Maximum	94,9	65,92	92,3	32,5	88,88	96,66	89,78
SD	28,34	28,86	41,39	12,38	33,55	15,37	30,11
				Control group			
n	4	4	4	4	4	4	4
Average	75,37	33,34	38,94	15,42	28,84	26,99	36,71
Median	73,88	31,78	45,23	0,3	28,3	24,09	33,88
Minimum	56,48	20,9	0	0	26,54	1,38	0
Maximum	97,24	48,9	65,3	61,08	32,2	58,38	79,08
SD	19,69	12,01	28,2	30,44	2,44	27,95	32,45
				Total			
n	11	11	9	11	11	11	11
Average	72,01	25,2	41,71	11,07	36,17	60,09	36,14
Median	80,54	20,9	38,32	0,62	28,86	61,82	34,6
Minimum	21,78	0	0	0	0	1,38	0
Maximum	97,24	65,92	92,3	61,08	88,88	96,66	89,78
SD	24,6	24,18	34,09	19,54	26,66	32,64	29,33
p	0,751[a]	0,315[b]	0,905[b]	0,527[b]	0,400[a]	0,026[a]	0,964[a]

[a]*t*-Student for independent samples, [b]Mann-Whitney.

In the long-term follow-up of both groups, no adverse events of any type, no infections, no skin necrosis, and no locoregional recurrences were observed.

The analysis of the satisfaction assessment questionnaire showed that all patients in both groups would choose to undergo breast reconstruction, and they were sufficiently informed about the fat grafting procedure. In both groups, the vast majority of patients were satisfied with the results of fat grafting ($p = 0.603$), would undergo the fat grafting procedure again ($p > 0.999$), and would recommend the fat grafting procedure to a friend ($p = 0.546$). When patients were allowed to freely give a score to their cosmetic result (self-assessment), scores ranged from 5 to 10 in SG and from 8 to 10 in CG ($p = 0.075$). These results show a strong trend in patients of the CG to be more pleased than patients in the SG. When satisfaction was evaluated through a visual analogue scale, patients of both groups were similarly satisfied ($p = 0.52$).

Initially, the 5-peer analysis showed disagreement in the pair-to-pair comparison and in the general comparison, with low values of kappa coefficient. So, changing the 5 subsets into 3 (worsened (–1), nothing changed (0), and improved (+1)), surgeons agreed to a minor degree (kappa = 0.131, confidence interval = 0.020; 0.242). Figures 3 and 4 show patients that were categorized as showing "improvement" by all peers. When computing the new scores, patients in the SG and in the CG received the respective scores (average) of 2.9 and 2.3 ($p = 0.60$) and, therefore, were regarded as presenting similar improvement.

4. Discussion

Taking into account age, BMI taken before and after fat grafting, time which elapsed between MRIs, and radiotherapy distribution, the groups are statistically similar. At the very beginning of the study design, in taking into account the sample size and radiotherapy as confounder factor, the stratified block randomization allowed an even distribution [33]. The high incidence of radiotherapy represents the great majority of patients seeking delayed breast reconstruction, and this is corroborated by other publications [37, 38]. The effects of radiotherapy on fat graft retention still are controversial. Rigotti et al. showed the damage to the microcirculation caused by radiotherapy and the benefits fat grafting promoted, including progressive regeneration and neovessel formation [39]. Khouri et al. recently showed that breast reconstruction after radiotherapy needed an average of 4.8 procedures compared to the 2.7 ones for the nonirradiated group [40], and this is in accordance with the work of Losken et al. [24] and, more recently, with the paper published by Longo et al. [41]. In turn, de Blacam et al. [25] showed the same rate of complications when fat grafting was used in secondary breast reconstruction with and without radiotherapy. Choi et al. published the same fat graft volume retention rate in reconstructed breasts with and without radiotherapy [42]. Regarding volumetric persistence and the incidence of complications, the present study showed no difference between the patients who had received radiotherapy and those who had not before fat grafting.

Table 4: Volumetry and fat graft complications.

	Breast volume Preoperative (cc)	Graft Volume (cc)	Breast volume Postoperative (cc)	Volumetric* Persistence	Fat Necrosis**
			Stem group		
1	630.45	45	618.15	−27.33%	Yes
2	527.11	92	589.83	68.17%	Yes
3	623.22	177	735.37	63.40%	No
4	885.96	137	1.005.43	87.20%	Yes
5	686.93	180	857.93	95.00%	No
6	1.048.12	147	1,454.60	276.51%	No
7	702.85	141	800.25	69.07%	No
8	547.30	117	584.71	31.97%	Yes
9	732.20	111	842.97	99.79%	No
10	254.14	171	377.69	72.25%	No
11	512.32	159	562.22	31.38%	No
Average		**134.3**		**78.8%***	**36,4%****
			Control group		
1	674.58	96	732.6	60.50%	No
2	470.92	75	525.57	72.86%	No
3	383.83	147	447.55	41.63%	No
4	785.21	111	861.3	68.60%	No
5	603	108	678.85	70.23%	No
6	552.97	115	584.7	27.65%	No
7	778.7	138	824.9	33.50%	No
8	253.25	102	290.2	36.20%	No
Average		**111.5**		**51.4%***	**0%****

*Mann-Whitney $p = 0.301$; **Fisher exact $p = 0.103$.

In our study, two patients in the SG behaved as outliers for volumetry. Patient 6 had a TRAM flap reconstruction and put on weight, 6 kg, by the time the postoperative MRI scan was conducted. According to Gutowski et al. [43], fat grafts may show volume change with weight fluctuation and, by taking into account that all patients with TRAM flaps in this series had high volumetric persistence rates and in this patient's case in particular, the weight gain might be responsible for the high volume determined. Patient 1 was irradiated and received the smallest volume of fat grafting. Considering the volumetric loss that all fat grafting may undergo and the internal error of the volumetric calculation tool in the pre- and postoperative MRI scan, the final volume ended up negative.

Inspired by Yoshimura et al.'s previous publications [11, 44], we developed a method that produced an enrichment rate (2 : 1 ratio) that is higher than CAL (1 : 1 ratio), which could be reproduced in the operating theater by other investigators with no difficulties. Based on previous papers and assumptions [13–15, 45, 46], the idea of adding more SVF cells into a fat graft that could render a better biological framework and warrant more volumetric persistence in the long term, considering that there would be more mesenchymal cells to differentiate into new adipocytes and secrete a greater amount of trophic factors, such as proangiogenic and antiapoptotic factors [47, 48], sounded appealing. However, despite all the authors' efforts to produce a substantial enrichment, the volumetric persistence in the SG did not reach a statistically significant difference when compared to that in the CG.

As much as we know, there is no published prospective and controlled study that objectively measured breast volumetry in the field of breast reconstruction using SVF enriched fat grafting. Our results are very optimistic since volumetric persistence as high as this one was only reported by Kølle et al. [15], who achieved persistence of 80.9% in the study group versus 16% in the control group, and Tanikawa et al. [16], who used CAL for the correction of craniofacial anomalies and achieved 88% of volumetric persistence in the study group when compared to 54% in the control group. The former employed a super enrichment rate with cultured-expanded cells injected in the arm, while the latter used manual CAL for correcting soft tissue defects associated with craniofacial microsomia. Concerning cell enrichment ratio, our study relies somewhere between these previous studies, and the answer to explain our results may be the variability in the type of breast reconstruction techniques, which present different amounts of scar tissue in the recipient bed.

Conversely, Choi et al. [42] used 3D-imaging volumetry to analyze volumetric persistence of centrifuged fat grafts, without enrichment, in secondary breast reconstruction. They found an average of 42% volume retention at 140 days after surgery, which is regarded as a short-term follow-up when it comes to fat grafting volume persistence. The RESTORE-2 study [30] and the study conducted by

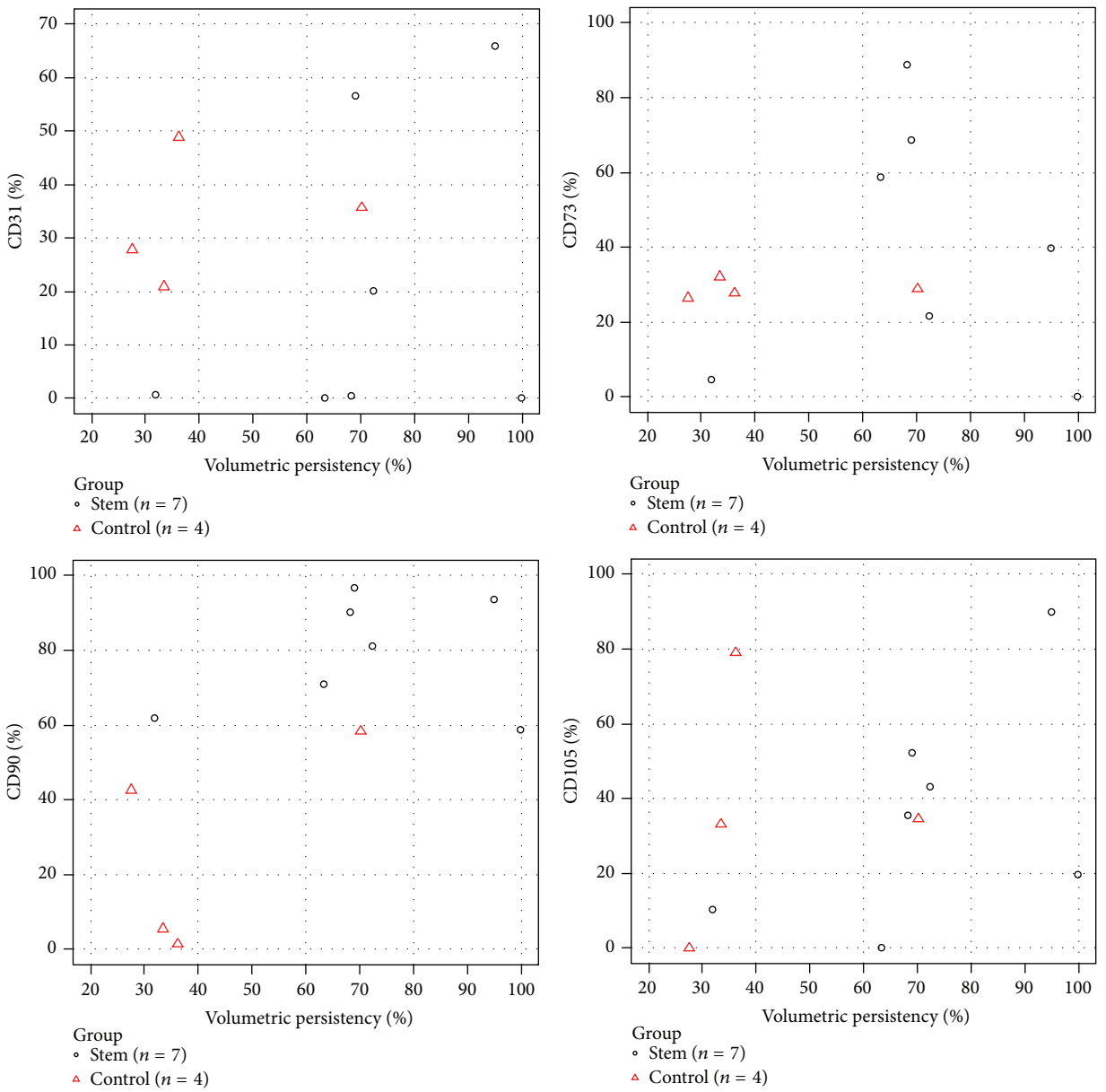
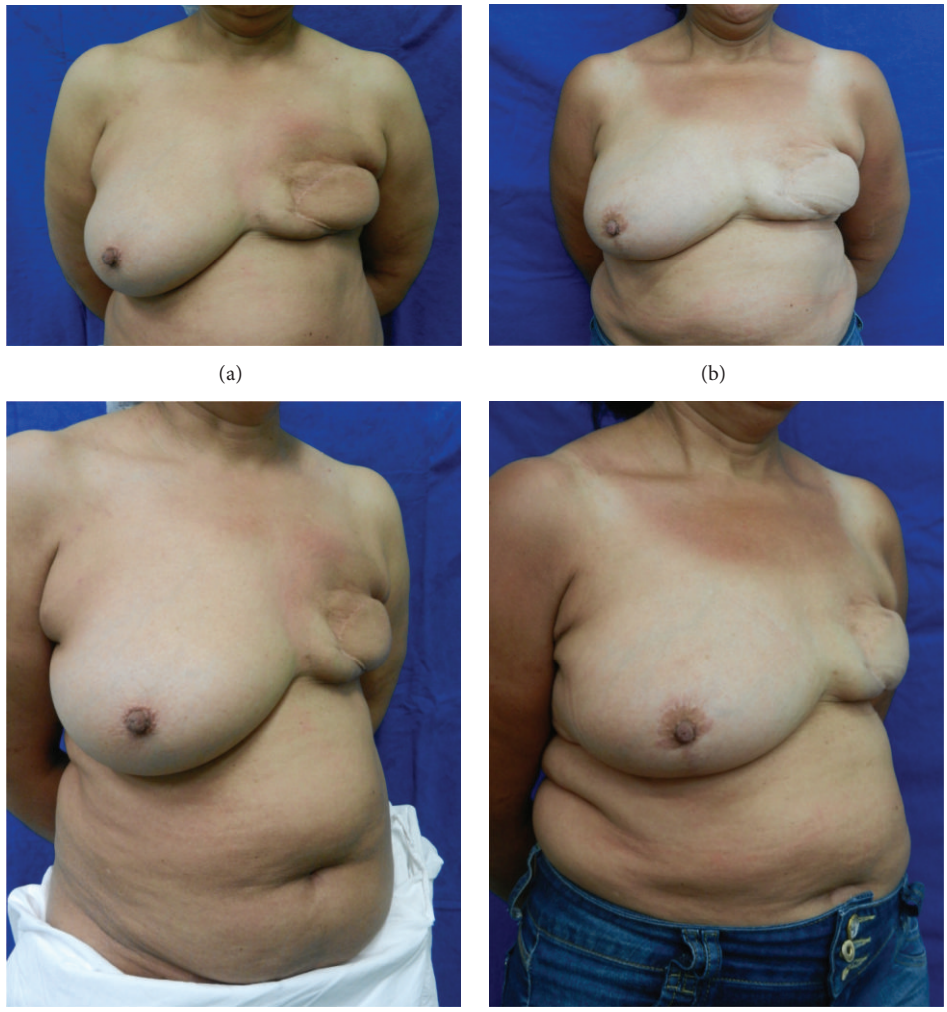


Figure 2: Charts show a positive correlation between surface markers expression and volumetric persistence for CD31, CD73, CD90, and CD105.

Gentile et al. [31] employed enriched fat grafting by means of the Celution system (Cytori, San Diego, USA) in secondary breast reconstruction, and, despite the good results published, these studies do not possess objective volumetry. Yoshimura et al. [49] used CAL as rescue for breast implant complications and had volume retention between 40 and 80%, and the main criticism to their study is that it does not have a control group.

Likewise, Peltoniemi et al. [17] used enriched fat grafts for primary breast augmentation. Patients of both groups in this controlled study had an average of 50% volumetric persistence, a similar retention rate obtained by other authors, who did not employ stromal cells enrichment but used the BRAVA system [50, 51]. Comparatively to Peltoniemi et al. work, Spear and Pittman [52] showed 39% volume retention in primary breast augmentation with conventional centrifuged fat grafting, and, based on the results published by Khouri and others [21, 23, 51], they drew attention to preoperative breast external expansion as a method of improving some important aspects of the recipient bed, such as neoangiogenesis and a favorable interstitial pressure, before cosmetic breast augmentation with adipose tissue.

The immunophenotyping of the fresh stromal vascular fraction in this study showed a similar surface marker profile compared to that published by Matsumoto et al. [46]. The great majority of studies have published immunophenotyping of the stromal vascular fraction cells after at least one expansion, and our study focused on the analysis of the fresh



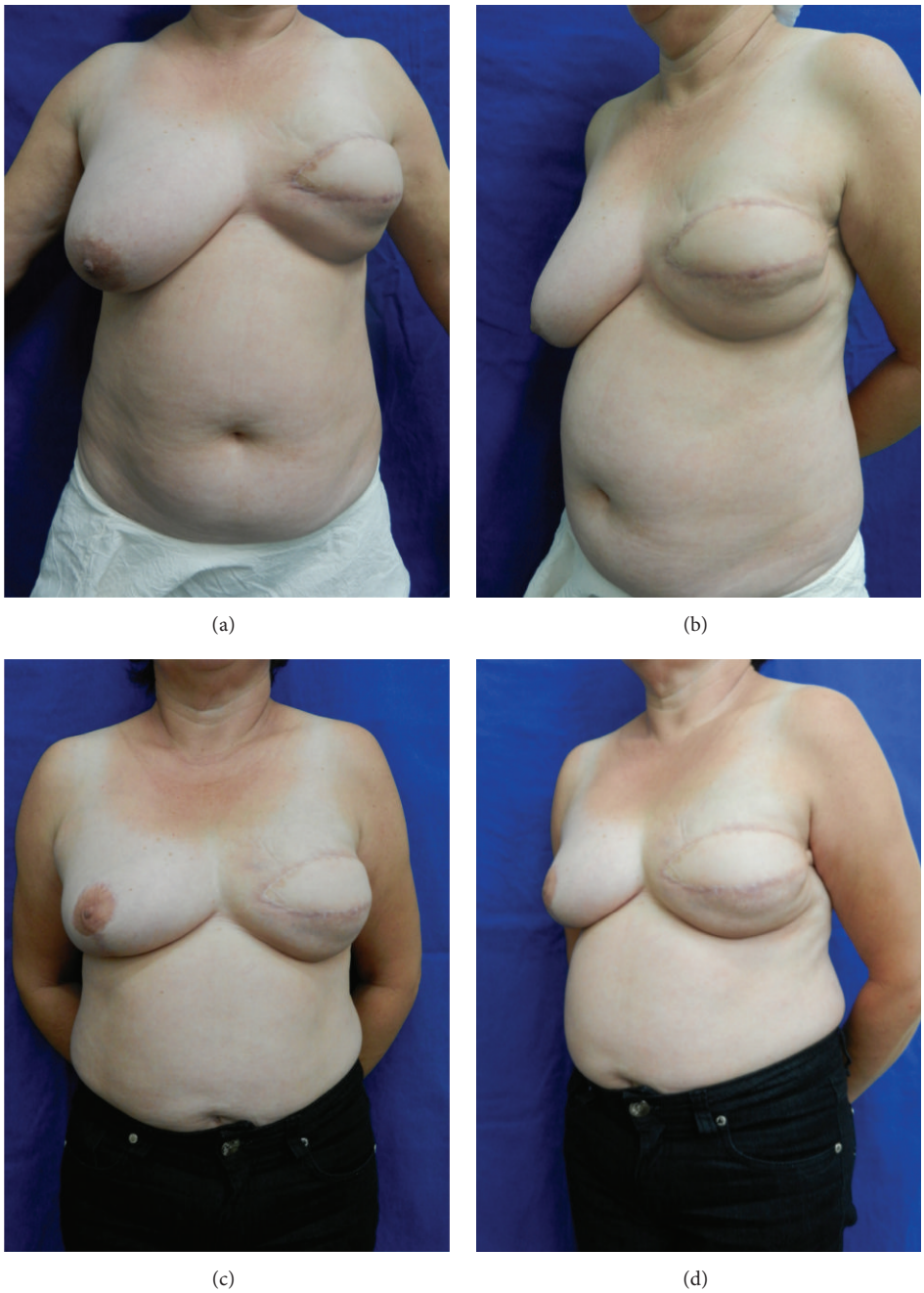
(a) (b) (c) (d)

Figure 3: (a, c) Preoperative frontal and oblique views of a CG patient in which 147 cc of fat was injected; (b, d) 12-month postoperative frontal and oblique views with volumetric persistency of 41.6%.

SVF. Thus, we could observe that the flow cytometric analysis showed a very individualized profile of surface markers expression. In such a way, no patient presented a similar profile. Patients in the SG expressed more CD90 than patients in the CG, and there was a positive correlation between the expression of CD90, a typical mesenchymal marker, and volume persistence. Meanwhile, patients in both groups, who presented high volumetric persistence, demonstrated high CD90 expression.

Modified Alderman's questionnaire showed the importance of breast reconstruction after mastectomy. Moreover, secondary breast reconstruction with fat grafting, with or without stromal cells enrichment, promoted a high level of patient satisfaction. Patients in the CG tended to become more satisfied than the patients in the SG, and the explanation for that is the incidence of fat necrosis that caused distress regarding local recurrence and led to reoperation in 3 patients. Fat necrosis only occurred in the SG and in patients who received radiotherapy. We speculate that even though stromal cells are more resilient to hypoxia and were present in greater number than that in the CG grafts, in some cases, together with mature adipocytes, they were not able to survive the hostile recipient bed, marked by intense fibrosis secondary to radiotherapy and surgical manipulation and damaged microcirculation [39, 45]. Another possible explanation is that the marked fibrosis present in the recipient bed could have misconducted these cells to another path of differentiation contributing to the formation of small nodules of fat necrosis [53]. Similarly, Yoshimura et al. [11] reported two cases of focal fibrosis on thorax and breasts when they injected SVF cells suspended in saline just after injecting fat for cosmetic breast augmentation. They discussed the possible absence of signaling from the adipose tissue, reassuring the importance of employing it as a vital living scaffold [53].

The kappa coefficient showed a weak agreement among raters; however, the evaluation of aesthetic results was positive, meaning that contour irregularities were improved by the fat grafting procedure in patients of both groups. In



(a) (b)

(c) (d)

Figure 4: (a, b) Preoperative frontal and oblique views of a SG patient in which 141 cc of enriched fat was injected; (c, d) 12-month postoperative frontal and oblique views with volumetric persistency of 69.1%.

the complete follow-up, patients have not presented infection, skin necrosis, or any donor site morbidity. In our study, despite not intending to be a long-term follow-up outcome, locoregional recurrences have not emerged in an average follow-up of 16 months in the CG and 36 months in the SG, and this data may contribute to the existing literature about ADSCs enriched fat grafting safety in secondary breast reconstruction. Three patients, 1 from the SG and 2 from the CG, were diagnosed with DCIS at the time of mastectomy, but none of them fulfilled the requirements published by Petit et al. [54] in a way to be considered as being at a higher risk for local recurrences. However, these patients still are under a regular and watchful follow-up. Our findings regarding oncological safety are in agreement with others previously published [43, 55–57].

Limitations to this study include the high incidence of radiotherapy among patients and the absence of randomization, which is justified by the fact that this study was carried

out in a single breast reconstruction unit without a large number of patients requiring refinements to be randomized in each group.

5. Conclusions

The results of this study are encouraging despite the fact that enrichment of fat grafts with SVF cells at a 2 : 1 proportion did not present a better volumetric persistence rate in the secondary breast reconstruction scenario. A real time higher supplementation rate of fat grafts with SVF cells, without expansion, can be done in the operating theater if appropriate material and personnel are available. Considering an average follow-up of 3 years, the enrichment of fat grafts with SVF cells has proved to not promote locoregional recurrences. The incidence of fat necrosis raises concerns over enriched fat grafts at a 2 : 1 proportion, and they may not be suitable for patients who have previously received radiotherapy. The adequate enrichment rate to ensure a higher volumetric persistence is to be determined by future studies.

Conflict of Interests

Authors have no conflict of interests to disclose.

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Research Article

Are They Really Stem Cells? Scrutinizing the Identity of Cells and the Quality of Reporting in the Use of Adipose Tissue-Derived Stem Cells

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There is an increasing concern that the term adipose tissue-derived stem cell (ASC) is inappropriately used to refer to the adipose stromal vascular fraction (SVF). To evaluate the accuracy and quality of reporting, 116 manuscripts on the application of ASC in humans and animals were examined based on the 2013 published International Federation for Adipose Therapeutics and Science (IFATS)/ International Society for Cellular Therapy (ISCT) joint statement and in reference to current guidelines for clinical trials and preclinical studies. It is disconcerting that 4 among the 47 papers or 8.51% (CI 2.37–20.38) surveyed after publication of IFATS/ISCT statement reported using ASCs but in fact they used unexpanded cells. 28/47 or 59.57% (CI 44.27–73.63) explicitly reported that adherent cells were used, 35/47 or 74.47% (CI 59.65–86.06) identified expression of surface markers, and 25/47 or 53.19% (CI 14.72–30.65) verified the multilineage potential of the cells. While there are a number of papers examined in this survey that were not able to provide adequate information on the characteristics of ASCs used with some erroneously referring to the SVF as stem cells, there are more room for improvement in the quality of reporting in the application of ASCs in humans and animals.

1. Introduction

The use of adipose tissue-derived stem cells (ASC) has gained popularity as alternative to bone marrow derived stem cells or to human embryonic stem cells, particularly as the manner by which the source tissue is collected is less invasive compared to the former and does not have serious ethical issues compared to the latter. In view of pronouncements on the beneficial use of stem cells in the popular media [1], acknowledging the real therapeutic potential of stem cells is yet to be made as the scientific community is just starting to unravel their efficacy and safety [2].

There has been a major confusion though in the use of the term adipose tissue-derived stem cells, with some authors referring to the heterogeneous stromal vascular fraction (SVF) after centrifugation as stem cells. To provide guidance on this, the International Federation for Adipose Therapeutics and Science (IFATS) and International Society for Cellular Therapy (ISCT) in 2013 published a joint statement regarding the characteristics and differences of the two portions when derived from the adipose tissue with recommendations on how both should be ascertained [3]. A number of other guidelines and expert opinions also have been published in relation to the use of stem cells in clinical trials and the importance of reporting guidelines for preclinical studies [4–9].

Our objective is to determine the exact identity of the ASCs used in human patients and animal subjects as reported in published papers and the quality of reporting in reference to existing guidelines and expert recommendations.

2. Materials and Methods

The search engine Pubmed (http://www.pubmed.org/) was used to come up with the list of manuscripts and publications related to researches or clinical reports employing ASC in human patients and animal subjects from January 2011 to June 2015. For a report to be included in this survey, it must indicate adipose tissue-derived stem cells in either the title or the abstract. The keyword "adipose tissue-derived mesenchymal stem cells" combined with "clinical trial," "therapy," or "patient" was used to generate the list. We excluded from the list review articles and those reports that utilize ASC for *in vitro* experimentation only. Relevant articles were initially identified by the title and abstract and subsequently each paper was examined further by verifying whether indeed the use of ASC was reported and applied in either human patients or animal subjects. It is acknowledged that this search method was not exhaustive as there are manuscripts in journals that are not included in Pubmed.

We evaluated the papers in four key characteristics to ascertain the identity of cells used in reference to the IFATS and ISCT recommendations. These include (1) the use of expanded cells as treatment regimen, alone or in combination with other agents, (2) explicitly mentioning plastic adherent cells that were used, (3) phenotyping of surface markers, and (4) conducting multilineage differentiation of the expanded cells. We divided the papers further into two groups to examine the impact of the IFATS and ISCT statement on how authors would report the identities of the cells used. These were the before IFATS/ISCT statement group, comprised of papers published during the period 2011–2013, and the after IFATS/ISCT statement group, comprised of papers published on 2014-2015.

For papers utilizing ASC in human patients, the key areas considered were ethics (reported undergoing ethical review and approval for the study via an institutional review, reported obtaining an informed consent, and reported clinical trial registration), safety (processing the cells in Good Manufacturing Practice or GMP facility, testing for genomic stability, and contaminants prior to use), and cell characteristics (viability, number of cell passages, and number of cells administered). For papers utilizing ASC in animal subjects, the key areas considered were ethics (reporting of oversight and approval of the study via the Animal Care and Use Committee), study design (allocation to groups/randomization, calculation of sample size, and blinding), experimental animals (species, sex, age, and group size), and cell characteristics (viability, number of passages, and number of cells delivered).

3. Statistical Analysis

Data are presented as number, proportion, and percentages with binomial 95% confidence interval. Proportions were analyzed using Fisher Exact Test and post hoc analysis using the statistical software GraphPad Prism. The results are presented as percentages with 95% confidence intervals and a value of $P < 0.01$ was considered to be statistically significant.

Table 1: Papers included in this survey that reported on the use of ASC for human and veterinary clinical trials.

Classification	Source of adipose tissue for ASC	Type of transplantation	References
Human clinical trial	Human	Autologous	[10–39]
		Allogeneic	[37, 40–44]
Veterinary clinical trial	Animal	Autologous	[45, 46]
		Allogeneic	[47]

4. Results

4.1. Overall Description of Selected Articles. The electronic search identified 623 articles. Based on the title, abstract, and description of the paper, a total of 149 papers were shortlisted after all the review articles, duplicates, and papers not relevant to the survey were removed. Only 116 papers were retrieved for evaluation after manuscripts not complying with our criteria were taken out further from the list [10–125]. All in all, 34 papers reported the use of ASC in human patients, 81 papers reported the use of ASC in animal subjects, and 1 paper reported the use of ASC in both human and animals. Based on the year of publication, 69 papers were grouped as before IFATS/ISCT statement group while 47 papers were determined to comprise the after statement group. 88.57% (31/35) of papers reporting application for human use were clinical trials while 11.43% (4/35) were case reports. 81.71% (67/82) of the papers involving animal use were human preclinical studies, 3.66% (3/82) were veterinary clinical trials, and 2.44% (2/82) were veterinary preclinical studies. 12.20% (10/82) were classified as basic research. Table 1 summarizes the specific papers in this survey that were classified as human and veterinary clinical trial.

We observed in this survey varying sources of adipose tissue from where the lipoaspirates and cells were derived. Overall, 27.59% (32/116) had human patients as sources, 26.72% (31/116) utilized tissues from human donors, 27.59% (32/116) derived the tissues from animal subjects and 12.07% (14/116) from animal donors, 0.86% (1/116) reported utilizing human adipose tissue-derived stem cell from a commercial source, 0.86% (1/116) utilized tissues from human patients and human donors, another 0.86% (1/116) derived the tissues from human donor and animal subjects, and 2.59% (3/116) derived the tissues from a human donor and an animal donor, while the remaining 0.86% (1/116) did not indicate the source of the tissue. We also classified the papers according to the nature of cell transplantation whether it will be autologous, allogeneic, or xenogeneic. Most of the papers on human application reported autologous use at 80% (28/35), with 14.28% (5/35) being allogeneic; 2.86% (1/35) were not classified as no source was indicated while the last 2.86% (1/35) were both autologous and allogeneic. For animal studies, most are allogeneic at 42.68% (35/82) or xenogeneic at 40.24% (33/82). 12.20% (10/82) are autologous and 4.88% (4/82) are both allogeneic and xenogeneic. Majority of the reports at 85.34% (99/116) indicated benefits results with the use of ASC while 12.93% (15/116) indicated otherwise.

Table 2: Summary of papers that reported human application of ASC according to the route of delivery of stem cells.

Route of delivery	References
Intra-arterial	[37]
Intra-articular	[17, 42]
Intradermal	[11, 35]
Intramuscular	[10, 27]
Intravenous	[13–15, 41, 43, 44]
Injection at submucosal, mucosal, and tract wall	[12, 16, 28, 40]
Subcutaneous	[13, 14, 18, 26, 33]
Transplanted with scaffold material or tissue graft	[21, 26, 31, 38]

Table 3: Summary of papers that reported human application of ASC according to solution used to resuspend ASCs prior to delivery of cells.

Carrier used	References
Culture medium with human albumin	[40]
Hyaluronic acid	[33]
Lactated Ringer's solution	[32]
Normal saline	[11]
Saline with 1% human albumin	[14]

Various routes of administration of ASC in humans were observed in this survey as indicated in the papers depending on which site the cells are expected to settle. These include intra-arterial, intra-articular, intradermal, intramuscular, and intravenous injection at the submucosal, mucosal, and subcutaneous layer or transplanted with the scaffold material and tissue graft. Table 2 summarizes the papers included in this survey that reported route of delivery of the ASCs. Table 3 presents the carrier used to resuspend the stem cells before use or administration of the cells in humans as reported by specific papers included in this survey. These include the use of culture medium with human albumin, stabilized hyaluronic acid, lactated Ringer's solution, and normal saline with or without 1% human albumin. Majority of the papers did not indicate the specific medium used.

4.2. Impact of IFATS and ISCT Statement. Figure 1 illustrates the percentage of papers in reporting the primary characteristics of mesenchymal stem cells. There are still a number of papers which failed to conduct appropriate tests to verify the identity of the cells they use. Fisher Exact Test results showed significant difference in proportions ($P < 0.05$) in the before ($n = 69$) and after statement groups ($n = 47$) when reporting the use of adherent cells. There were no significance differences in the reporting of the surface markers and multilineage differentiation but there are a number of papers that failed to report such characteristics at the after statement group. 28/47 or 59.57% (CI 44.27–73.63) explicitly reported adherent cells (Figure 1(b)) were used and 35/47 or 74.47% (CI 59.65–86.06) identified expression of surface markers (Figure 1(c)). Meanwhile, 25/47 or 53.19% (CI 38.08–67.89) verified the differentiation potential of the cells (Figure 1(d)). Of these 25, 1 paper reported verifying only the osteogenic potential while 4 papers did the differentiation tests but nevertheless used unexpanded cells for the treatment groups. While there was no significant difference observed when comparing the before and after statement groups as far as the use of expanded cells is concerned (Figure 1(a)) disaggregating the data according to human (before statement, $n = 22$, after statement group $n = 13$) and animal (before statement group $n = 47$, after statement group $n = 35$) application showed significant difference ($P < 0.001$) and post hoc analysis indicates the difference when comparing papers with animal application to papers with human application of ASC (Figure 2). All of the 11 papers in this survey that did not use cultured and expanded cells but indicated they were using ASC instead of SVF were reports of application in humans. There is no significant difference between the human before statement group (7/22 or 31.82%, CI 13.87–54.87) and human after statement group (4/13 or 30.77%, CI 9.09–61.43). Table 4 summarizes the frequency of papers that reported information on the characteristics of the cells used.

Table 4: Comparison on reporting of data on cell characteristics between papers grouped prior to and after publication of IFATS/ISCT statement on ASCs.

Cell characteristic	Description of data reported	Number of articles	
		Prestatement ($n = 69$)	Poststatement ($n = 47$)
Expanded cells	Use of expanded cells	52	42
	Did not use expanded cells	7	4
	Implied use	10	1
Adherent cells	Reported use	47	28
	Did not report	7	0
	Implied use	15	19
Phenotyping of surface markers	Phenotyping done	48	35
	No phenotyping	21	12
Differentiation potential	Conducted	34	25
	Did not conduct	35	22

4.3. Quality of Reporting on ASC Use in Humans. Figure 3 shows the percentages with 95% confidence interval of papers reporting on human application of ASC ($n = 35$) on selected parameters. There are 4/35 or 11.43% (CI 3.20–26.74) papers that did not indicate obtaining approval from an institutional review board. Of these 4, 3 reports were case studies while 1 paper was a preclinical study and, of the 4 case reports included in this survey, 1 paper reported having obtained an approval. 5/35 or 14.29% (CI 4.81–30.26) did not report having obtained informed consent from either patient or tissue donors. Of these 5, 2 papers were case reports while 3 papers were clinical studies. Only 10/35 or 28.57% (CI 14.64–46.30) reported processing the tissues and cells in GMP conditions. Of the 20/35 or 57.14% (CI 39.35–73.68) conducted tests for

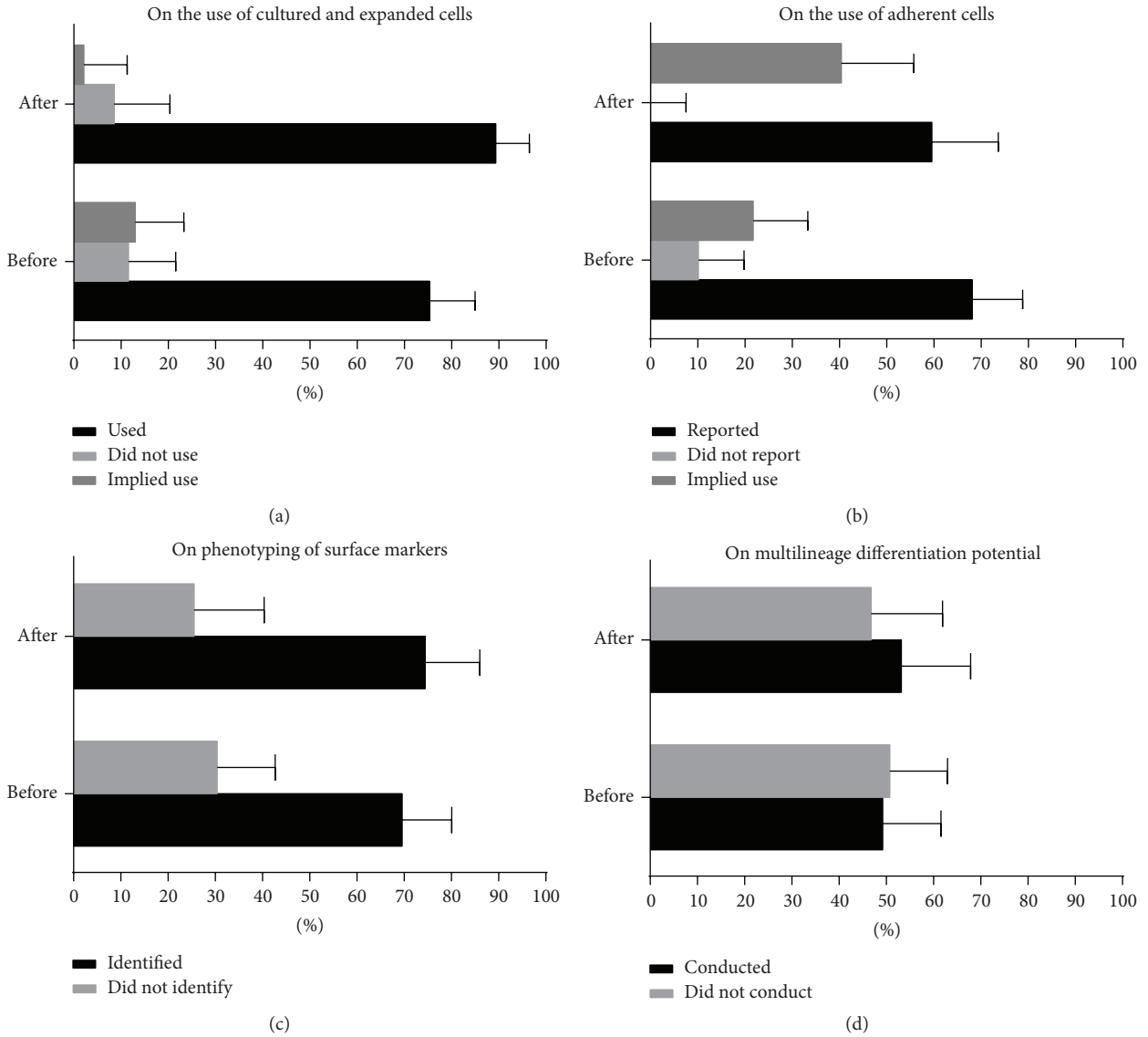

Figure 1: Impact of IFATS and ISCT on reporting of the characteristics of the ASC. Papers reporting on the characteristics of the cells transplanted or delivered to human patients or animal subjects were grouped and assessed before ($n = 69$) and after ($n = 47$) publication of the IFATS and ISCT statement.

possible contamination before using the expanded cells, 1 paper reported testing for 5 different types of contaminants (viral, bacterial, fungal, mycoplasma, and endotoxin), 1 paper reported testing for 4 types, 6 papers reported testing for 2-3 types, and 11 papers reported testing for 1 type of contaminant. Of these 11, 10 papers tested for bacterial contamination, while 1 conducted endotoxin testing. More than half of these papers surveyed (19/35 or 54.28%, CI 36.35–71.89) did not report conducting cell viability while only 7/35 (20%, CI 8.44–36.94) checked the genomic stability of the cells. As to the number of cells delivered during transplantation 24/35 (68.57%, CI 50.71–83.15) reported actual cell numbers while 8/35 (22.86%, CI 10.42–40.14) indicated the number of passages at the time the expanded cells were used. Table 5 summarizes the frequency of papers that reported on selected key areas and parameters of ASC use in humans.

4.4. Quality of Reporting on ASC Use in Animals. Figure 4 presents percentages with confidence interval of papers reporting on animal application of ASC on selected parameters. Among the 82 papers reporting on animal application of ASC, there are 35 papers of those that utilized human adipose tissue as source for mesenchymal stem cells. Of these 35, 24 or 68.57% (CI 50.71–83.15) reported on obtaining informed consent from human donors while a lesser number of 16/35 (45.71%, CI 28.83–63.35) reported on having obtained approval from an institutional review board for the use of human samples. 7/82 or 8.54% (CI 3.5–16.80) did not report obtaining an approval from an animal welfare ethics committee. All papers reported the species used but 35/82 or 42.68% (CI 31.82–54.10) reported the age of their subjects while 69/82 or 84.15% (CI 74.42–91.28) reported whether male or female animals were used. 2/82 or 2.44% (CI 0.30–8.53)

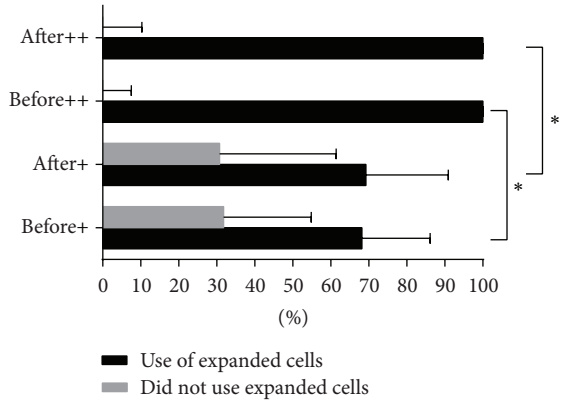

Figure 2: Disaggregated proportions of papers on human (+) and animal (++) application of ASCs that reported the use of expanded and unexpanded cells before and after IFATS/ISCT statement (before statement, human $n = 22$; after statement, human $n = 13$; before statement, animal $n = 47$; after statement, animal $n = 34$), percentage and 95% confidence interval, Fisher Exact Test $P < 0.001$ with post hoc analysis, and *significant difference $P < 0.01$.

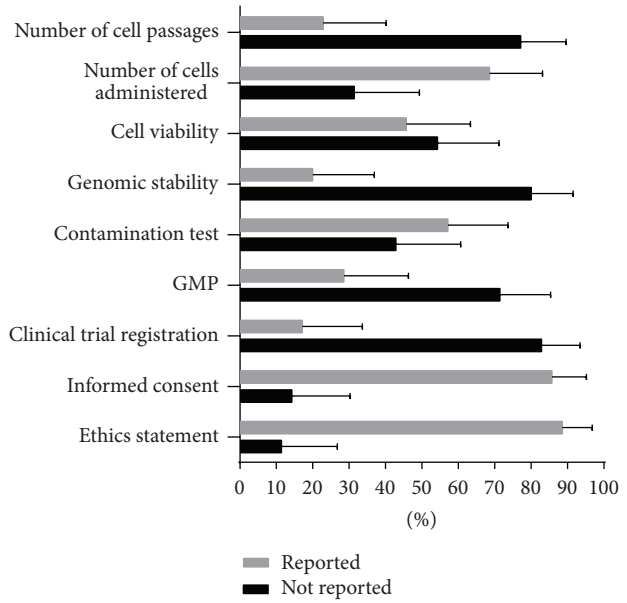

Figure 3: Quality of reporting papers on application of ASC in humans using selected parameters in ethics, safety, and cell characteristics ($n = 35$).

indicated how the sample size was calculated while 44/82 or 53.66% (CI 42.30–64.75) reported randomizing assignment of animals to treatment groups while 38/82 or 46.34% (CI 35.25–57.70) indicated that blinding techniques were done. No paper reported on conducting genomic stability tests for the stem cells used, 2/82 or 2.44% (CI 0.30–8.53) reported conducting testing for possible contaminants, 78/82 or 95.12% (CI 87.98–98.66) indicated the number of cells delivered, 25/82 or 30.49% (CI 20.80–41.64) reported checking the cell viability, and 51/82 or 62.20% (CI 50.81–72.68) reported the number of passages at the time the cells were used. Table 6 summarizes the frequency of papers that reported on selected key areas and parameters of ASC use in animals.

Table 5: Summary of reporting on selected key areas and parameters in papers that reported the use of ASC in humans ($n = 35$).

Key area	Information	Number of articles	
		Yes	No
Ethics	Approval from review board	31	4
	Informed consent	30	5
	Clinical trial registration	6	29
Safety	Processing the cells in GMP	10	25
	Testing for genomic stability	7	28
	Screen contaminants	20	15
Cell characteristics	Viability	16	19
	Number of cells administered	24	11
	Number of cell passages	8	27

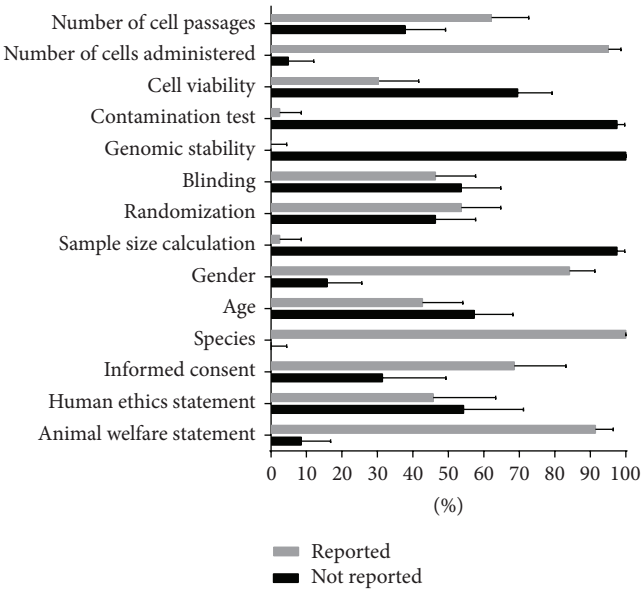

Figure 4: Quality of reporting papers on application of ASC in animals using selected parameters in ethics, safety, study design, and cell characteristics ($n = 82$).

5. Discussion

Transparency in reporting of research results is encouraged to hasten the standardization and reproducibility of the use of ASC for therapeutic purposes while highlighting the value of patient safety and product efficacy [126]. We assert further that accuracy in reporting the true identity of cells is very important especially that cell characteristics may vary according to source of tissue, species, site, physiological and disease condition, or age, may behave differently in a varying substrate or environment, and may provide differing outcomes when used for therapy [53, 67, 99, 104, 127, 128]. While the stem cell is a subset of the SVF it certainly is not one and, the same as the latter, contains more different types of cells other than the stem cells [3]. Expansion entails culturing

Table 6: Summary of reporting on selected key areas and parameters in papers that reported the use of ASC in animals ($n = 82$).

Key area	Information	Number of articles	
		Yes	No
Ethics	Approval from animal ethics committee	75	7
	Human ethics statement*	16	19
	Informed consent from tissue donors*	24	11
Animal characteristics	Species	82	0
	Age	35	47
	Gender	69	13
Study design	Random allocation to groups	44	38
	Calculation of sample size	2	80
	Blinding	38	44
Safety	Testing for genomic stability	0	82
	Screen contaminants	2	80
	Viability	25	57
Cell characteristics	Number of cells administered	78	4
	Number of cell passages	51	31

* For papers that reported utilizing human tissue ($n = 35$).

plastic adherent cell after centrifugation to retrieve adequate cell population that is needed for transplantation purposes and we considered this as the primary parameter to gauge if indeed ASC was employed. Results in this survey show that despite guidance provided by IFATS and ISCT to ascertain the cells true identity still some authors claim that they used stem cells where in fact they did not. One paper goes to the extent of highlighting a rapid way of retrieving stem cells from the lipoaspirate in just 3 to 4 hours without any culturing done prior to injection. Although we cannot discount that using SVF may produce better or poorer results than using expanded mesenchymal stem cells, transparent reporting of the true identity of the cells should always be done. When we compared how reporting fared between papers on application of ASC in humans and in animals (Figure 2), reporting on human applications tend to misreport the identity of the cells used. How potentially serious the misreporting of stem cells instead of SVFs is shown by the 95% confidence interval for the proportion of these particular papers which was calculated to be at 2 to 20 percent.

Compliance to reporting standards and to expert's recommendation has been always a major concern even in the top-tier scientific journals [129]. The current survey showed that parameters on ethics, safety, study design, and cell viability in the application of stem cells to human patients as well as in animal subjects were not fully considered during reporting. Although the information we gathered from this survey is just based on the author's reports it may mean that these data were not reported as they were not done at all or were not reported but nevertheless were done. Poor quality in reporting will have an impact on our understanding of the real benefits of ASC and may seriously affect its potential to become a standard treatment. In addition, it may to some extent negatively affect, if not endanger, the patient when results from poor reporting are used as basis. For example, obscure quality and quantity of stem cells may lead to transplanting inadequate cells resulting in ineffective results, but if the cells are more than the required number, they may potentially circulate to unintended locations and form cell aggregates that could cause pulmonary emboli or infarctions [41, 112]. Patients are on the receiving end whenever medical institutions and companies processing stem cells for commercial purposes disregard proper protocols and standards to take advantage of the hype and exaggerated promotion of stem cells as a wonder treatment for intractable disease conditions. As to safeguarding the rights of recipients, it is not enough that an informed consent is obtained but an evaluation of the patient's understanding of the risks should also be secured [130]. The results of this survey showing misreports on the use of ASC may serve not only as a guide for readers to carefully examine published research results but also as a reminder for authors to prevent further misuse.

In the light of these survey results, we continually support the need for authors, reviewers, and editors to comply with the recommendations and guidelines. Such compliance not only should be reflected during reporting but must begin as early as the research planning or conceptualization stage until implementation. This survey is also a compelling evidence for the need to further disseminate the information and guidance provided by ISCT and IFATS or conduct further discussions in seminars or conferences. Researchers actively pointing out this similar oversight to editors will be helpful [131]. More research is required to fully understand the therapeutic potential, the effectiveness, and safety of ASC and a unified global effort to comply with the existing standards would definitely provide rapid and reliable results.

6. Conclusion

We determined in this survey that a substantial number of published reports were not able to provide information on the characteristics of ASCs used with some erroneously referring to the SVF as stem cells. In addition, survey results suggest that there is more room for improvement in the quality of reporting in the application of ASC in humans and animals in reference to existing guidelines and recommendations.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Ernesto Balolong and Soojung Lee contributed equally to this work and should be considered co-first authors. Jeong Ik Lee and Judee Grace Nemeno contributed equally to this work.

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Research Article

Adiponectin Isoforms and Leptin Impact on Rheumatoid Adipose Mesenchymal Stem Cells Function

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Adiponectin and leptin have recently emerged as potential risk factors in rheumatoid arthritis (RA) pathogenesis. In this study we evaluated the effects of adiponectin and leptin on immunomodulatory function of adipose mesenchymal stem cells (ASCs) derived from infrapatellar fat pad of RA patients. ASCs were stimulated with leptin, low molecular weight (LMW) and high/middle molecular weight (HMW/MMW) adiponectin isoforms. The secretory activity of ASCs and their effect on rheumatoid synovial fibroblasts (RA-FLS) and peripheral blood mononuclear cells (PBMCs) from healthy donors have been analysed. RA-ASCs secreted spontaneously TGFβ, IL-6, IL-1Ra, PGE2, IL-8, and VEGF. Secretion of all these factors was considerably upregulated by HMW/MMW adiponectin, but not by LMW adiponectin and leptin. Stimulation with HMW/MMW adiponectin partially abolished proproliferative effect of ASC-derived soluble factors on RA-FLS but did not affect IL-6 secretion in FLS cultures. ASCs pretreated with HMW/MMW adiponectin maintained their anti-inflammatory function towards PBMCs, which was manifested by moderate PBMCs proliferation inhibition and IL-10 secretion induction. We have proved that HMW/MMW adiponectin stimulates secretory potential of rheumatoid ASCs but does not exert strong impact on ASCs function towards RA-FLS and PBMCs.

1. Introduction

Adiponectin and leptin have been recently considered to be important factors in rheumatoid arthritis (RA) pathogenesis [1]. Both these adipokines play well known metabolic role but also exert impact on immune system. Leptin is a proinflammatory factor stimulating innate and acquired immune response and its concentration increases during infection and inflammation. Adiponectin exerts opposite effects to leptin; however, many reports about its action are conflicting, probably due to the existence of several isoforms of this adipokine [2]. In plasma, adiponectin circulates in four isoforms: low molecular weight (LMW) isoform, middle molecular weight (MMW) isoform, high molecular weight (HMW) isoform, and globular isoform, exhibiting different effects on immune system. HMW isoform is thought to possess proinflammatory activity, whereas LMW isoform is thought to possess anti-inflammatory activity [2].

RA is an autoimmune disease characterized by synovial fibroblasts (FLS) hyperproliferation, overactivation of lymphocytes, synovial membrane inflammation, cartilage destruction, and bone erosion. In the rheumatoid joint, apart from synovium, cartilage, and bone, fat pads are affected by inflammatory process [3]. Intra-articular adipose tissue is a local source of proinflammatory adipokines [4] as well as of adipose mesenchymal stem cells (ASCs) possessing immunoregulatory and regenerative properties. These cells are very promising in terms of cell therapy of autoimmune and degenerative diseases [5]. Mesenchymal stem cells (MSCs) have been shown to suppress T cells activation and to induce T regulatory cells (Tregs) generation. Their immunoregulatory effects are mediated by various factors, for example, transforming growth factor β (TGFβ), interleukin 6 (IL-6), interleukin 10 (IL-10), interleukin 1 receptor antagonist (IL-1Ra), and prostaglandin E2 (PGE2) [6]. Because MSCs immunosuppressive activity is dependent

on stimuli provided by local environment [6], we hypothesize that adipocytokines present in rheumatoid joint may affect ASCs function. The aim of this study was to evaluate the effects of leptin and adiponectin isoforms on function of ASCs derived from rheumatoid infrapatellar fat pad (IPFP).

2. Materials and Methods

2.1. ASCs Donors and ASCs Treatment. Infrapatellar fat pads (IPFPs) were obtained from 29 RA patients undergoing total knee joint replacement surgery. All patients were selected from the Rheumoortopaedic Clinic of the Institute of Rheumatology in Warsaw and gave their written informed consent according to the Declaration of Helsinki. The study was approved by the Institute of Rheumatology Ethics Committee. ASCs were isolated and cultured in medium composed of DMEM/F12, 10% fetal calf serum, and antibiotics. Cells showed differentiation capacity to the chondrogenic and osteogenic lineages and had the $CD105^+CD90^+CD73^+CD45^-CD34^{+/-}CD19^-CD14^-$ phenotype. ASCs (2×10^4/well/1 mL) at passages 3–5 were seeded in culture medium and were treated or not with 10 ng/mL of recombinant human (rh) leptin (PeproTech, London, UK), 10 μg/mL of rhLMW adiponectin, or 10 μg/mL of rhHMW/MMW adiponectin (BioVendor, Brno, Czech Republic). Concentrations of abovementioned adipocytokines were selected on basis of their concentration in serum of RA patients [7].

2.2. Immunoenzymatic Assays. After 24 h of stimulation, culture supernatants (SNs) from ASCs were harvested. Concentrations of the following factors in SNs were measured by specific ELISAs: IL-6, IL-8 (using own procedures as previously described [8]), TGFβ, VEGF, IL-1Ra (DuoSet kits, R&D Systems, Minneapolis, MN, USA), and PGE2 (Parameter kit, R&D Systems).

2.3. Conditioned Media Preparation. For ASC-conditioned media (CM) preparation, ASCs were seeded and cultured for 24 h with addition of adipocytokines as described above; then, medium in stimulated ASCs cultures was replaced with the fresh one, deprived of any stimuli. After additional 24 h of culture, SNs (in volume of 900 μL) were harvested and used for RA-FLS cultures.

2.4. Rheumatoid FLS Treatment. Rheumatoid synovial fibroblasts (RA-FLS), isolated from rheumatoid synovial membranes and cultured as described previously [8], were seeded (2×10^4/well/1 mL) in CM or cocultured with ASCs (2×10^4/well/1 mL). SNs from cocultures were harvested after 48 h, whereas in CM-treated cells the medium was replaced with the fresh one after 24 h and harvested after additional 24 h. Concentration of IL-6 was measured in SNs as described above. For proliferation assay, RA-FLS (2×10^4/well/0.1 mL) were cultured in CM or with ASCs (1×10^4/well/0.1 mL) for 48 h. Proliferation was evaluated by bromodeoxyuridine (BrdU) cell proliferation assay (Millipore Corporation, Billerica, MA, USA).

2.5. Peripheral Blood Mononuclear Cells Treatment. Peripheral blood mononuclear cells (PBMCs), isolated from buffy coats of healthy male donors according to routinely applied procedure using Ficoll-Paque (GE Healthcare, Uppsala, Sweden), were treated or not with 2.5 μg/mL of phytohaemagglutinin (PHA) and then cultured (4×10^5/well/1 mL) for 24 h with ASCs (2×10^4/well/1 mL). SNs from cocultures were harvested after 24 h and concentration of IL-10 was measured in SNs (Ready-SET-Go kit, eBioscience, San Diego, CA, USA). For proliferation assay, PBMCs (1×10^5/well/0.1 mL) were seeded onto ASCs (1×10^4/well/0.1 mL) and cultured for 72 h. PBMCs proliferation was evaluated using ^{3}H-thymidine incorporation assay.

2.6. Statistical Analysis. Statistical analysis was performed using STATISTICA 10.0 software (Stat Soft Inc., Tulsa, OK, USA). Obtained data was not normally distributed, as assessed by Shapiro-Wilk test. The differences between samples were evaluated using the nonparametric Wilcoxon signed-rank test. All results are shown as arithmetic mean and standard error of a mean. Differences were considered statistically significant for $^{*}p < 0.05$, $^{**/\#\#}p < 0.01$, and $^{***}p < 0.001$.

3. Results

Immunomodulatory properties of ASCs are associated with several soluble factors secretion [6]. As depicted in Figure 1, RA-ASCs secreted TGFβ, IL-6, IL-1Ra, and PGE2 as well as IL-8 and VEGF which are related to angiogenesis process. Importantly, HMW/MMW adiponectin enhanced secretion of all factors, whereas LMW adiponectin and leptin exerted weaker or no effect, respectively (Figure 1). Given that among applied adipokines only HMW/MMW adiponectin affected ASCs secretory profile, we decided to test if HMW/MMW adiponectin-stimulated ASCs exert any effects on RA-FLS and PBMCs from healthy donors.

ASC-conditioned media (CM) contributed to upregulated proliferation of RA-FLS, which has already been reported by our group [9]. This effect was partially abolished by ASCs pretreatment with HMW/MMW adiponectin (Figure 2(a)), but not with LMW isoform or leptin (own data, not shown). Interestingly, proliferation of cells in cocultures stayed unchanged (Figure 2(b)). Both CM from ASCs and ASCs presence caused modest downregulation of IL-6 production, but it was independent of ASCs prestimulation. Upon treatment with CM from HMW/MMW adiponectin-stimulated ASCs, IL-6 secretion was slightly decreased comparing to CM from unstimulated ASCs; however, this difference did not reach statistical significance (Figure 2). LMW adiponectin- or leptin-treated ASCs did not exert any impact on IL-6 production in FLS cultures (own data, not shown).

Despite the strong impact of HMW/MMW adiponectin on secretory potential of ASCs, this adipokine did not affect ASCs immunosuppressive potential against PHA-activated PBMCs from healthy donors. Proliferation of PBMCs was moderately inhibited by the presence of unstimulated ASCs, but pretreatment of ASCs with HMW/MMW adiponectin

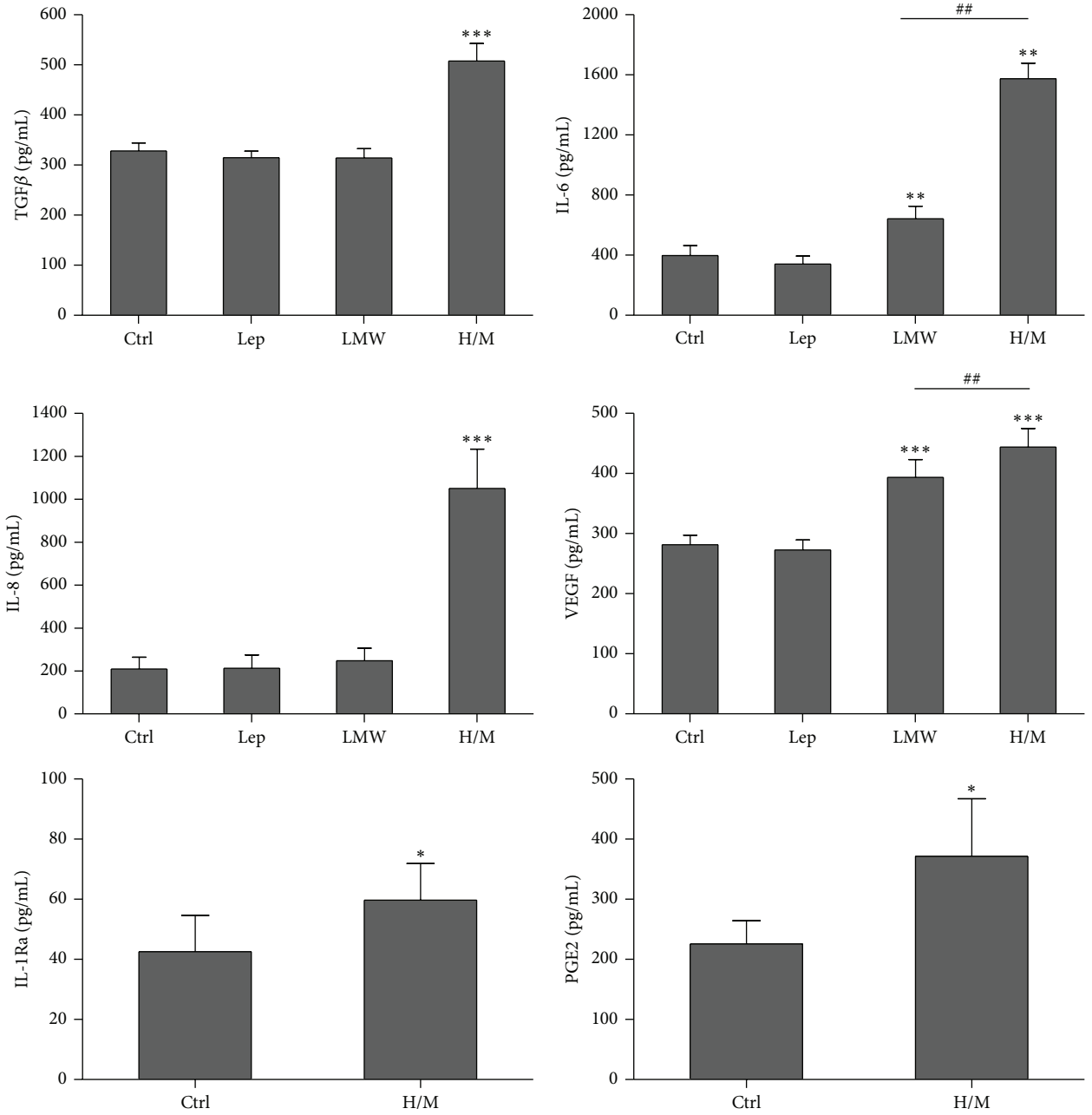

Figure 1: Adipokines effects on secretory activity of ASCs derived from rheumatoid infrapatellar fat pad. Unstimulated (Ctrl) or stimulated with 10 ng/mL leptin (Lep), 10 μg/mL of LMW adiponectin, or 10 μg/mL of HMW/MMW adiponectin ASCs from RA patients were cultured for 24 h. Concentrations of select factors were measured in supernatants collected from ASCs cultures using specific ELISA tests (n = 14–18). Asterisks (∗) indicate statistically significant differences *versus* Ctrl; hash marks (#) indicate statistically significant differences between indicated data. $^{*}p < 0.05$, $^{**/\#\#}p < 0.01$, and $^{***}p < 0.001$.

did not alter their antiproliferative effect (Figure 3(a)). Similarly, induction of anti-inflammatory IL-10 in resting PBMCs by ASCs was not further affected by prestimulation of ASCs with HMW/MMW adiponectin (Figure 3(b)).

4. Discussion

In this *in vitro* study, we have analysed the impact of select adipokines on function of human RA-ASCs derived from intra-articularly localized infrapatellar fat pad. Previously, we have demonstrated that RA-ASCs express genes coding for adiponectin (AdipoR1 and AdipoR2) and leptin (ObRb) receptors [10]. Here, we proved that HMW/MMW adiponectin acts as a strong stimulator of ASCs secretory activity, whereas leptin and LMW adiponectin do not exert considerable effects on ASCs. It is worth noting that some factors, such as IL-6, TGFβ, and PGE_2, responsible for immunosuppressive ASCs function [6] might be detrimental

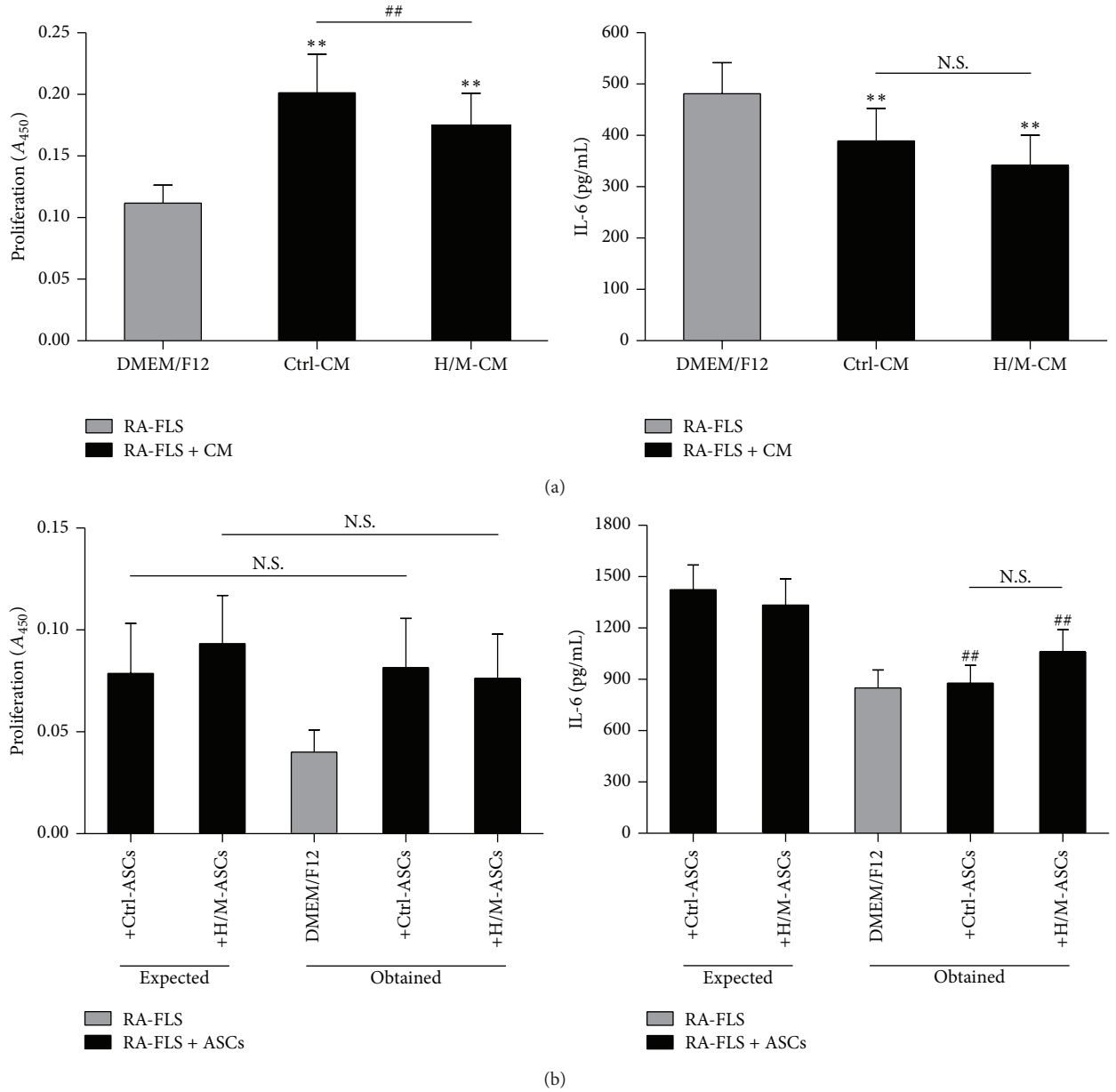

Figure 2: Effects of unstimulated and HMW/MMW adiponectin-stimulated RA-ASCs on RA-FLS function. RA-FLS were cultured either in conditioned media (CM) collected from RA-ASCs (a) or in the presence of RA-ASCs (b). For coculture experiments, unstimulated ASCs (+Ctrl-ASCs) or ASCs stimulated with 10 μg/mL of HMW/MMW adiponectin (+H/M-ASCs) were used, whereas CM from respective cultures of unstimulated (Ctrl-CM) and stimulated (H/M-CM) ASCs were added to FLS. RA-FLS cultured separately (DMEM/F12) served as a control. Proliferation of cells was determined by BrdU incorporation assay. Absorbency of samples was measured at 450 nm (A_{450}). IL-6 was detected by specific ELISA test. (a) The effects of ASC-CM on RA-FLS proliferation ($n = 14$) and IL-6 secretion ($n = 17$). (b) Proliferation ($n = 6$) and IL-6 ($n = 9$) secretion in ASCs-FLS cocultures. Expected: the sum of the values obtained for separate FLS and ASCs cultures; obtained: real values stated in the cocultures. Asterisks (∗) indicate statistically significant differences *versus* DMEM/F12; hash marks (#) indicate statistically significant differences between indicated data or between expected and obtained values; $^{**/\#\#}p < 0.01$; N.S.: nonsignificant.

in RA pathogenesis [11, 12]. In this view, observed in this study, HMW/MMW adiponectin-induced IL-6, TGFβ, and PGE_2 secretion increase may be recognized as an intensification of ASCs anti-inflammatory activity and also as a contribution of these cells to the inflammation in the rheumatoid joint. Moreover, upregulation of IL-8 and VEGF, important proangiogenic factors associated with arthritis, seems to confirm this suggestion.

Studies about adiponectin isoforms effects on MSCs are not available; however, it is known that HMW/MMW adiponectin strongly upregulates secretion of cytokines and chemokines by RA-FLS, while LMW adiponectin does not

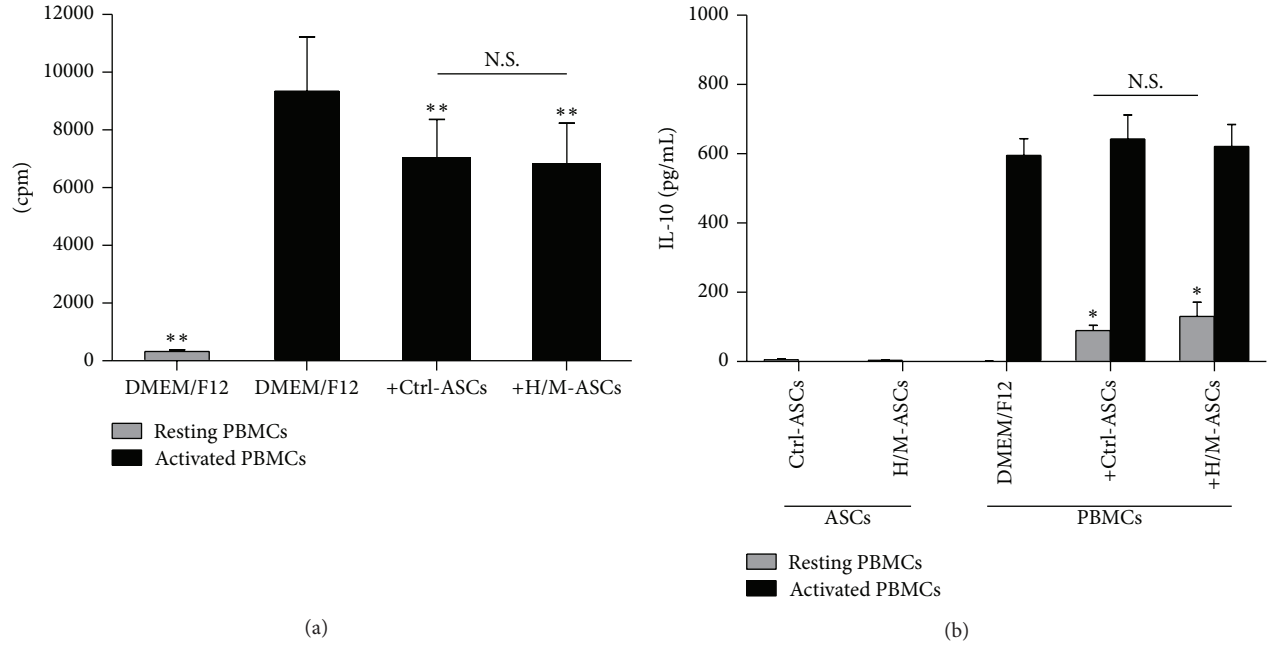

Figure 3: Effects of unstimulated and HMW/MMW adiponectin-stimulated RA-ASCs on proliferation of PBMCs from healthy donors and IL-10 secretion by these cells. PBMCs either were prestimulated with 2.5 μg/mL of phytohaemagglutinin (activated PBMCs) or were left untreated (resting PBMCs) and then cultured in the presence of unstimulated ASCs (+Ctrl-ASCs) or ASCs stimulated with 10 μg/mL of HMW/MMW adiponectin (+H/M-ASCs). Resting and activated PBMCs cultured separately in DMEM/F12 medium served as controls (DMEM/F12). Proliferation of cells was determined by ^{3}H-thymidine incorporation assay. Radioactivity of samples is expressed in counts per minute (cpm). Concentration of IL-10 was measured in culture supernatants by specific ELISA test. (a) Proliferation of PBMCs cocultured in the presence of ASCs ($n = 9$). (b) IL-10 secretion in ASCs-PBMCs cocultures ($n = 6$). Asterisks ($*$) indicate statistically significant differences *versus* activated (a) or resting (b) PBMCs cultured separately; $^*p < 0.05$; $^{**}p < 0.01$; N.S.: nonsignificant.

have this impact, which was described independently by two groups [7, 13]. Moreover, HMW/MMW adiponectin was reported to be less potent in stimulating FLS from osteoarthritis patients [13]. Together with present results it shows that HMW adiponectin, but not LMW adiponectin and leptin, strongly affects rheumatoid cells and suggests this isoform contribution to RA pathogenesis.

Surprisingly, in spite of stimulating effect of HMW/MMW adiponectin on RA-ASCs secretory activity (Figure 1), we did not demonstrate that ASCs treated with this adipokine modify significantly their functions towards other cells. To evaluate HMW/MMW adiponectin impact on ASCs function we performed *in vitro* cocultures or cultures in ASC-conditioned media with FLS from rheumatoid patients and PBMCs from healthy donors. We aimed for *in vitro* setting in which only ASCs would derive from pathologic environment and responder cells would not be affected by inflammatory process. Nonetheless, in case of FLS, it was not possible due to ethical reasons.

In the course of RA, the synovial membrane inflammation develops and persists; FLS are overactivated, proliferate excessively, and produce proinflammatory mediators and tissues degrading enzymes [14]. We observed that soluble factors released by RA-ASCs exert proproliferative effects on RA-FLS, but it was partially abolished when ASCs were prestimulated with HMW/MMW adiponectin. Possibly, it was due to antiproliferative factors released by ASCs upon HMW/MMW adiponectin stimulation, for example, IL-6, TGFβ, and PGE2 [6]. Interestingly, the lack of cell proliferation increase in coculture may indicate cell contact-dependent reciprocal inhibition of trophic factors production.

Interleukin 6 is associated with chronic inflammatory response in RA [12]. We have already shown that unstimulated ASCs moderately decreased IL-6 release in RA-FLS culture [9]. Here, we demonstrated that HMW/MMW adiponectin-pretreated ASCs exerted similar effect. Thus, despite postulated proinflammatory role of HMW adiponectin, we did not observe that this adipokine promotes proinflammatory ASCs function towards RA-FLS in respect of IL-6 secretion. This is in contrast to TNF, which, as we have recently reported, triggers proinflammatory capabilities of ASCs leading to upregulation of IL-6 and metalloproteinase 3 release by RA-FLS [9].

In the rheumatoid joint, mononuclear lymphoid cells infiltrate into synovial membrane and form ectopic lymphoid tissue. T lymphocytes activated in germinal centers of ectopic lymphoid follicles proliferate excessively and contribute to the chronic inflammation by proinflammatory cytokines secretion [15]. In our study, unstimulated ASCs limited proliferation of PHA-activated PBMCs and induced IL-10 production by resting PBMCs. Because ASCs pretreated with HMW/MMW adiponectin did not alter their impact on PBMCs (Figure 3), it is clear that this adipokine does not

affect ASCs immunosuppressive activity towards lymphoid cells.

It must be underlined in this section that present study has several limitations. Due to ethical reasons, we were unable to obtain the IPFP and synovial membrane from healthy donors. Thus, we could not provide responder FLS from healthy individuals and our analysis was limited to unstimulated *versus* stimulated rheumatoid ASCs. Comparing IPFP-derived ASCs with ASCs from subcutaneous adipose tissue (obtained from lipoaspirates) would be also questionable, as it is known that different adipose tissue depots have distinct secretory potential possibly influencing resident cells, and ASCs derived from different fat source exhibit diverse characteristics (i.e., proliferative and regenerative potential) [16–18].

In conclusion, we demonstrated that HMW/MMW adiponectin, but not LMW adiponectin and leptin, stimulates considerably secretory potential of rheumatoid ASCs suggesting its contribution to RA pathogenesis. However, regardless of postulated proinflammatory role of HMW adiponectin, we did not prove that this adipokine induces proinflammatory ASCs activity towards RA-FLS and PBMCs.

Conflict of Interests

The authors declare no conflict of interests.

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