Techniques and Processing Methods to Isolate Stem Cells and Stromal Vascular Fraction Cells

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Introduction

The field of regenerative medicine is continuously growing and is becoming nowadays a key component in the practice of medicine. Many scientific advances in this area have been done during the last two decades. Regenerative medicine relies mainly on the use of human cells with the aim of replacing or regenerating damaged tissues or organs to restore normal function. Other disciplines like tissue engineering (using scaffolds) or the use of growth factors also belong to the wide field of regenerative medicine.

This approach of using cells has its rationale in emulating our body's physiological regenerative capabilities in adding healthy cells where there is a deficit or malfunction due to diverse causes.

A great variety of different cell types are available with this purpose, from allogeneic (from a different individual) purified cultured stem cells to the use of freshly isolated autologous (from same person) cells from different tissue sources.

Stem cells offer a tremendous potential for use in regenerative therapies to halt or reverse the effects of degenerative diseases. A stem cell is a cell that can divide to give rise to both a new copy of itself (self-renewal) and at least one specialized cell type (differentiation capacity).

We can classify the different stem cells into three main categories: embryonic stem cells, induced pluripotent stem cells, and adult stem cells [1].

Embryonic stem cells are obtained from the inner cell mass of the human blastocyst, a ball-like structure that is formed about 5 days after fertilization of the human egg. These pluripotent stem cells are grown and expanded in the laboratory and can give rise to all tissues derived from endoderm, mesoderm, and ectoderm [2].

Induced pluripotent stem cells are cells engineered in the lab by converting terminally differentiated specific cells (such as fibroblasts skin cells) into undifferentiated cells, equivalent to embryonic pluripotent stem cells. These cells are genetically reprogrammed to become a stem cell achieving pluripotency. The cells are modified in the lab introducing genes that encode four transcription factors (Oct4, Sox2, Klf-4, and c-Myc) [3].

Adult stem cells comprise a wide range of different progenitor cells that can be isolated from the great majority of all tissues in humans [4].

This group includes hematopoietic stem cells (HSCs) residing in bone marrow, mesenchymal stem cells (MSCs) from different tissues, muscle satellite cells, etc.

Adult mesenchymal stem cells (MSCs) are probably the cell type closer to the clinical reality

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due to their presence in many different adult tissues and their role in tissue regeneration and immunomodulation. The main tissue sources for MSCs isolation are bone marrow and adipose tissue, being the latter the one that provides the best yield in these cells per gram of tissue [5].

In cell-based therapies, there is still a great debate about what is the best cell type for a given clinical indication. This is caused by the development of different cellular products from biotech companies. Different cellular products or cell types have significant biological differences among them, which include: expression of cell surface markers, differentiation capacity, angiogenic potential, etc. However, the ideal cell type and/or tissue source would be the one that is autologous, abundant, easy to isolate, biologically potent, and affordable for the proposed final clinical use.

Different cell types can be obtained, either in the laboratory or fresh in the operating room, from different tissue sources and by using a huge variety of isolation methods or systems.

Certainly, the use of cells expanded in culture allows researchers to have a more homogeneous cell population (relatively pure in stem cells after several passages); however, the use of freshly isolated cells with minimal manipulation is favored in clinical practice due to reduced costs and procedural simplicity. The available preclinical literature does not show clear evidence favoring any of these approaches, and comparative studies are still necessary to clarify this matter [6].

This chapter aims to review and summarize different cell isolation techniques that can be developed in a short time intraoperatively, focusing on bone marrow and adipose tissue. A brief discussion is also included concerning some of the available clinical information and the promising future of cell-based and regenerative therapies.

Cell Isolation Techniques from Human Lipoaspirates

In the 1960s, Rodbell and collaborators developed a method to isolate cells using rat adipose tissue samples [7]. They basically extracted and minced the rat fat pads, washed several times the tissue parcels with saline solution, and then incubated the tissue pieces with collagenase to break the collagen-rich extracellular matrix, creating a dissociated tissue sample. A centrifugation step separated a yellow floating layer containing oil and adipocytes, and all other cells formed a pellet at the bottom of the sample tubes. The cellular pellet contained the stromal vascular fraction (SVF), a very heterogeneous cell population comprised of many different cell types: blood derived cells (erythrocytes, lymphocytes, monocytes, etc.), endothelial cells, fibroblasts, and other progenitor cells (including MSCs) [8]. This simple procedure allowed the separation of all mature adipocytes (tissue parenchyma) from all other supporting cells (stroma). See Fig. 1.

It was almost 40 years later when a group of scientists (led by plastic surgeons) working in Pittsburgh in 2001 demonstrated that after culturing SVF in vitro, the cells able to adhere and grow in culture were multipotent [9]. They reported that those cells (which they called PLA—processed lipoaspirate cells) had the capacity to differentiate toward the adipogenic, chondrogenic, osteogenic, and myogenic lineages. Those cells are now known as ASCs (adipose stromal/stem cells) and can be characterized by phenotypic and functional criteria: selected by adhesion to plastic (Fig. 2), proliferative potential, presence or absence of specific cell membrane markers, and the capacity to differentiate into other cell types [10]. The relative abundance of ASCs within SVF cells can be as high as 5–10% of all nucleated SVF cells obtained depending on cell isolation method used and efficiency.

During the last decade, we have seen an impressive increase in the number of publications concerning SVF focused on different features: mechanisms of action, regenerative capabilities on in vitro and in vivo models, isolation techniques, etc.

There is a huge variety of different techniques aiming to extract or isolate adipose-derived cells, which result in different cellular outputs and hence in a different biological response that affects clinical outcomes.

According to the method used to release the cells, all these techniques can be categorized into two different groups: mechanical and enzymatic

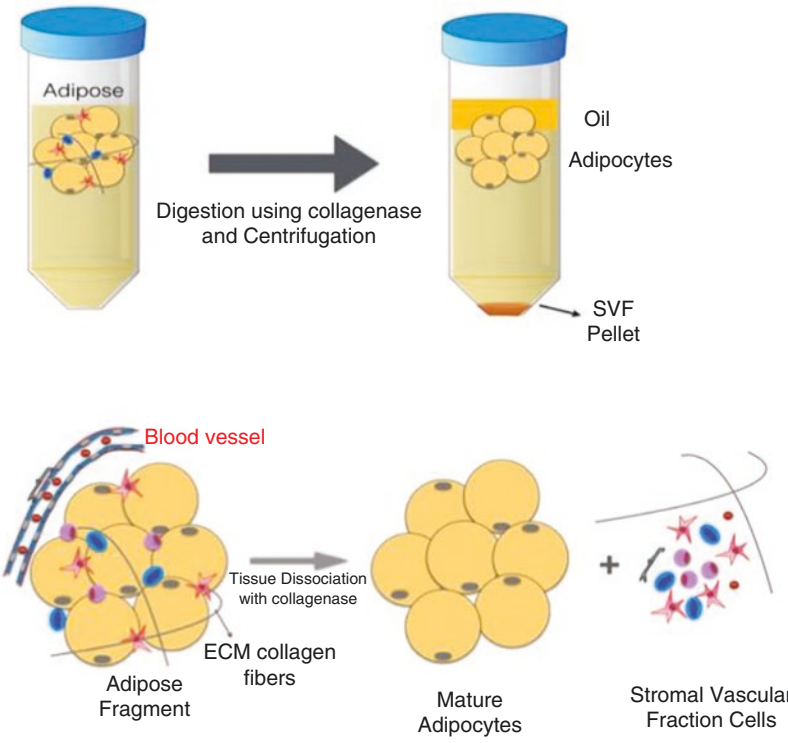

Fig. 1 Overview of SVF isolation procedure: adipose digestion in Falcon tubes showing tissue disaggregation, cell release, and concentration of SVF pellet via centrifugation

ENZYMATIC COLLAGENASE DISSOCIATION OF ADIPOSE TISSUE

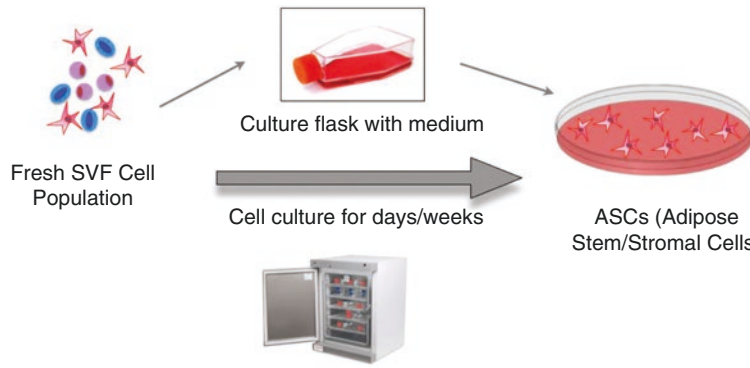

Fig. 2 Image showing ASC enrichment from SVF through cell culture expansion

CELLS CULTURE SELECTION OF ASCs

methods. Nevertheless, both can be combined within the same procedure.

Mechanical methods are based on different physical methods to promote cell release from tissues. This category includes, among others, shaking, centrifugation, filtration, etc.

Enzymatic methods utilize proteolytic enzymes (proteases) to break down the tissue extracellular

matrix disaggregating the scaffold that holds the cells together. Different proteases or mixtures of them can be potentially used, but the most common and powerful one is bacterial collagenase [11].

Mechanical Cell Isolation Methods

Mechanical methods for cell isolation or concentration using lipoaspirate samples are performed using a great variety of different techniques. This has been reviewed recently by several authors [11, 12].

It is very important to point out that some of them physically extract isolated stromal vascular fraction cells, while others only decrease the relative amount of adipocytes by removing most of them in the product finally obtained.

The first group is based mainly on vortexing, vibration, or shaking followed by centrifugation to concentrate the cells that are released due to these processes [13–16].

The second group includes devices or methods that allow a relative concentration of stromal cells per volume. This is due to the removal of most adipocytes due to mechanical forces using manual techniques or point-of-care devices [17].

Some of these techniques have become very popular because of the simplicity/easiness and short-processing time, but the rationale and scientific support is still lacking or very poor [18].

Enzymatic Cell Isolation Methods

Enzymatic tissue dissociation using bacterial collagenase was the first described and is clearly the method that achieves the highest yield of isolated cells using adipose tissue samples. It is important to point out that the specific procedure determines the cell isolation efficiency and the biological characteristics of the final product. Many different factors play an important role in the cell isolation process: potency of a specific collagenase blend, concentration used, digestion time, shaking method, incubation temperature, and many more.

There are a variety of isolation systems commercially available in the market for SVF isolation, and the number is continuously increasing. It is important to highlight that their clinical use is regulated in a different way depending on each country. The regulatory framework is still not clearly defined and is still subjected to changes due to new scientific and clinical findings.

Some of these systems simplify the whole process by using specific medical devices [19] or almost fully automated systems working in a closed system [20], while others rely on a completely manual procedure using plastic disposables [21].

On last years, we are starting to see several comparative studies using some of these methods, which are helpful to the practicing physician to choose which ones would be more advantageous [22, 23].

Among the various factors that are important to compare among different systems, we would like to emphasize the following: availability of supporting scientific and clinical information, disposable cost, processing time, and user-friendliness. Regarding the scientific and clinical information, it is desirable to have as much information as possible about quality control and safety analysis of the final cellular product. This includes scientific reports about flow cytometry characterization, cell yield, collagenase residual activity, endotoxin levels, etc.

Comparison between Mechanical and Enzymatic Methods

The availability of so many possibilities and point-of-care medical devices to isolate cells from adipose tissue samples highlights the importance of analyzing critically all possibilities at hand in order to choose the best one according to the final clinical use.

In order to make an objective decision, there are different factors that should be taken into account, being the most important ones those related to the quality, safety, and potency of the final obtained cellular output. The level of automation is another factor to bear in mind, since there are manual, semi-automated, or fully automated commercial systems, which result in different processing times and simplicity of use.

Regarding the safety of a specific cellular product, it would be important to know if the processing technique is performed under strict aseptic conditions using a closed system. Moreover,

the availability of data about the absence of microbial contamination by microbiological culture, low endotoxin levels, or negligible residual collagenase activity is critical [22].

With respect to the quality parameters, the most important ones to bear in mind would be the cell yield (measured as number of nucleated cells obtained per gram of tissue processed), the cellular viability, and the phenotypical cell characterization using specific membrane markers by flow cytometry.

Biological potency assays or bioassays can provide an objective measure of biological activity by evaluating specific cellular products within a living biological system, which includes in vivo animal studies, ex vivo models, or in vitro cell culture systems [24].

These biological potency assays might give information about immunomodulatory functions, angiogenic activity, or the capacity to secrete different growth factors with regenerative properties.

There is substantial evidence that enzymatic methods yield more nucleated cells from the same amount of tissue. There is also a significant increase in the frequency of stromal/stem cells (positive for CD34 marker in vivo) with respect to the total cell population obtained. These data are summarized in Table 1.

Table 1 Comparison between mechanical and enzymatic methods for SVF isolation regarding yield, viability, and gross cell characterization. Mean cell yield values for enzymatic methods included in this table is 659.800 nucleated cells per gram, whereas for mechanical methods was only 49,571 nucleated cells per gram.

Yield (Nucleated cells per gram adipose)	Viability (%)	Method	Publication Date	First Author	Journal	CD34 + cells (%)	CD45+ cells (%)
480.000	NA	Collagenase digestion	2013	Shah	Cytotherapy [13]	81,2	27,7
25,000	NA	Mechanical (wash and centrifugation)	2013	Shah	Cytotherapy	23,7	81,7
25,000	65	Mechanical (RBC lysis and centrifugation)	2014	Markarian	Biotechnology Letters [14]	NA	NA
125,000	NA	Mechanical (shaking and centrifugation)	2014	Raposio	Plastic and Reconstructive Surgery [15]	5%	95%
1,310,000	NA	Collagenase digestion	2006	Yoshimura	Journal of Cellular Physiology [25]	20–40	20–40
719,000	83%	Collagenase digestion	2014	Dos-Anjos	Cytotherapy [19]	NA	NA
560,000	90	Collagenase Enzymatic	2016	Chaput	Plastic and Reconstructive Surgery [26]	21,45	30,59
80,000	54	Mechanical (Vortexing and Centrifugation)	2016	Chaput	Plastic and Reconstructive Surgery	5,81	41,17
50,000	45	Mechanical (Intersyringe dissociation)	2016	Chaput	Plastic and Reconstructive Surgery	38,11	19,17
230,000	80–90	Collagenase	2014	Conde-Green	Plastic and Reconstructive Surgery	NA	32
12,000	80–90	Mechanical (vortexing and centrifugation)	2014	Conde-Green	Plastic and Reconstructive Surgery	NA	70–85
30,000	More than 90	Mechanical (mystem)	2015	Gentile	PRS GO	NA	NA

Mechanical methods, in general, offer several advantages such as being less expensive and time consuming. However, enzymatic methods might be better for the clinical setting due to higher yield and the clearly superior cellular composition of the cells isolated.

Cell Isolation Using Bone Marrow Aspirate

The use of bone marrow and cancellous bone in an intraoperative clinical setting is very common, especially among orthopedic surgeons for bone healing applications.

Bone formation, remodeling, and healing depend on the recruitment of endothelial progenitor cells (EPCs), hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and their supporting accessory cells to the injured site [27].

The bone marrow is found within the central cavities of axial and long bones. It consists of hematopoietic tissue islands and adipose cells surrounded by vascular sinuses distributed within a mesh of trabecular bone [28]. The bone marrow is the major hematopoietic organ in humans, responsible for the production of all blood cells (leucocytes, erythrocytes, and platelets) and participates in bone turnover and remodeling.

Bone grafting is widely used in hospitals to repair injured, aged, or diseased skeletal tissue. However, bone autograft material for bone regeneration is limited in quantity, and its harvesting requires an additional surgical intervention with associated morbidity, pain, and secondary complications [29].

The use of autologous bone marrow mononuclear cells (containing MSCs) and the relatively simple and noninvasive method to harvest them by aspiration is becoming very popular. The idea of extracting and concentrating bone marrow aspirate was pioneered by Hernigou in 2002 [30]. This procedure can be performed rapidly during the same surgical act using point of care medical devices [31, 32].

At present, one of the most straightforward bioregenerative strategies, specifically for those clinical conditions that cannot be addressed by standard of care treatments, is the use of autologous bone marrow aspirate concentrate (BMAC). This approach, based on the concentration of bone marrow mononucleated cells using CE-marked kits or commercially available devices, is performed in the operating room during surgery. This procedure does not involve any substantial cellular manipulation and when the cells are injected within the same histological environment (intraosseus) also would comply with a homologous use. Thus, this therapy could avoid the classification and specific regulations associated with advanced therapy medicinal products (ATMPs), regulated in Europe by European Medicines Agency (EMA). Moreover, the safety of these procedures, as well as significant clinical evidence, has been confirmed by many authors previously [33–35].

The use of bone marrow aspirate concentrate is among the different bioregenerative therapies in the field of musculoskeletal injuries, being specially used by orthopedic surgeons.

The final result of centrifuging a bone marrow aspirate is a concentrate of mononucleated cells, which includes MSCs at low frequency (0.001–0.01% of all nucleated cells). The BMAC also includes platelets, which might also be relevant in the clinical response observed.

The scientific evidence have demonstrated that its use as a single or complementary regenerative therapy enhances the physiological bone repair capacity, allowing a better and faster patient recovery.

This strategy has been used for several clinical indications, such as bone fractures, pseudoarthrosis (non-unions), avascular necrosis of the femoral head (AVN), or osteochondral lesions.

How Is BMAC Obtained?

The aspiration of bone marrow concentrate is usually performed under sedation and local anesthesia in the operating room. A percutaneous aspiration with a 13 G trocar, placed at the anterior part of the iliac crest, is performed on the ipsilateral side of the lesion to be treated.

After perforating the iliac crest, the trocar is introduced about 5 cm in depth. The trocar posi-

tion must be changed continuously (in depth and direction), aspirating a maximum of 5 cc at a time. The aspiration technique is critical in order to obtain the highest number of progenitor cells and avoid the contamination with peripheral blood.

This step is crucial, because aspirating from a single point only using high volumes might contaminate the aspirate with peripheral blood, and we would not be getting the progenitor cells that are stuck in the bone or around the walls of the blood vessels (osteoprogenitor cells and MSCs) [36]. Sometimes, several aspiration points are used on the iliac crest to improve the aspiration technique.

Following the aspiration procedure, the aspirate is filtered in order to discard blood clots or bone chips. Usually around 60–120 ml of bone marrow aspirate are obtained, which are then centrifuged and resuspended to get 8–16 cc of BMAC. The BMAC is aspirated at the lower plasma phase including the buffy coat, avoiding most of the red blood cells at the bottom.

Finally, the injection of BMAC is performed by minimally invasive techniques, either directly intraosseous through a trocar or as an adjunct to other surgical procedures such as arthroscopic subchondral bone microfractures with or without a scaffold [37].

Advantages and Disadvantages Over Other Therapeutic Procedures

Comparing the use of BMC with PRP (platelet rich plasma), for the same therapeutic indications, this procedure allows the inclusion of progenitor cells residing in bone marrow. There are several commercially available point-of-care devices for extracting and concentrating bone marrow aspirates [31].

Regarding the cell obtention from other tissues such as adipose, the main advantage is that it is easier and more convenient to perform an aspiration of the iliac crest by an orthopedic surgeon than to perform a liposuction.

Comparing the use of BMAC with conventional surgical procedures such as prosthetic surgery or osteosynthesis in pseudoarthrosis, this procedure allows the physician to obtain satisfactory clinical results (pain improvement and functional recovery) without adding aggressiveness to the surgery.

About the disadvantages, it is well described on the scientific literature that the number MSCs obtained per gram of adipose tissue is much higher compared to one cc bone marrow aspirate [5]. Adipose stromal vascular fraction also contains higher amounts of other cell types with angiogenic potential, such as endothelial progenitors or pericytes. Some authors suggest that adipose might be a better source for MSCs due to the superior phenotype and functional capacities of isolated cells (i.e., osteogenic differentiation). However, this debate is still controversial since conflicting results have been reported [38].

Another hurdle that requires careful analysis is the "contamination" of bone marrow aspirate with peripheral blood, which is called peripheral blood admixture. This happens when the volume of bone marrow aspirate increases or when most volume is obtained from the same location. This could lead to a significant increase in the percentage of nucleated cells coming from blood in the bone marrow aspirates [36].

Clinical Use of Bone Marrow Concentrate

There are several clinical reports that support the use of bone marrow concentrate for hip avascular necrosis (AVN) treatment after decompression of the femoral head, with promising results, especially in the earliest stages of the disease (grades I-II of AVN) [33].

The summarized procedure for AVN is the following: the patient is placed on the traction table, and using a fluoroscope, the guiding needle is percutaneously placed in the center of the necrotic lesion in the anteroposterior and axial hip planes. Then a tunnel is created with a 4 mm drill to inject the BMAC into the affected area of the femoral head.

The patient is discharged on the same day with partially loaded crutches, and heparin prophylaxis for 10 days [39].

Moreover, several studies have reported its use for pseudoarthrosis and non-union, where the fractures with best results were those that had a greater number of progenitor cells [40]. Pseudoarthrosis (non-union) is defined as an abnormal union formed by fibrous tissue after a bone fracture that has bone healing problems. In these cases, the non-healing lesion is perforated using drills, traversing the proximal and distal fracture sites. Through the channel created at the non-union site, the cannula is introduced and the BMAC injected, in the focus, proximally and distally, without using any type of osteosynthesis material [41].

Promising results are also being reported for knee osteoarthritis (OA) in early–moderate stages (I-II) [42]. This response could be dose dependent as supported by some authors according to the final cell dose used [43].

The pathogenesis of osteoarthritis is very complex and not fully understood. As proposed by several authors, it seems that the subchondral bone plays an important role and would affect the articular cartilage degeneration. Thus, the approach of injecting the BMAC or any other biological product intraosseus at the subchondral affected bone is advantageous. This infiltration could be more efficacious even for more severe OA, promoting the inhibition of cartilage-degrading cytokines, stimulating chondrogenesis or the production of hyaluronic acid and lubricine by chondrocytes [44].

Human Clinical Studies Using Adipose Freshly Isolated Cells (SVF)

There is substantial and encouraging preclinical and clinical information that supports the use of freshly isolated autologous cells from adipose tissue samples [45, 46]. Adipose SVF cells are currently being used in different clinical settings. As of April 2016, a total of 75 clinical studies are registered in clinicaltrials.gov, being USA and Europe the most active regions worldwide (Fig. 3). Forty-two of the total number were registered as active studies recruiting patients.

The main and more studied targets for intervention are soft tissues (radiation wounds, diabetic ulcers, etc.), musculoskeletal tissues (bone defects, tendinopathies, osteoarthritis, etc.), ischemic injuries, and immune disorders.

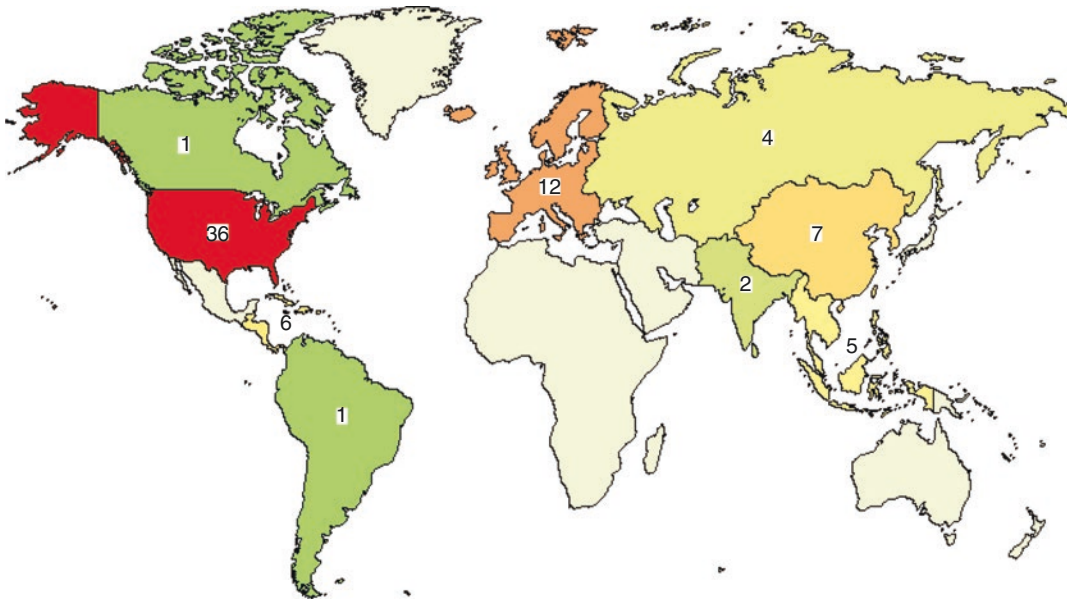

Fig. 3 Clinical studies using SVF colored by number including locations around the world. Source:https://ClinicalTrials.gov

The field of orthopedics is being most active for clinical SVF application. In this regard, many studies have reported significant clinical improvements for osteoarthritis, particularly in the knee and hip joints [47–51]. Recent clinical reports also suggest a beneficial effect of SVF cells for Achilles tendinopathy [52] and bone regeneration [53, 54]. The SVF application has been also successfully performed in patients with burn wounds [55] and other chronic wounds associated with peripheral vascular disease or diabetes [56].

Safety and feasibility is clearly demonstrated in all these studies. Patient clinical efficacy is also frequently reported. However, well-designed randomized clinical trials including controls are still needed to confirm this initial but compelling evidence. Moreover, any clinical use of cells must comply with applicable regulations.

Concluding Remarks

The field of regenerative medicine and biological therapies is evolving very rapidly and constantly changing. Many findings (preclinical and clinical) on different medical fields support this new paradigm of using cell-based therapies for treating patients. This is especially important when current traditional approaches do not provide satisfactory clinical results.

However, there is still no agreement on which tissue source or cellular product is the best for each clinical indication. Furthermore, many different devices or methods are available for the same purposes, creating more confusion. Any clinical decision based on biological or cellular products must be based only on scientific and clinical evidence. Different approaches will yield very different final products. The physician is responsible for choosing the best cost-effective method for a given clinical indication based on disease severity focusing on patient safety and all scientific information available to provide the best possible patient care.

There is currently a great opportunity to continue the scientific progress by addressing these questions developing comparative studies using different cell isolation methods or approaches for specific medical problems. The field of plastic and aesthetic medicine can be on the front of these advances. All assessments must be based on well-designed cell quality and potency assays while keeping patient safety at the highest levels. Both basic science and clinical research should complement each other in this fascinating endeavor.

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