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"MEDICINA SPERIMENTALE E RIGENERATIVA"
XXX Cicle

THE ROLE OF ADIPOSE STEM CELLS SEEDED ON A CROSS-LINKED BOVINE TENDON COLLAGEN AND GLYCOSAMINOGLYCAN (CHONDROITIN-6-SULFATE) SCAFFOLD IN THE TREATMENT OF ULCERS OF THE LOWER LIMBS.

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1. INTRODUCTION

Tissue engineering is an interdisciplinary field of bio-medical research focusing on the restoration of tissue defects or even on the replacement of a complete organ[1-3]. A well-established approach for the generation of tissue constructs is the seeding of cells onto different biomaterials, which serve as three-dimensional scaffolds. To ideally promote the function and regenerative capacity of seeded cells, scaffolds should mimic the natural extracellular matrix [4-7]. In this case, three components are necessary: 1) the stem cells; 2) the scaffold supporting formation of tissue architecture and cell function; 3) the appropriate cocktail of growth factors and other molecules with trophic, surviving and pro-angiogenic properties[8-9].

Stem cells have the ability to regenerate themselves and to differentiate in one, or several, differently specialized cells. They are present in many human adult tissues, such as blood, bone marrow, adipose tissue, musculoskeletal tissue, epidermis, brain and vascular tissue, and they are important for tissue repairing and for maintaining cellular homeostasis (regenerative capacity). It has been shown that these stem cells are able to differentiate, through specific stimuli, not only in the cells of the tissue from which they were isolated, but also in other not related phylogenetically to the tissue in which they reside. For example, epidermal stem cells are able to differentiate itself both in keratinocytes, nerve cells and muscles. The recognition that even stem cells found in adult individuals (i.e. not only fetal or embryonic tissues) possess a multipotent differentiation therefore represents a major scientific discovery that could open new research prospective both in tissue bioengineering and in the treatment of different pathologies.

One of the main sources of stem cells is adipose tissue, in which recent studies have demonstrated the presence of multipotent and pluripotent stem cells, all able to regenerate themselves and to differentiate in various specialized cell types. Due to its abundance and minimal-invasive accessibility, adipose tissue is an attractive source for harvesting stem cells with minimum discomfort for the patient.

Fat transfer, in fact, is an easy technique, with limited invasiveness, executable in Day-Hospital. It is repeatable with high compliance and is poor in complications. Since 1893 it was recommended to use fat cells to fill areas of depression where the structure had a deficit. Since then the sampling technique and injection have been gradually improving until the consolidated method by Coleman in 1998. Fat transfer (the so called "lipofilling") has been used for many purposes such as treatment of tissues depression, burns, scars, restoration of facial and body volume in cosmetic surgery, breast reconstructive or aesthetic surgery, treatment of difficult wounds etc..

In these past years it has been possible to realize that lipofilling had not only a filler function, but also a "curative" one. In fact, in patients undergoing this surgery was easy to recognize an improvement in quality, texture and appearance of the treated areas, this thanks to the presence within the fatty tissue of stem cells and growth factors. For this reason, lipofilling has been used previously by some authors for the treatment of chronic wounds refractory to common cure in use, such us post traumatic lesion, bed sores at first stage or skin ulcers of various etiology. In these studies, however, fat was always used in combination with other regenerative factors, such as mesenchymal cells or platelet rich plasma. ADSCs contribute to healing of the ulcer through stimulation of processes that involve the formation of a neo-

epithelial barrier similar to the skin, together with angiogenetic factors and antiapoptotic factors such as monobutyril, VEGF, leptin, and KGF.

2. REGENERATIVE MEDICINE AND TISSUE ENGINEERING

Various healthcare programs have been implemented by nations in order to address the increasing medical needs of dealing with chronic disease. The current standards of care are largely based on palliative therapies and the use of pharmaceutical drugs. Whereas, allotransplantation, autologous tissue transfer, and the use of synthetic materials are presently used for treating many acute and chronic medical conditions, such as organ failure, tissue loss due to trauma, cancer ablation or even congenital structural anomalies for which no adequate treatment is available[10-11]. However, these therapeutic approaches have limitations and risky side effects, including organ shortages, donor site morbidity, allergic reactions, and immune rejection[12].

Regenerative medicine and tissue engineering interventions aim to treat the root cause of the disease linked to progressive cell destruction and irreversible loss of tissue function with the promise to meet the two most urgent needs of organ transplantation: the identification of a new, potentially inexhaustible source of organs and immunosuppression-free transplantation[13]. In other words, instead of simply mitigating the symptoms as traditional (drugs) therapy approaches do, regenerative medicine aims to repair the underlying pathobiology or restore/replace the native cellular architecture and organ function.

Regenerative medicine is a new and rapidly developing interdisciplinary branch of medicine, typically characterized by a convergence of disciplines such as cell biology, biochemistry, molecular embryology, immunology, advanced materials science, and engineering, such as the 3D bioprinting[14]. The goal of regenerative medicine is to replace or regenerate human cells, tissues,

or organs in order to restore or establish normal function. It achieves this by delivering functional cells, supporting scaffolds, growth promoting and signal molecules or DNA encoding these molecules. On the other hand, tissue science and engineering is the use of physical, chemical, biological, and synthetic processes to control and direct the aggregate behaviour of cells, thereby tissue engineering can be considered a subcategory of regenerative medicine[15]. In a broad sense, regenerative medicine encompasses some of the knowledge and practice of tissue science and engineering but also includes self-healing through endogenous recruitment or exogenous delivery of appropriate cells, biomolecules, and supporting structures. The field has already made headway in the synthesis of structural tissues such as skin, cartilage, bone, and bladder [16]. The classic regenerative medicine is to isolate specific cells through a biopsy from a patient, to grow them on a three-dimensional biomimetic scaffold under precisely controlled culture conditions, to deliver the construct to the desired site in the patient's body, and to direct new tissue formation into the scaffold that can be degraded over time [17-18].

3. ROLE OF STEM CELLS IN REGENERATIVE MEDICINE

Within regenerative medicine, stem cells have shown great promise. Tissue engineering and regenerative medicine are undertaking the quest of finding the most suitable type of stem cells that could be employed for therapy, and various types of stem and progenitor cells are in meantime being employed in various clinical trials to replace or regenerate damaged organs[19-20].

Stem cells are believed to be part of the internal repair system of the body, where they replace cells that are lost due to normal turnover or pathological conditions. They are unspecialized cells capable of dividing asymmetrical, thereby continuously renewing themselves and giving rise to specialized cell types [21].

Exogenous stem cells can be originated either from the embryo, extra-embryonic fetal tissues, or from adult tissues. Embryonic Stem Cells (ESCs) are pluripotent cells derived from the inner cell mass of the blastocyst within the first 5-7 days after an egg is fertilized by sperm. They can produce derivatives of all three embryonic germ layers: endoderm, ectoderm and mesoderm[22]. Although ESCs could be used in principle for the treatment of many genetic and non-genetic diseases, the enthusiasm about their use has faded away with time, for ethical issues (destruction of embryos), immune rejection[23], and the possibility of tumour formation[24].

More recently, great attention has been given to induced Pluripotent Stem Cells (iPSCs), which were first generated from adult somatic cells (mouse fibroblasts) through retroviral-mediated expression of four “stemness” genes (KLF4, SOX2, OCT4, and cMYC)[25]. Subsequently, cMYC was omitted as its contribution concerned only accelerated proliferation and in further works, iPSCs were obtained after using LIN28 as the fourth gene[26-27]. These iPSCs

showed similar function and molecular phenotype characteristics to ESCs and since then many methods employing viral and nonviral vectors have been used to obtain iPSCs[28-29]. Since constitutive expression of reprogramming transgenes interferes with iPSCs differentiation into lineages of all three primary germ layers[30], and aberrant expression of some or all of the reprogramming factors could lead to tumorigenesis in vivo[31] and may affect global gene expression[32], methods to obtain iPSCs free of reprogramming transgenes have been searched. This could be achievable using non-integrating vectors, or even direct protein delivery, but the efficiencies were exceedingly low that prevent reliable application for reprogramming disease-specific adult human somatic cells[33-36]. Moreover, the genetic instability of iPSCs in long-term culture, and consequently their potential tumorigenicity[37], are likely the main reasons preventing their approval by the US-Food and Drug Administration (FDA) and the European Medicines Agency (EMA). This drawback has also been proved in humans with age-related macular degeneration treated with iPSCs differentiated from retinal pigment epithelium (RPE). In one individual, transplantation achieved a very encouraging clinical outcome, while the second subject did not receive a transplant due to the detection of gene mutations in the RPE cells differentiated from the autologous iPSC lines[38].

Fetal stem cells are derived from extra-embryonic tissues (amniotic fluid, placenta, umbilical cord blood and Wharton's jelly), exhibit less growth capacities than ESC, demonstrate low immunogenicity in vivo[39], and do not give rise to tumours. These cells are still poorly characterized and are being presently evaluated in the field of regenerative medicine[40-41].

Adult stem cells are multipotent and are able to differentiate into a limited number of cell types, often those originating from the same germ layer. A type of adult somatic stem cells is mesenchymal stem cells (MSCs), derived from

the mesodermal embryonic tissue. MSCs is the more common term used for stem cells with a self-renewal capacity and multipotent ability, that are precursors of cartilaginous, osseous, adipose and other mesenchymal tissues[42]. Even if the bone marrow (BM) is the most common source, MSCs have been identified in skeletal muscle, pancreas, synovium, skin, blood vessels, adipose tissue, and placenta[43-44]. MSCs isolated from different sources share similar characteristics, although recently it has been recognized that subsets of MSCs with differences in protein and gene expression can be identified in the various tissues[45].

MSCs have generated substantial interest in the medical areas of transplant, regenerative medicine and cancer treatment because of their multi-potency and multi-functionality. Besides the induction of angiogenesis[46], these mesodermal cells are potential modulators of hostile injury microenvironments through their immunomodulatory and anti-inflammatory properties with the result of limiting inflammatory damage to the tissues[47-51]. Among the immunomodulatory activities, MSCs have been described to suppress T- and B-cell responses, to modulate the functions of regulatory T cells, and to inhibit the maturation, activation and antigen presentation of dendritic cells[52]. Anti-inflammatory effects by MSCs are gained through the production of a host of molecules, such as for example tumor necrosis factor (TNF)-stimulated gene-6, interleukin-10, prostaglandin-E2, and other bioactive molecules that act on macrophages[53-54]. Because of their versatility, MSC- based therapies are increasingly brought to the clinics and include stem cell implantation or infusion to treat hematopoietic disease, cardiac conditions, Parkinson’s disease, respiratory diseases, as well as rheumatology and orthopaedic morbidities[55].

4. ADIPOSE DERIVED STEM CELLS

MSCs in the adipose tissue, termed adipose- derived stem cells (ADSCs), have been shown in large to display same biological capabilities as the BM-MSCs[43]. The advantages of ADSCs over BM-MSCs and other adult stem cell types are that ADSCs are relatively easy to obtain from liposuctions performed in local anesthesia, can be obtained in large numbers, are capable of maintaining their phenotype and plasticity after long term in vitro culture and they comprise a low immunogenicity[55]. Based on this, ADSCs have generated great interest and are perceived as the most preferred cell type for tissue engineering and regenerative medicine[56].

Zuk et al.[57] were the first to investigate whether human adipose could be an alternative source of MSCs. These authors obtained human adipose from liposuction aspirate and used collagenase to release stromal cells from the extracellular matrix by processing the so-called stromal vascular fraction (SVF), containing a variety of different types of cells including ADSCs. The isolated adipose stromal cells were cultured with defined media to induce adipogenic, osteogenic, or chondrogenic differentiation. It was observed that adipose stromal cells were capable of developing intracellular lipid stores, alkaline phosphatase expression, or proteoglycan expression, markers indicative of adipose, bone, and cartilage tissues, respectively. In order to determine if the isolated adipose stromal cells were indeed stem cells, Zuk et al.[58] examined surface antigen expression and differentiation capacity of clonogenic cultures. Using flow cytometry, the authors observed that the clonogenic cells expressed surface antigens similar to marrow MSCs. Table 1 presents the main phenotypic characteristics of ADSCs.

Table 1 Phenotypic markers of undifferentiated ASCs

	Expression	References
Adhesion molecules		
• β1 integrin (CD29)	+	[76,78]
• α4 integrin (CD49d)	+	[58,76]
• Vascular cell adhesion molecule (VCAM; CD106)	+/-	[58,76]
• Intercellularadhesionmolecule (ICAM-1; CD54)	+	[76]
• Activated leukocyte adhesion molecule (ALCAM; CD166)	+	[58,76]
• Tetraspan protein (CD9)	+	[76]
• Endoglin (CD105)	+	[76]
Receptors		
• Hyaluronan receptor (CD44)	+	[58,76]
• Transferrin receptor (CD71)	+	[58]
• Triiodothyronine (T3) receptor α	+	[79]
Surface enzymes		
• Neutral endopeptidase (CD10)	+	[76]
• Aminopeptidase (CD13)	+	[76]
• Ecto 5' nucleotidase (CD73)	+	[77]
Cytoskeleton proteins		
• α-smooth muscle actin	+	[58, 76]
• Vimentin	+	[57]
Others		
• HLA-ABC	+	[76]
• CD34	+	[58,76]
• Decay accelerating factor (CD55)	+/-	[58,76]
• Protectin (CD59)	+	[58,76]

NOTE:+, positive association; –, negative association

Moreover, besides the mesenchymal lineage differentiation, the clonal cells were capable of differentiating into neuron-like cells, as judged by morphology and phenotypic marker expression. ADSCs are also prone to stimulate angiogenesis[59-60], an essential feature for regenerative purposes. Their

neurotrophic and angiogenic properties have been shown to be due to the secretion of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell- derived neurotrophic factor (GNDF), vascular endothelial growth factor-A (VEGF-A) and angiopoietin-1[61]. Indeed, the secretome of ADSCs is complex, ADSCs having the property of secrete proteins involved in angiogenesis, wound healing, tissue regeneration and immunomodulation[62]. Moreover, ADSCs have the capacity to differentiate into Schwann cells[63], pancreatic beta cells[64], and hepatocytes[65-67]. Interstingly, recente work on ADSCs from pediatric patients has shown their ability to give raise to non-mesenchymal cells, consistent with significant plasticity. Indeed, both mesenchymal lineages (adipogenic, chondrogenic and osteogenic) and neural and epithelial lineages can originatefrom clonal lines that like the parental line express markers of pluripotency[66].Thus, ADSCs are endowed with properties essential in wound healing and might be an interesting source of stem cells for tissue engineering and regenerative medicine for many medical and surgical applications. For example, Phase I and II clinical trials are evaluating safety and efficacy of ADSCs in the setting of myocardial infarction[68]. Other medical and surgical conditions are being treated with ADSCs, demonstrating ADSC's positive effects in tissue engineering and regenerative medicine[69].

4.1. LOCALIZATION AND ORIGIN OF ADSCs

ADSCs have been shown to be associated with small vessels. Based on the positivity to the CD34 antigen, Zimmerlin and colleagues[70] identified two different perivascular subpopulations in SVF: pericytes (CD146+/CD34-/CD31-) and the so-called supraadventitial adipose stromal cells, SA-ADSC (CD146-/CD34+/CD31-). Other studies have shown that pericytes and ADSCs share many cell surface markers, including smooth muscle β-actin, platelet-

derived growth factor (PDGF) receptor-β, and neuro-glial proteoglycan 2[71-72], thus bringing to hypothesize that pericytes could be precursor cells for ADSCs *via* a transitional cell[70]. It has been proposed that these SA-ADSCs may replace the terms "pre-adipocytes" and "adipose-derived (AD)-MSCs" because the latter terms confounded several distinct populations[73-74]. These considerations bring to the heterogeneity of ADSCs and for this reason we will use this term in the following discussion. To further complicate this issue, it has been shown that the adult human subcutaneous adipose tissue contain a small percentage (4-9%) of stem cells, called multilineage-differentiation stress-enduring (Muse) cells, which are of mesenchymal origin, are positive for stage-specific embryonic antigen-3 marker (SSEA-3), characteristic of ES cells, and can differentiate into cells of mesodermal, endodermal, and ectodermal lineages *in vitro*, and have the ability of self-renewa[74]. Adipose-Muse cells were found to be negative for CD34 and C146, and thus may represent a distinct population of ADSCs or a subset with very interesting features for regenerative medicine. Indeed, they were nontumorigenic when injected in immune-deficient testes and presented low telomerase activity[74].

4.2. SIMILARITIES AND DIFFERENCES WITH BM-MSCs

Since bone-marrow-MSCs (BM-MSCs) have been the gold standard for MSCs for many years, most of the properties of ADSCs have been described in comparison with those cells. Following the indications made by Dominici *et al.*[75], BM-MSCs and ADSCs share a fibroblast-like morphology and immunophenotype for mesenchymal markers[76]. Like BM-MSCs, ADSCs express higher levels of stromal-associated markers (CD13, CD29, CD44, CD73, CD90, CD105, CD166) by the later stages of culture[77]. Nevertheless, ADSCs and BM-MSCs differ as concerning the isolation yield, the frequency

of colony forming units and the differentiative capacity. Indeed, ADSCs are usually isolated from the stromal-vascular fraction (SVF) of the adipose tissue[58-57] in numbers which are approximately from 40 to 100-500 fold higher than BM-MSCs[80-81]. Moreover, it appears that ASCs and BM-MSCs display some differences at genomic and proteomic levels, as well at functional level[82-85].

4.3. ENZYMATIC AND NON-ENZYMATIC ISOLATION OF SVF

The most widely used technique for the isolation of SVF from lipoaspirate is by digestion of the fatty portion of the lipoaspirate with collagenase, separating the contents into two distinct phases: the floating mature adipocytes fraction, and the cellular components of interest in the lower aqueous fraction[86-87]. This separation can be enhanced by centrifugation; nevertheless, comparable separation can be achieved by gravity-based phase separation and filtration[88]. Although centrifugation is more efficient, it will also pellet down all the cells present, while filtration can be designed to capture only the important cell types based on size, thus enriching the specific cellular cocktail.

Centrifugation of the aqueous fraction yields a reddish pellet which contains SVF cells. Erythrocytes, a major contaminant present in the SVF pellet, can be lysed to isolate a purer population of ADSCs and/or SVF cells if intended for in vitro expansion[89-90].

In view of the regulatory questions relating to enzymatic isolation, it is important to look into alternative methods for isolating SVF and compare these with the conventional methods[91-93]. Most of these techniques involve mechanical agitation which breaks down the adipose tissue and releases the stromal cells. As expected, the cellular yield from mechanical procedures are

much lower compared to enzymatic methods, as cells of the adipose tissue tightly bound by collagen will not be easily released by mechanical action alone[92].

A novel method of mechanical agitation was recently defined by Tonnard et al.[94]. The injectable product, termed as “nanofat”, was obtained by emulsification and filtration of the lipoaspirate. Although termed as nanofat grafting, in effect no viable adipose cells survived the emulsification process, but the graft was rich in CD34+ ADSCs. The efficacy and properties of nanofat have been demonstrated in multiple case studies related to skin rejuvenation, scar healing, skin grafting for wound management, and treating vulvar lichen sclerosus (VLS), a chronic inflammatory disease of the anogenital area, and also by standard ADSC-related phenotypic and differentiation studies[94-96]. Owing to the simplicity of the technique, it might be amenable to scaling up by simply using the desired volume of syringe and/or using multiple syringes as required.

The effect of the emulsification process on other cells of interest, normally found in enzymatically processed SVF, remains to be seen. Combining such techniques with centrifugation or filtration can yield products highly concentrated with ADSCs, thus eliminating enzymatic digestion, reducing process time, cost, and respective regulatory constraints.

4.4. DIFFERENTIATION CUES

In order to determine if adipose tissue is a comparable source of MSCs to bone marrow, yields and differentiation capacities of cells isolated from each tissue were compared. For example, De Ugarte et al.[97] found no significant differences in the number of culture adherent cells per gram of stromal cells

obtained from human marrow or adipose tissue. Yet more than double the average mass of adipose tissue (17 g) could easily be isolated from each patient compared to bone marrow (7 g). The authors also cultured isolated cells in various differentiation media, finding no difference in the number of cells that developed lipid droplets (adipogenic cells), or the alkaline phosphate activity of osteogenic cells. However, when induced to differentiate into cartilage, adipose derived cells stained positive for chondrogenesis while marrow derived cells did not. Using similar methods, several other investigations have compared the ability of marrow and adipose cells to differentiate along these lineages and have demonstrated that cells from either tissue possess an equal capacity to become adipose, bone, and cartilage [98-99]. Overall, these results may be the outcome of different culture conditions and/or the isolation of different subsets of MSCs, highlighting that the potential of ADSCs to differentiate into either osteoblasts or chondrocytes is controversial at minimum.

Although, as cited above, ASCs and BM-MSCs show some differences in the differentiation capability, ASCs display the same capacity of BM-MSCs to give rise in vivo to osteophytes and chondrophytes when injected in the knee of severe combined immunodeficient (SCID) mice[85]. Overall, ADSCs need growth/morphogenic factor supplementation from the tissue environment to be appropriately differentiated to mesodermic lineages.

More recently, stimulated ADSCs seeded onto fibrin conduits were shown to boost axon regeneration and angiogenesis in a rat sciatic nerve injury model[100], much alike as BM-MSCs[101], although a direct comparison was not made. However, it seems that neurite outgrowth enhanced by BM-MSCs occurred independently of BNDF[101]. Other studies have shown that human ADSCs express a range of neurotrophic factors which can enhance neurite outgrowth of neuronal cell lines[102-104]. Thus, the precise mechanism of neurite outgrowth mediated by ADSC remains to be elucidated.

Other studies have shown that human ADSCs used in animal experimental models can promote tissue formation and enhance graft retention as a result of enhanced vascularity[105-107]. In this context, however, the precise mechanisms are not known. It can be that engraftment of ADSCs into the novel vessels and paracrine factors are at work together. Choi and colleagues[106] showed that co-implantation of ADSCs with rat cardiomyocytes in a vascularised tissue engineering chamber resulted in integration of ADSCs into the endogenous vascularisation of the construct. On the other hand, the same group demonstrated that while increased levels of the pro-angiogenetic chemokine interleukin (IL)-8 were observed *in vitro* when ADSCs were grown on a rat cardiac extracellular matrix (ECM) extract-derived hydrogel (cardiogel), the presence or absence of cardiogel had no effect on neoangiogenesis in the tissue chamber model[94]. In general, these results suggest that the right combination of ADSCs and ECM scaffold should be further explored for tissue engineering purposes *in vivo*.

Taken together, the above evidence demonstrates that compared to bone marrow, a high number of MSCs capable of multi- lineage differentiation can be obtained from adipose tissue, and that more cues to their differentiation should be found in in vivo models or in vitro models recapitulating in vivo situation.

Due to their interesting properties, ADSCs have been found useful for plastic surgery applications, including fat grafts, management of difficult wounds, regeneration of local soft tissue defects, bone reconstruction, recovery from acute tissue ischemia of vascular origin, and scar management[106-108]. The optimal delivery system has to be likely tailored for each morbidity to cure and that is why plenty of methods have been evaluated for ADSC treatment, including systemic administration, local injection, topical applications, and different scaffolds[108].

5. PLATED-RICH PLASMA IN REGENERATIVE MEDICINE

The current practice of regenerative medicine encompasses not only the use of mesenchymal stem cell therapy but also platelet-rich plasma (PRP), a concentration of blood-derived human platelets in a small volume of plasma. Platelets are non-nuclear cellular fragments derived by megakaryocytes localized in the bone marrow. Mitochondria and the dense tubular system are responsible for providing energy, messengers for reactivity and platelet functions. Platelets are crucial for prevention of blood loss after vessel injury, a process known as haemostasis. They contribute to normal haemostasis in several different ways. First, they adhere to the extracellular matrix of the wounded vessel and prevent blood loss by acting as a physical barrier, and the effectiveness of this mechanism is increased by the ability of platelets to bind to each other in an interaction called aggregation. In addition, platelets contribute to haemostasis by secreting vasoactive substances such as thromboxane A2 that contributes to haemostasis by constricting the wounded vessel[109]. It has also been established that platelets are important for blood coagulation induced by vessel injury with consequent release of tissue factor (TF). Briefly, TF induces a cascade of events where proteases serially cleave each other, which results in the production of a blood clot composed of fibrin. The fibrin clot contributes to the physical blocking of blood loss through the wounded vessel. Platelets affect the process of blood coagulation by acting as an attachment site for coagulation proteases. This facilitates the interactions between coagulation proteases and it also protects the coagulation proteases from degradation by protease inhibitors[110].

Besides haemostasis, platelets are crucial for tissue repair and vascular remodelling[111-113]. They produce cytokines, chemokines and growth factors

promoting recruitment, adhesion, and proliferation of adult stem cells. Moreover, platelets provide survival signals to monocytic, endothelial, and neural stem cells[114]. The first stage of normal wound healing, immediately following injury or insult, is inflammation, where activated platelets adhere to the site of injury releasing growth factors (Table 2).

Platelet-derived growth factors

Growth factor	Function
Transforming Growth Factor (TGF-β)	promotes formation of extracellular matrix and regulates bone cell metabolism
Platelet-Derived Growth Factor (PDGF)	promotes cell replication, angiogenesis, epithelialization and granulation tissue formation
basic Fibroblast Growth Factor (bFGF)	promotes proliferation of endothelial cells and fibroblasts and stimulation of angiogenesis
Epidermal Growth Factor (EGF)	promotes cell differentiation and stimulates re-epithelialization, angiogenesis and collagenase activity
Vascular Endothelial Growth Factor (VEGF)	promotes angiogenesis
Connective Tissue Growth Factor (CTGF)	promotes angiogenesis, vessel permeability, and stimulates mitogenesis for endothelial cells

Table 2. Platelet-derived growth factors.

The theoretical concept that concentrating platelets at the injured site could accelerate and optimize the healing mechanisms set the rationale for the development and continued research into the use of PRP in the clinical application for regenerative medicine[115].

6. FAT GRAFTING

Historically, the use of fat grafts to correct congenital deformities and complex traumatic wounds with soft-tissue loss after radical oncological surgery was proposed in 1893 by Neuber, in 1912 by Hollander, in 1921 by Neuhof, and in 1931 by Josef[116]. The liposuction technique, introduced by Fisher in 1974, followed by the tumescent technique, introduced by Klein in 1985, accelerated the development of the lipofilling technique. The tumescent technique allowed patients to undergo liposuction under local anaesthesia administered using small cannulas[117]. In 1987, Coleman[118] introduced a new technique to decrease traumatic handling of fat during liposuction. Coleman's technique consisted of three steps: manual lipoaspiration under low pressure, centrifugation for 3 min at 3400 rpm, and reinjection in small aliquots in a "fanning-out" pattern to varying depths in the soft tissue only during withdrawal of the cannula. This technique remains the gold standard for liposuction and lipofilling, but has undergone some technical modifications[119]. Since the 1980s, autologous fat transplantation has been one of the most popular procedures performed by plastic surgeons[120]. In 2009, fat grafting represented 5.9% of all non-surgical aesthetic procedures[121]. However, because the results of lipofilling are variable, optimization of the procedure is required. The long-term results of fat grafting are often disappointing because of unpredictable partial absorption of up to 70% of the volume of the fat graft. A number of studies have reported resorption rates of 30-70% within a year[122]. Thus, autologous fat grafting has unpredictable success rates, and there is no agreement among physicians as to the ideal method for the harvesting and handling of fat grafts[119-122] The Coleman technique should be considered as the standard and preferred method for harvesting and processing. However, one of the problems observed is a decrease in the number of fat cells because of damage caused during the aspiration and centrifugation steps. Another limitation is the requirement to

infiltrate cells in direct contact with well-vascularized tissues[123]. Furthermore, the Coleman technique can be operator dependent and time-consuming if performed by less-experienced surgeons. Numerous modifications of the Coleman have been attempted in order to improve the survival of the injected fat, including atraumatic fat-harvesting, fat washing to eliminate inflammatory mediators, centrifugation, and incubation of fat grafts with different bioactive agents. Fat is a filler with ideal properties: it naturally integrates into tissues, is autologous, and is 100% biocompatible. However, this is not the only function of lipofilling; fat is an active and dynamic tissue composed of several different cell types, including adipocytes, fibroblasts, smooth muscle cells, endothelial cells, and adipogenic progenitor cells called "preadipocytes"[124-126] Adipose-derived stem cells (ADSCs) have a differentiation potential similar to that of other mesenchymal stem cells as well as a higher yield upon isolation and a greater proliferative rate in culture when compared to bone marrow-derived stem cells. Because of these properties and because these cells can be easily harvested in great amounts with minimal donor-site morbidity, ADSCs have proved to be particularly promising for regenerative therapies[127-129].

It is widely accepted that less-traumatic methods of fat harvesting result in increased adipocyte viability and graft survival. Several techniques have been proposed for fat harvesting, and there is an ongoing debate in the literature as to which method produces more viable and functional adipocytes. The main techniques are vacuum aspiration, syringe aspiration, and surgical excision. Recent experimental as well as some clinical studies support direct fat excision over aspiration. Fagrell et al.[130-132] introduced a technique called "fat cylinder graft," in which fat is drilled out in cores by a punching device, whereas Qin et al. [133] recommended the core graft for block grafting because it maintains the structure and viability of harvested fat tissue by avoiding damage to the adipocytes. Pu et al. [134] found significantly impaired adipocyte function in

conventional liposuction aspirates compared with fresh fatty tissue samples and syringe-aspirated fat. Low negative-pressure lipoaspiration may yield fat faster than syringe aspiration and can be used when a large volume of fat is required, as in breast surgery. The high vacuum pressures of conventional liposuction may cause structural disruption in up to 90% of adipocytes[130-135]. Cannula size may also affect the viability of harvested fat[136]. Erdim et al.[137] reported higher graft viability with lipoaspirates that were obtained using a 6-mm cannula rather than a 4-mm or 2-mm cannula.

Coleman et al.[138] described a technique for fat harvesting that minimized trauma to the adipocytes. With a 3-mm, blunt-edged, 2- hole cannula connected to a 10-mL syringe, fat is suctioned manually by withdrawing the plunger. The cannula is pushed through the harvest site, as the surgeon uses digital manipulation to pull back on the plunger of the syringe and create a gentle negative pressure. A combination of slight negative pressure and the curetting action of the cannula through the tissues allows parcels of fat to move through the cannula and Luer-Lok aperture into the barrel of the syringe. When filled, the syringe is disconnected from the cannula, which is replaced with a plug that seals the Luer-Lok end of the syringe. The plunger is removed from the syringe before it is placed into a centrifuge.

There are different natural fat deposits in the body; surgeons should identify the most suitable area after an accurate examination of the patient. The abdomen is the most common site of fat harvesting; the second is the trochanteric region (saddlebags) and the inside of the thighs and knees[139-140]. The harvesting of fat grafts can be performed via a “wet” method or a “dry” method. In 1993, Klein et al.[141] described the “wet” method, which involves the injection of the donor site with a fluid solution (Klein's solution) containing 0.9% NaCl, epinephrine, and a local anaesthetic. Illouz and de Villers[142] highlighted the fact that the wet technique causes hydrodissection and enlarges

the target fat layer, thus facilitating the subsequent aspiration, with decreased pain and ecchymosis. It has been observed that low shear stress leading improves graft survival; in fact, the shear stress exerted on harvested fat has been determined to be a factor affecting adipocyte viability[143]. Alternatively, a "dry" method without the tumescent fluid could be used. However, the "dry" technique may lead to a greater requirement for analgesics.

The most commonly used methods to prepare fat grafts are sedimentation, filtering, washing, and centrifugation. Fat processing is necessary because the lipoaspirate contains not only adipocytes but also collagen fibres, blood, and debris. These elements can cause inflammation at the recipient site, which can be detrimental for the fat graft. Blood must be extracted because blood accelerates the degradation of the transplanted fat. Moreover, the injection of debris gives an erroneous impression of the volume of correction because the debris will be absorbed after a few hours[144-145]. In animal experiments, no significant differences have been observed in the weight or architecture of fat grafts obtained using the centrifugation, filtration, or sedimentation methods[146-148]. In contrast, studies conducted in patients have demonstrated more favourable outcomes with centrifugation rather than gravity separation[149]. Comparative studies investigating the effects of fat processing with centrifugation, washing, and filtration have shown no significant differences in fat retention; however, filtration resulted in nodule formation, whereas centrifugation did not[149-151]. Ferraro et al. demonstrated[123] that centrifugation with a force greater than 50 g resulted in damage to the structural integrity of adipose tissue, increased necrosis and apoptosis of cells, and decreased adipogenic differentiation capacity and tubule formation. Tubule formation during angiogenesis provides blood supply and nutrients to adipose tissue and ultimately sustains the fat graft for long-term retention. Higher centrifugation speeds have also been correlated with increased fluid portion, reduced

injectable tissue volume, and increased oil portion, which are associated with damage to adipocytes. Coleman suggested a processing method that has gained popularity and has been since integrated in many fat-transfer clinical protocols. Aspirated fat in syringes is spun at 3000 rpm for 3 min to isolate the fat. After the centrifugation, three layers are observed: the first layer includes lipids, which can be poured off using absorbent material; the second layer consists of fatty tissue; and the third layer contains blood, tissue fluid, and local anaesthetic and is ejected from the base of syringe. The middle layer is routinely used for adipose tissue grafting [152-153] (Fig. 1).

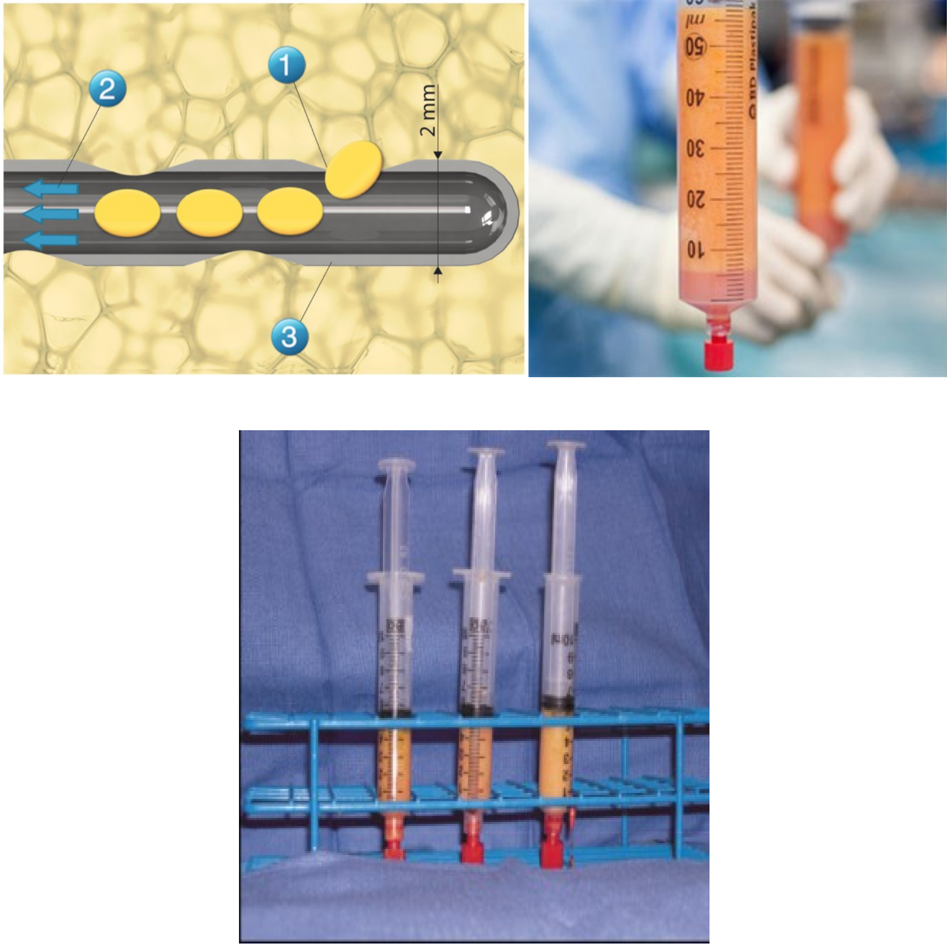

Fig.1 A: 1-adipose tissue, 2-negative pressure, 3-cannula; B: Lipoaspirate C: The “Middle Layer”, lipid and blood have been removed after centrifugation.

Despite a long history of clinical use and the evolution of fat-transfer

techniques, no consensus exists on the best technique and the longevity of results, yet the principles of fat reimplantation are based on optimal recipient-site vascularity for increased fat survival. Through a skin incision of a size corresponding to the diameter of the cannula, the fat graft is inserted at the level of the anatomical region affected. Small-gauge cannulas are thought to reduce trauma to the recipient site, thus reducing the risks of bleeding, haematoma formation, and poor graft oxygen diffusion[154]. Therefore, fat reinjection in multiple small-volume sessions is preferred over one single injection. Usually, through multiple access sites, multiple tunnels are created on insertion, but fat is injected only during withdrawal of the cannula in a "fanning-out" pattern. Fat grafts are distributed in small aliquots and fanned out to varying depths in the soft tissue to avoid excessive interstitial pressure at the recipient site and overcrowding of the transplanted adipocytes.

Application of fat grafting:

1. Breast Reconstruction
2. Scars
3. Burns
4. Radiodermatitis
5. Chronic Wounds
6. HIV- associated lipodystrophy
7. Facial and Hand Rejuvenation
8. Breast Augmentation/Asymmetry
9. Rhinoplasty
10. Gluteal Augmentation

The possible complications include bruising, swelling, haematoma formation, paraesthesia or donor-site pain, infection, hypertrophic scarring, contour irregularities, and damage to the underlying structures for example due to the intraperitoneal or intramuscular penetration of the cannula[154-161].

Complications of the recipient site include fat necrosis, oil cyst formation,

calcification (in breast lipofilling), cellulitis, infection, fat reabsorption, temporary dysaesthesia [155-163].

7. THE DERMAL REGENERATION TEMPLATE

Skin is the largest organ of the body with many essential functions. Since its direct contact with the external environment, which makes it extremely prone to damage and/or injury, the skin plays a crucial role as a barrier against exogenous substances, pathogens and mechanical stresses. Damages to this barrier lead to loss of water and protein, and bacterial invasion to the underlying tissue. Hence, a quick regeneration after an injury is necessary to avoid complications[164], a wide range of biomaterials has been used by the medical practitioners to manage the chronic wounds[165].

Many efforts have been made by the researchers to promote the regeneration of the skin and many studies demonstrate the usefulness of allografts or autografts. For the past few decades, different polymeric biomaterials were developed. These materials, which can act as smart "skin substitutes" by performing many of skin's functions, are made with varied combinations of synthetic and/or biologic substances. Depending on the product characteristics, skin substitutes could replace skin either temporarily or permanently. These substances are an alternative to the standard wound coverage in circumstances when standard therapies are not desirable[166]. Skin substitutes are used to help wound healing, alleviate pain and replace the function of the skin and have an important role in the treatment of deep dermal and full thickness injuries of various aetiologies[167]. A comprehensive list of skin substitutes, which are currently available, is given in Table 3.

Skin substitutes can be made of humans tissue (allografts), animals' one (xenografts), or using membranes developed from natural or synthetic polymers. Even if there is no ideal skin substitute available, tissue engineering and bioengineering are trying to create an ideal one that fulfils the best properties.

Substitute type	Commercial forms	Description	Uses
Autografts	Epicel®	Cultured epidermal autograft	Severe deep dermal, full thickness burns
	MySkin™	Cultured epidermal autograft	For burns, ulcers and other non-healing wounds
	Cultured skin substitutes	Cultured composite autograft	For large burns and other congenital skin disorders
	Bioseed®-S	Autologus keratinocyte fibrin glue suspension	Treatment of chronic leg ulcers
	CellSpray®	Cultured epithelilal autograft suspension	To treat superficial burns
	Stratagraft®	Cultured composite autograft	Burns and severe skin wounds
	Recell®	Autologus cell therapy device	To treat burns, scalds, traumatic wounds, scars
Allografts	Lyphoderm™	Lysate of cultured human keratinocyte	For chronic leg ulcers
	ICX-SKN	Cultured dermal allograft	To cover surgically excised partial thickness burns
	Alloderm®	Cadaver skin with acellular dermal matrix and intact basement membrane	For ENT/head and neck plastic reconstruction
Acellular allograft	OASIS®	Processed dermal xenograft	For partial and full thickness wounds and trauma wounds
Xenografts and biosynthetic grafts	Permacol™	Processed dermal xenograft	For temporary coverage of partial thickness burns
	Matriderm®	Bovine dermal collagen and elastin	For burns and reconstruction
	Biobrane®	Porcine dermal collagen bonded to semipermeable silicone membrane	To cover partial thickness burns and skin graft donor sites
	Integra®	Two layered skin substitute comprsisng bovine collagen and an outer silicone layer	For surgically excised deep and full thickness burns
	EZ Derm™	Porcine derived xenograft with collagen crosslinked to an aldehyde	For partial thickness wounds, donor sites, and sandwich autografts and full thickness wounds

Tab. 3 List of currently available skin substitutes

It is possible to recognize three types of skin substitutes: those consisting only epidermal equivalents, those involving dermal components from processed skin and those made both of dermal and epidermal components. Temporary skin substitutes provide immediate physiological conditions for the wound

closure, including protection from mechanical trauma, physical barrier to bacteria and creation of a moist wound environment[168]. Permanent skin substitutes are used to permanently achieve wound closure, replace the skin components and provide a higher quality skin replacement than the thin autologous skin graft. Biological skin substitutes act temporarily like natural skin with the advantages of being relatively abundant in supply. These are not very expensive and have a more intact and native extracellular matrix (ECM) structure, which may allow the construction of a more natural new dermis. They also show excellent reepithelialisation characteristics due to the presence of a basement membrane[169]. The most widely used biological substitute worldwide is cadaveric skin allograft, porcine skin xenograft, amnion and cultured epithelial autografts (CEA).

Xenograft : Porcine skin allograft is the widely used xenograft in modern practice of burn wound care. Prior to the grafting, pig skin has been specially treated and contains only the dermis layer. Xenografts are mainly used for the coverage of partial thickness burns. The disadvantages include its risks of rejection and infection[169].

Allografts: The cadaveric skin allograft is the most commonly used skin substitutes in burn wound management[169]. Depending on the methods of processing and storage, there are two main types of cadaveric skin allografts, cryopreserved allograft and glycerol-preserved allograft (GPA). The GPA is more popular and commonly used in clinical practice[170].

Amnion: a thin semi-transparent tissue found in the innermost layer of the foetal membrane. It has been used as biological dressings for burns since 1910. Because it is made from human placenta, amnion is one of the most effective substitutes to be used in healing or covering partial thickness burn wounds. Efficiency of amniotic membrane to protect wound bed as well as to reduce

bacterial load in contaminated wounds is comparable with that of human skin allografts. In converse, its poor mechanical stability makes it more difficult to handle[171].

Cultured epithelial autografts (CEA): are grown from patient's own skin. Keratinocytes can be grown in culture to produce thin epithelial sheet grafts[172]. The autologous keratinocytes are isolated, cultured and expanded into sheets over periods of 3–5 weeks. The use of suspension keratinocytes in fibrin glue has reduced the time for clinical use to 2 weeks[173]. These grafts can act as a permanent skin replacement for patients who have deep dermal or full thickness burns.

Synthetic skin substitutes: are made of synthesized materials that are made on demand with specific characteristics required in each case. They are constructed from non-biological molecules and polymers that are not present in human skin[174]. These constructs should be safe, stable, biodegradable and provide an adequate environment for the regeneration of tissue. Biodegradation should preferably take place after this period. An earlier product in this category is Biobrane™ (Smith & Nephew, Largo, Fla), which is still used throughout the burn community often as a temporary cover for burn and perhaps equally as often as skin donor site coverage. It is composed of two layers of silicone and a nylon mesh to which collagen is bonded. Dermagraft ® (Advanced BioHealing, Westport, Conn) is a synthetic product that can be used as temporary skin substitutes. Dermagraft® uses either polygalactic or polyglycolic acid meshes combined with neonatal fibroblast to enhance wound healing. Apligraft® consists of living cells and structural proteins; the lower dermal layer combines bovine type 1 collagen and human fribroblasts, which produce additional matrix proteins, the upper epidermal layer is formed by promoting human keratinocytes. Matriderm® is an acellular dermal substitute used in a one-step procedure in combination with a split-thickness skin graft.

Matriderm® is made of native collagen scaffold and elastin. OrCel® is a bilayered cellular matrix in which normal human allogenic skin cells are cultured in two separate layers into to a type I bovine collagen sponge. Hyalomatrix® bi-layered wound device which comprised entirely of HYAFF® and semipermeable silicone membrane and requires a graft after 14-21 days after application. Integra® is a bilayer membrane made of a porous matrix of fiber of cross-linked bovine tendon collagen and glycosaminoglycan (chondroitin-6-sulfate) [175].

Acellular skin substitutes recruit fibro-vascular tissues from the wound bed and may consist of either biopolymers, such as collagen and chondroitin-sulfate or elastin (Integra® Dermal Regeneration Template[176]; MatriDerm®[177]), decellularized human dermis (AlloDermTM)[178], derivatized hyaluronic acid (Hyalomatrix®)[179], or polyurethane (BioTemporizing Matrix, "BTM")[180-181]. Each of these materials protects open wounds, promotes ingrowth of fibrovascular tissue, and may suppress granulation tissue and scar. However, the biologic materials (i.e., acellular dermis, collagen, hyaluronic acid) are prone to microbial contamination in the absence of antimicrobial agents due to their properties as biological ligands for bacteria and degradation by enzymatic activities[182-183]. In comparison, synthetic polymers (i.e., polyurethane, poly-glycolic/poly-lactic acids) are often degraded by hydrolysis, have fewer microbial binding sites, and are less prone to microbial contamination. If used as dermal substitutes, the acellular materials may require 2 to 4 weeks to vascularize sufficiently. However, if used as a scaffold for cell transplantation, 2 weeks or longer for vascularization would negatively impact cell survival and reduce cellular engraftment and wound closure. Allogeneic human fibroblasts and/ or keratinocytes have been combined with degradable scaffolds (i.e., partially denatured collagen (ApligrafTM; StrataGraftTM)[184-185], poly-glycolic/poly-lactic acids (DermaGraftTM) which deliver growth factors

and extra- cellular matrix to wounds to promote autologous healing but do not persist more than a few days to weeks. Autologous keratinocytes have been applied as cultured cell sheets (EpiCelTM)[186], sprayed cell suspensions prepared during surgery (ReCellTM)[187], with culture- expanded fibroblasts as the dermal component[188], or in combination with a polymeric dermal scaffold populated with autologous culture-expanded fibroblasts[189-190]. Limitations of keratinocyte sheets have included poor durability and ulceration[191-192] and with sprayed keratinocyte suspensions a requirement for co-application with widely meshed skin autograft[193], which reduces the conservation of donor skin and increases scarring after wound closure. Preclinical investigations have reported more complex models that also include melanocytes[194-196], microvascular endothelial cells[197-199], mesenchymal stem cells[200-202], adipocyte stem cells[203], sensory nerve cells[204], hair follicle progenitor cells[205-206], or induced pluripotent stem cells (iPSCs)[207-209]. These kinds of models promote activation of biological signaling pathways, which may stimulate more rapid and complete healing, or drive expression of additional phenotypes to correct anatomic deficiencies.

As biologic complexity increases and phenotypes are restored, engineered tissues gain structures and functions that do not result from mechanisms of wound healing. These added properties may derive from embryonic or fetal mechanisms that regulate tissue morphogenesis, in addition to the mechanisms of wound healing. Together, the combination of developmental biology, wound healing, and biomedical engineering constitute the emerging field of regenerative medicine[210].

To ideally promote the function and regenerative capacity of seeded cells, scaffolds should mimic the natural extracellular matrix[4-7].

As scaffold material we decided to use Integra® dermal regeneration template

that is a bilayer membrane system for skin replacement. The dermal replacement layer is made of a porous matrix of fiber of cross-linked bovine tendon collagen and glycosaminoglycan (chondroitin-6-sulfate) that is manufactured with a controlled porosity and defined degradation rate[211]. The epidermal substitute layer is made of thin polysiloxane (silicone) layer to control moisture loss from the wound (Figure 2).

Integra® is frequently used as scaffold material for experimental tissue engineering studies, because it easily enables cellular seeding due to its porous structure with pore sizes ranging between 20 and 125 µm[212].

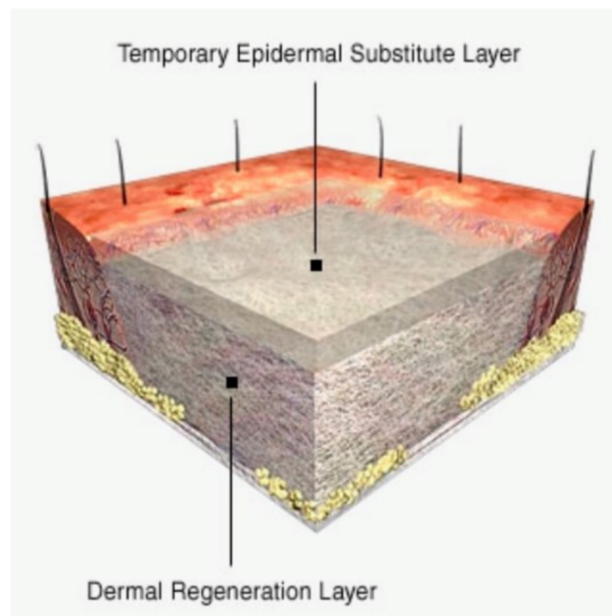

Fig. 2. The two layer of Integra®

Matrix Properties

Porus matrix fibers of cross-linked Collagen/Glycosaminoglycan (GAG) are represented in Figure 3 and display the following features:

- Pore volume fraction of 98%
- Molecular weight between cross-links of 12.000 +/- 5.000 dalton
- Average pore diameter of 30 to 120 um
- Collagen/GAG ratio 92/8 (type 1 bovine tendon collagen/chondroitin 6-sulphate)
- Specific resistance to degradation by collagenase
- In vivo degradation rate, T=30 days

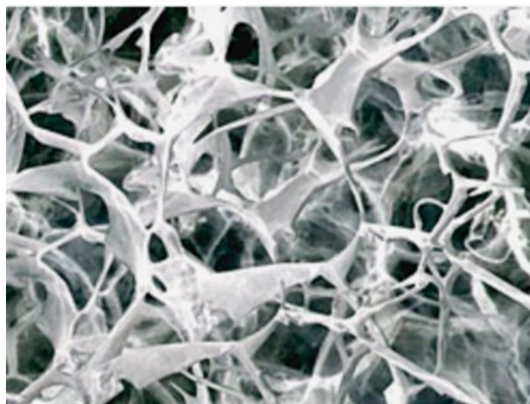

Fig. 3. Microscopic structure of Integra®

Benefits of Integra® are:

- The permanent regeneration of dermal skin
- Thinner skin graft resulting in less scarring in donor site
- Faster healing donor site
- Immediate physiological wound closure without risking the loss of donor material
- Patients can begin rehabilitation with Integra® Template in place

- No reports of rejection
- No immediate need to create donor site wounds
- Donor site heal faster and can be harvested more frequently
- Post excisional use of Integra® Template does not require the use of temporary coverings such as allograft, xenograft, or synthetic dressings

Indications for use:

- Burns; post-excisional treatment of life threatening full-thickness or deep partial-thickness thermal injuries where sufficient autograft is not available at the time of excision or not desirable due to the physiological condition of the patient
- Reconstruction; for the repair of scar contractures o in post-oncologic surgery when other therapies have failed, or when donor sites for repair are not sufficient or desirable due to physiological condition of the patient

When is placed on a wound Integra® provides the needed framework for the blood vessel and dermal skin cells to re-grow into a new skin layer. The silicone outer layer temporarily closes the wound to ward off infection and control fluid and heat loss. As skin cells migrate into the matrix the collagen is slowly absorbed into the body and replaced with proteins that are naturally produced by the skin. In approximately 14 to 21 days, new dermal skin is produced and silicone layer can be removed. A thin skin graft is applied to the wound area to complete the procedure.

8. AIM OF THE STUDY

The ultimate aim of this study is to strengthen the usefulness of a regenerative intervention in the treatment of pathological conditions like chronic ulcers refractory to conventional treatments, using an alternative model of wound dressing.

In our study, we would like to create a new model of wound dressing, combining the aforementioned fat regenerative capacities with the already known ability to accelerate healing of dermal substitutes. To do this, we have first characterized ADSCs obtained from lipoaspirates according to Colemsn's technique. Several parameters were considered: phenotype, growth kinetics, and differentiation capacity. These studies brought the notion that ADSCs can readily differentiate into adipocytes but not towards osteoblastic and chonodrobalstic lineages, unless appropriate signals are provided through the conditioned medium of differentiated cells. We thus turned to a more patient-oriented study by using a cross-linked bovine tendon collagen and glycosaminoglycan (chondroitin-6-sulfate) scaffold sprinkled with ADSCs and PRP taken from the same patient. Once this method, for which I present preliminary results, will be further studied and optimized, we expect to see an important wound reduction or a completely wound healing, together with pain reduction and an improvement of the quality of life of these patient. An immediate reduction of pain symptoms, which allows reduction in hospitalization, the absence of adverse reactions and the achievement within a reasonable time of tissue regeneration are the key points of this approach. The expected results will lead to an improvement in the management of chronic ulcer in the lower limbs and a reduction of the treatment time for these patients, generally characterized by numerous medications for periods of months or years.

9. MATERIALS AND METHODS

9.1 Fat harvesting

Fat harvesting was performed in the Department of Plastic Surgery of the University of Foggia. Lipoaspirates were obtained only from patients operated under local anesthesia for different morbidity conditions and to be treated with lipofilling with autologous fat. Each participant gave written informed consent to the study, which was performed in accordance with the principles of Declaration of Helsinki. Ten subjects participated in this study (mean age 40 years, range 18-62 years). To obtain fat, the standard Coleman technique was used by injecting the abdominal subcutaneous layer with 500 to 1000 mL lactated Ringer's solution with the addition of 0.5 mL epinephrine and 25 mL 1% lidocaine. After 10 minutes, waiting for local vasoconstriction induced by epinephrine, two small incisions in the abdominal region (4 mm each) allowed adipose tissue suction using a Ø3mm cannula, blunt-edged, 2- hole cannula connected to a 10-mL syringe, fat is suctioned manually by withdrawing the plunger. The so isolated adipose tissue was then sent to the Laboratory of Experimental and Regenerative Medicine, Department of Medical and Surgical Science of the University of Foggia for processing procedures.

9.2 Isolation and culture of human adipose-derived mesenchymal stromal cells

The first objective of the project was isolation, long term culture, characterization and study of differentiation capability of ADSCs. To isolate the SVF, fat tissue was added with equal volumes of phosphate-buffered saline (PBS), centrifuged at 300xg for 10 min at 18-22°C and the resulting middle

phase was digested at 37°C for 45 min with 0.2% collagenase solution. Enzyme activity was neutralized with Dulbecco's modified Eagle's medium (DMEM), containing 10% FBS, and centrifuged at 300 xg for 10 min to obtain a SVF pellet. The final pellet was resuspended in DMEM: F12 media supplemented with 40% (v/v) heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1% Antibiotic Antimycotic (ABAM) and 10 ng/ml epidermal growth factor (EGF) (all reagents were purchased from Sigma-Aldrich), plated on T75 flasks for cell culture (Corning) and incubated at 37°C and 5% CO_2. The following day the medium was aspirated to remove the floating cells, the plate was washed with PBS to remove the remaining debris and the attached cells were maintained in DMEM low glucose supplemented with 10% FBS, 1% L-glutamine, 1% ABAM and 10 ng/ml EGF at 37°C and 5% CO_2. Every time cells reached 80% of confluence, cells were detached with Accutase Solution (Invitrogen), washed, counted with a Bürker chamber and replated in a new plastic flask at a density of 1×10^5 cells/cm^2 in order to calculate their growth curve. Doubling time was calculated inserting times and cell counts on the website www.doubling-time.com/compute.php

9.3. Characterization of ADSCs by flow cytometry

Flow cytometry analyses were performed on cultured ADSCs at passages 0, I, II, III, IV and V. Briefly, cells were detached from culture flask using Accutase solution and, after washing, were incubated with PBS 2% FBS for 10 min at 4°C in order to block non-specific sites on cell membrane. Cells were then stained at 4°C for 30 min with the following monoclonal antibodies (moabs) against CD14, CD29, CD34, CD45, CD73, CD105 (all from Invitrogen), and CD44 (Becton Dickinson Biosciences, BD, Franklin Lakes, NJ, USA). All moabs were conjugated with fluorescein isothiocyanate (FITC), except the

moab against CD73 that required an additive incubation with secondary antibody (FITC goat anti- mouse; Sigma-Aldrich) for 30 min at 4°C.

9.4. Culture of human dental pulp stem cells

Dental pulp stem cells (DPSCs) were isolated as previously described[213]. DPSCs were seeded into T75 flasks and cultured in DMEM low glucose containing 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin. Cultures were maintained at 37°C in 5% CO_2, the supplemented medium was changed every 3 days and the cells were passaged when 80% confluent using Accutase Solution (Invitrogen). In order to induce osteogenic differentiation in ADSCs, we collected CM from DPSCs for 2 weeks in osteogenic medium: DMEM low glucose supplemented with 2% FBS, 1% penicillin/streptomycin, 50 μg/ml ascobate-2-phosphate, 10^{-8} M dexamethasone, 10^{-2} M β-glycerophosphate.

9.5. Isolation and culture of human chondrocytes

Human auricular cartilage was acquired from patients hospitalized at the Division of Plastic and Reconstructive Surgery, Hospital of Foggia, and who gave written informed consent. Cartilage fragments were removed from apparently healthy auricular regions by surgical biopsy. The cartilage was transported to the laboratory immediately after harvesting in sterile tubes containing PBS (Ca^{2+} and Mg^{2+} free) with penicillin/streptomycin and ABAM. A sterile scalpel was used to cut the cartilage into small pieces, which were then transferred to a Petri dish and maintained overnight at 37°C, 5% CO_2 in DMEM containing 10% FBS, 100 μg/ml L-ascorbic acid, 1% L-glutamine, 1% penicillin/streptomycin and 1% ABAM (growth medium) to ensure sterility of

the specimens prior to enzyme digestion. The day after, the cartilage pieces were washed in PBS (Ca^{2+} and Mg^{2+} free) containing penicillin/streptomycin and ABAM and then incubated in the growth medium containing trypsin (0.25% w/v) at 37°C, 5% CO_2 for 30-60 min (the time of enzymatic digestion was adapted to the size of tissue fragments). Supernatants were discarded and the cartilage was further digested in growth medium containing 1.0 mg/ml collagenase II for 4 h at 37°C, 5% CO_2. The digested tissue was passed through a nitex nylon filter (mesh width 70 μm) and then centrifuged at 1500 xg for 10 min. The cell pellet was washed three times in PBS (Ca^{2+} and Mg^{2+} free) containing penicillin/streptomycin and ABAM and re-suspended in the growth medium. Cultures were maintained at 37°C in 5% CO_2, and the supplemented medium was changed every 3 days. When chondrocytes reached 70 to 90% confluency, the cells were passaged using Accutase Solution and replated in a new plastic flask. In order to induce chondrogenic differentiation in ADSCs, we collected CM from chondrocytes cultured for 3 weeks in chondrogenic medium: DMEM low glucose supplemented with 1% FBS, 1% penicillin/streptomycin, 6.25 μg/ml insulin, 10 ng/ml TGF-β3, 50 nM ascobate-2- phosphate.

9.6. Differentiation of hADSCs towards different lineages

9.6.1. Adipogenic differentiation.

To induce adipogenic differentiation, ADSCs at passage II were harvested and plated on cell culture plate (Corning) at a density of $5x10^4$ cells. Cells were then cultured for two weeks in adipogenic differentiation media: DMEM low glucose supplemented with 2% FBS, 1% antibiotic solution, 0.5 mM isobutyl-methylxanthine, 1 μM dexamethasone, 10 μM insulin, 200 μM indomethacin.

The presence of adipose elements in induced cultures was determined by Oil-Red-O (Sigma) staining as follows: cells were washed in PBS, then fixed in 10% formalin for 5 min at room temperature, washed in isopropanol 60% and air- dried. Cells were then incubated with Oil-Red-O staining solution for 10 min, then washed several times in PBS and observed with an inverted microscope Eclipse TS100 (Nikon, Tokyo, Japan) equipped with a DS-FI1 CCD camera (Nikon).

9.6.2. Osteogenic differentiation.

In order to induce osteogenesis, ADSCs at passage II plated on cell culture plate at a density of $5x10^4$ were treated for 2-4 weeks with osteogenic medium: DMEM low glucose supplemented with 2% FBS, 1% antibiotic solution, 50 μg/ml ascorbate-2-phosphate, 10^{-8} M dexamethasone, 10^{-2} M β-glycerophosphate. The presence of calcium deposits in induced cultures was determined by Alizarin Red (Sigma) staining as follows: cells were fixed in 10% formalin for 10 min, then washed in PBS (pH 4.2) and incubated for 10 min at 37°C with Alizarin Red 2% in water. The cells were finally washed several times to remove the excess of staining and analyzed as described above. In another set of experiments, ADSCs were incubated with CM obtained from DPSCs. CM was filtered and fresh FBS (10%), penicillin/streptomycin (1%) was added.

9.6.3. Chrondrogenic differentiation

In order to evaluate the chondrogenetic potential, ADCSs at passage II plated on cell culture plate at a density of $5x10^4$ were maintained in chondrogenic

induction medium: DMEM low glucose supplemented with 1% FBS, 1% antibiotic solution, 6.25 μg/mL insulin, 10 ng/mL TGF-β3, 50 nM ascobate-2-phosphate. After two-four weeks the presence of proteoglycans in induced cultures was determined by Safranin O (Sigma) staining as follow: cells were fixed in 10% formalin for 20 min at room temperature, then washed in PBS and acetic acid and incubated for 5 min at room temperature with 2% Safranin O in water. The cells were finally washed several times to remove the excess of staining and analyzed as described above. In another set of experiments, ADSCs were incubated with above CM obtained from differentiated chondrocytes. CM was filtered and fresh FBS (10%), penicillin/ streptomycin (1%) was added.

9.7. Culture of bone marrow-derived MSCs and their differentiation

Bone marrow (BM) aspirates obtained from two healthy volunteer donors were diluted 1:1 in PBS and subjected to Histopaque 1077 (Sigma, St Louis, MO, USA) density gradient centrifugation at 1700 rpm for 30 min without brake. The obtained buffy coat cell fraction was centrifuged at 1700 rpm for 10 min and resuspended in mesenchymal stem cells medium (Stem Cell Technologies) supplemented with 10% FBS (Gibco Limited, Uxbridge, UK), 100 UI/ml penicillin and 100 μg/ ml streptomycin, at cell density of $4x10^5$ cells/cm^2. After 24 h the non-adherent fraction was discarded and the BM- derived MSCs obtained from the adherent fraction were expanded at confluence until the second passage[217]. For the differentiation toward the mesodermic lineages, the cells at passage 2 were plated at a density of $1x10^3/cm^2$ and maintained in differentiating induction medium for two weeks, as described above for the ADSCs.

9.8. Seeding of Integra® with ADSCs

For all the experiments with Integra®, ADSCs were isolated from lipoaspirates obtained from eight different subjects, who gave written informed consent, and used at passage II.

For cell seeding, commercially available Integra® (Integra Lifesciences, Rozzano, Italia) was used. After unpacking, Integra® (10×12.5 cm) was removed from the packing and left to drip dry without applying any mechanical force, such as squeezing or shaking. Integra® was then washed twice in 500 ml sterile 0.9% NaCl solution. Prior to seeding, the Integra® matrix was cut into pieces of 1× 1 cm.

In a first set of experiments, pieces of Integra® were sprinkled with ADSCs (1 × 105) resuspended in complete medium. As negative controls, only medium was added to Integra®. Seeded pieces were evaluated at different time points (1 hour, 24 hours, 48 hours, 72 hours, 6 days, 10 days and 15 days).

In another set of experiments, the same procedure described above was performed with 1 × 106 ADSCs, that were seeded onto Integra® pieces in complete medium. Seeded pieces were evaluated at different time points (1 h, 6 h, 24 h, 48 h, 6 days and 10 days).

In the third set of experiments, ADSCs were isolated by the emulsification of lipaspirated fat. Emulsification of the fat was achieved by shifting the fat between two 10-cc syringes connected to each other by a female-to-female Luer-Lock connector[218]. The emulsified fat was processed as before by enzymatic digestion.

ADSCs obtained from emsulfied fat (1×10^6) were seeded onto Integra® in the

presence of medium deprived of FBS and supplemented with PRP obtained from the same patient. To do this, before liposuction, from each patient we collected blood in 6 tubes containing buffered solution of sodium citrate 0.109 mol/l (3.2%), theophylline, adenosine and dipyridamole. Tubes were then centrifuged at 3000 rpm for 10 minutes in order to separate cells from the plasma portion. This plasma was centrifuged again at 3000 rpm for 10 minutes, and the resulting PRP was used in the following experiments. Preliminary experiments were carried out by seeding ADSCs on culture flasks in the presence of either 8%, or 4% or 2% of PRP in culture medium. Gelification of medium and arrest in ADSC growth was obtained with both 8% and 4% PRP, but not with with 2% PRP, which was then used to culture ADSCs in Integra®. Sheets were evaluated after different time points (1 hour, 24 hours, 48 hours, 7 days, 10 days and 15 days).

9.9. Histology and immunohistology

Both seeded and unseeded Integra® pieces were fixed in 4% buffered paraformaldehyde. Sections (4 µm) were cut from formalin-fixed, paraffinembedded blocks and they were mounted on glass slides coated with poly-Llysine, dried at 37°C overnight, deparaffinised in xylene, washed in ethanol, and finally washed in PBS (pH 7.4). Preparations were stained with hematoxylin and eosin.

Immunohistochemical (IHC) method on 4-µm serial sections was performed by using Ventana Benchmark® XT autostainer and standard linked streptavidin-biotin horseradish peroxidase technique (LSAB-HRP), according to the best protocol for the antibody used in Laboratory of Pathological Antatomy, University of Foggia. Primary mouse monoclonal antibodies anti CD31 (clone JC70), anti vimentin (clone V9), anti actin-smooth muscle (1A4),

anti S-100 (clone 4C4.9) were used as pre-diluted in PBS (Ventana, Medical Systems Inc, Tucson, AZ). All antibodies were from Roche Tissue Diagnostics (Hoffmann-La Roche, Oro Valley, USA). Negative control slides without primary antibody were included. Immunoreactions products were assesed following incubation at room temperature for 5-10 min with diaminobenzidine (DAB). Slides were counterstained with Gill's type II haematoxylin, and then examined under light microscopy by two independent observers. Immunostained slides were acquired by digital camera and analysed by CellSens V1.9® Olympus image analysis software at original magnification of 10x or 20x.

10. RESULTS

10.1. Isolation and characterization of ADSCs

After plastic adhesion, a fibroblastic morphology very similar to that described for mesenchymal cells isolated from bone marrow was displayed (Fig. 4A). ADSCs adhered and proliferated on tissue culture plastic and could be kept until passages V. All the ADSCs cultured at all passages until the V passage presented the same morphology. We did not find any difference in ADSCs morphology among the different subjects. We assayed the growth kinetic in three subjects. The average doubling time calculated over 42 days of culture and until the passage IV was 11.73 days (Figure 4B).

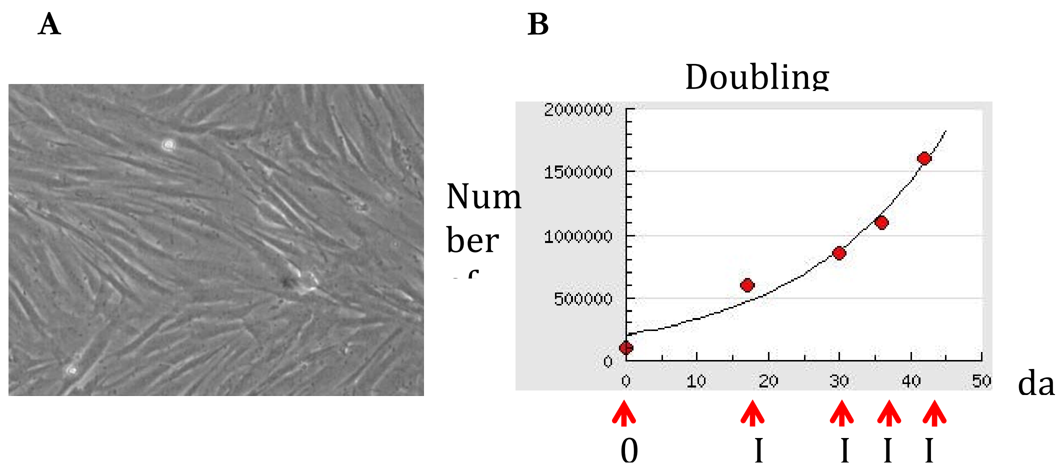

Fig. 4. *ADSC morphology and growth.* ***A)*** *Cell morphology at passage one, original magnification 10X (Bar = 30 µm).* ***B)*** *Growth kinetics of ADSCs in a representative culture obtained from one patient. Three patients gave similar results. 0 = adherent cells after isolation; I, II, III, and IV indicate cells at the first, second, third and fourth passage.*

ADSCs cultures obtained from seven patients were analyzed for expression of cell-surface markers from the isolation to the fifth passage (Figure 5). ADSCs expressed mesenchymal lineage markers, such as CD29 (92-98%), CD105 (89-

93%), CD73 (24-72%), but very low levels of hematopoietic lineage markers CD14 (0.5-3.0%) and CD45 (1-4%). CD34, a marker of stem/progenitor hematopoietic and endothelial lineages, was expressed by 10% of freshly isolated cells and its expression lowered to 1% of positive cells in the subsequent passages. No statistically significant differences in the expression of phenotypic markers among different passages were found.

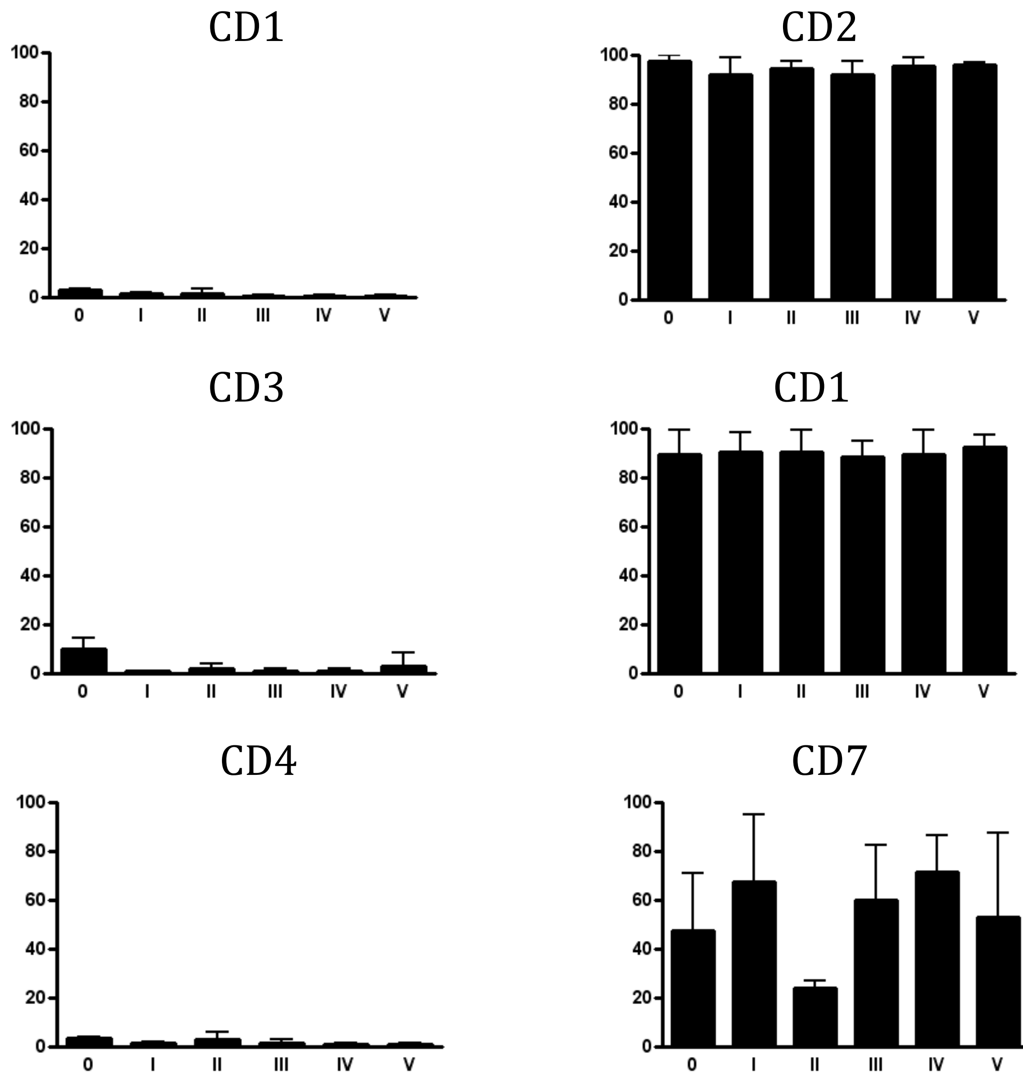

Fig. 5 Phenotypic markers of ADSCs at different passages. Data are expressed as mean ± SD.

10.2. Differentiation of ADSCs towards the adipocyte lineage

ADSCs incubated in adipogenic media were reproducibly induced toward the adipogenic lineage as early as 1 week post-induction. Freshly isolated ADSCs showed a clear differentiation pattern in the adipocytes as indicated by a significant fraction of the cells containing multiple lipid-filled droplets that stained with oil red O (Fig. 6). No difference in the adipocytic differentiation was noted in four different subjects.

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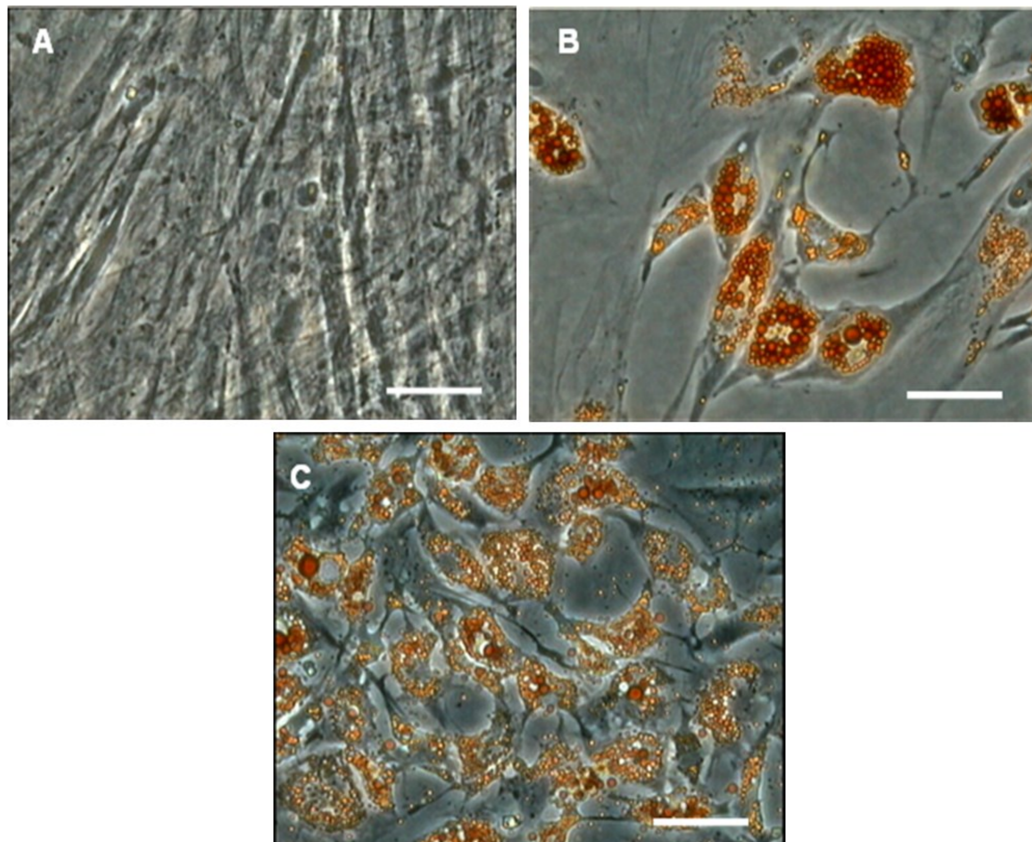

Fig. 6. *In vitro differentiation capability of ADSCs towards the adipogenic lineage. Representative images of ADSCs grown in differentiating medium (**B** and **C**). **A**) represents negative controls (i.e. uninduced cells grown in basal medium). Original magnification: A) 10X (Bar = 30 µm); B) 20X (Bar = 15 µm); C) 10X (Bar = 30 µm). Four patients were analysed. Images obtained in one representative experiment are shown.*

10.3. Differentiation of ADSCs toward the osteoblast lineage

ADSCs cultured in osteogenic medium changed morphology but did not produce calcified extracellular matrix after 2 weeks of induction (Fig. 7A and B). This result was obtained even when ADSCs obtained from other two individuals were cultured in differentiation medium for either 3 or 4 weeks (data not shown). In order to see whether paracrine factors from osteoblasts could be involved in the acquirement of osteogenetic behaviour, ADSCs were cultured in conditioned media from DPSCs. In the first place, DPSCs were induced to differentiate to osteoblasts with a defined osteogenic medium for 2 weeks. After this time, they were able to produce calcified extracellular matrix, as shown by alizarin red S staining (Fig. 7C). ADSCs were treated with the CM from these differentiating DPSCs every 2 days for 2 weeks. Only with this treatment, ADSCs were shown to secrete a calcified matrix (Fig. 7D).

10.4. Differentiation of ADSCs toward the chondrocytic lineage

After incubation of ADSCs in chondrogenic defined medium, the cells showed morphological changes but not proteoglycan deposition. This result was obtained even when ADSCs obtained from other two individuals were cultured in differentiation medium for either 3 or 4 weeks (data not shown). Chondrocytes were isolated from auricle cartilage and cultured in a growth medium. After 2 weeks in culture, they dedifferentiated, i.e. they acquired a fibroblastic morphology as previously shown[219]. However, these cells exposed to chondrogenic medium for 3 weeks displayed positivity to proteoglycan deposition and formed typical micromasses. On the other hand, ADSCs incubated in CM obtained from differentiated chondrocytes show proteoglycan secretion after 2 weeks of induction. No micromasses were found in the CM-

induced ADSC cultures.

10.5. Differentiation of BM-MSCs

As a positive control for differentiation, BM- MSCs were subjected to the same conditions as those for ADSCs promoting adipogenesis, osteogenesis, and chondrogenesis. After two weeks, BM-MSCs exhibited multiple intra-cellular lipid filled droplets in the majority of cells. Differentiation of BM-MSCs into osteocytes was demonstrated after two weeks by change in morphology and by the presence of calcium deposits. BM-MSCs differentiated in chondrocytes after two weeks of induction as demonstrated by change in morphology and the occurrence of staining for proteoglycans. No micromasses were observed.

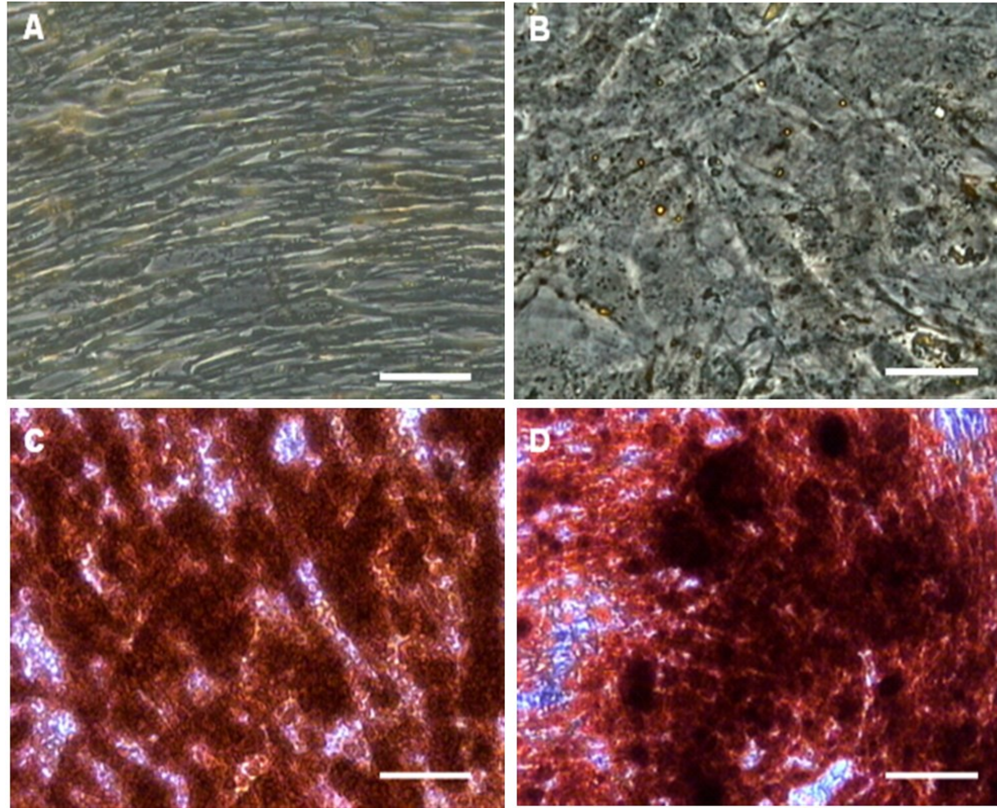

Fig. 7. *In vitro differentiation capability of ADSCs towards the osteogenic lineage. Cells maintained in basal media did not show any change in their morphology and no calcium deposit (**A**, magnification 10X. Bar = 30 µm); freshly isolated ADSCs maintained in differentiating medium showed a clear change in their morphology but no calcium deposit (**B**, magnification 20X. Bar =15 µm) in contrast to DPMSCs (used as positive control) that showed a clear osteogenic differentiation pattern (**C**, magnification 10X. Bar = 30 µm). ADSCs incubated in conditioned medium obtained from differentiated DPMSCs show calcium deposits after 2 weeks of induction (**D**, magnification 10X. Bar = 30 µm). Four patients were analysed. Images obtained in one representative experiment are shown.*

10.6. Survival of ADSCs within Integra® in the presence of complete medium

This part of the study was aim to understand the behavior and survival of the ADSCs within a scaffold used in skin regeneration. For this reason we have seeded ADSCs, taken from lipoaspirate, on a cross-linked bovine tendon collagen and glycosaminoglycan (chondroitin-6-sulfate) scaffold, i.e. Integra®.

Sheets of scaffold (1 cm x 1cm) sprinkled with passage II-ADSCs (1 X 10^5) were then examined under a microscope to check cell adhesion. Because of the

porosity of the scaffold (from 20 to 120 µm), it was difficult to see adherent cells on the surface of the sheet, even in samples collected within minutes after seeding. Our hypothesis is that ADSCs immediately enter the scaffold colonizing its interior. Supporting this thesis, we did not observe cells in suspension.

To demonstrate the presence of cells within the matrix, we fixed (in PFA 4% in PBS), at different time points, samples of seeded sheets and sheets without cells as negative control. Samples were then cut into thin slices and stained with hematoxylin/eosin (HE) solution for histological assessment under microscope.

Histological analysis showed the presence of cells inside the matrix at 1 hour (Figure 8A), that were vimentin-positive (Figure 8B). Cells displayed a rounded shape.

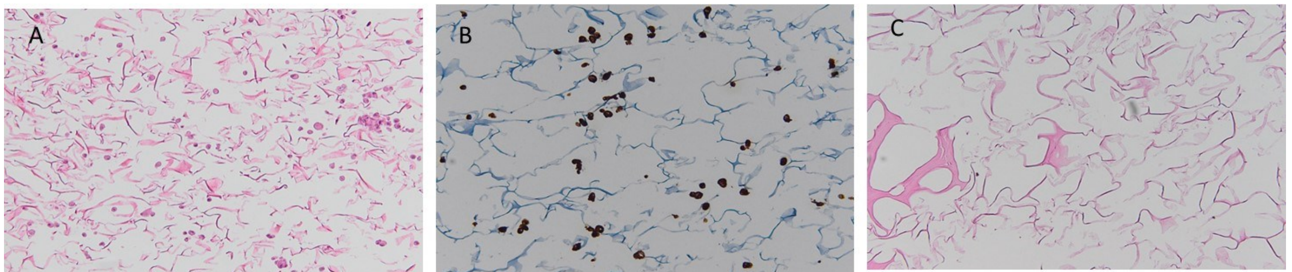

***Fig. 8**. Integra scaffolds with 1x10^5 ADSCs at 1 h. A: HE, 10x; B: vimentin, 20x; C: HE, unloaded scaffold.*

Scaffolds evaluated at 24 hours (Figure 9A), 48 hours (Figure 9C), and 72 hours sample (data not shown) displayed less cells than at previous time points. At these time points, cells began to show an elongated shape.

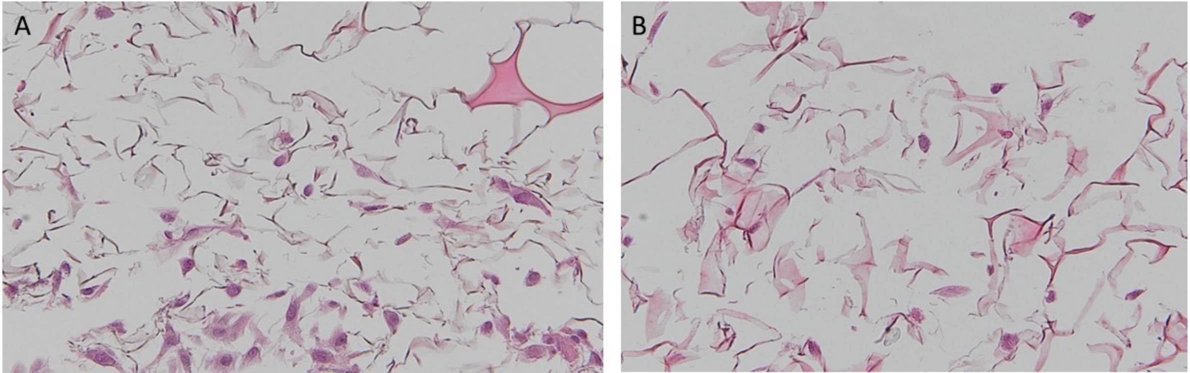

Fig. 9. *Integra scaffolds with $1x10^5$ ADSCs at 24 h (A) and 48 h (B). A: HE, 20x; B: HE, 20x.*

In 6, 10, and 15 days samples, the number of cells within the scaffold progressively decreased until their total absence.

To assess whether the progressive decrease in the number of cells in the scaffold was due to unsuitable conditions or an inadequate number of implanted cells, we repeated the microscopic analysis of sheets of scaffold seeded with 1 X 10^6 ADSCs and collected at 1 hour (Fig. 10 A,B), 6 hours (Fig. 11 A,B), 24 hours (Fig. 12 A,B), 48 hours (Fig. 13), 6 days (Fig. 14 A) and 10 days (Fig. 14 B,C). While HE staining gives an overall overview of the cell presence, vimentin, that is a specific marker of mesenchymal cells, testifies that the cells maintain their original characteristics.
Firstly, sprinkled sheets were colonized in a more organized way by a larger number of cells. Even cell survival has improved: we have found a larger number than the previous experiment even in samples at 6 and 10 days.

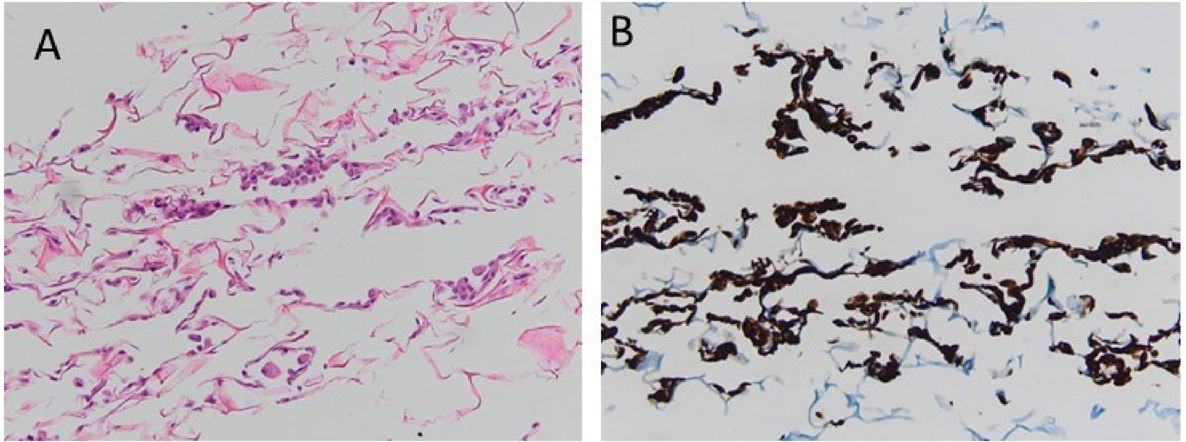

Fig. 10. *Integra scaffolds with 1x10^6 ADSCs at 1 h: A: HE, 10x; B, vimentin, 10x.*

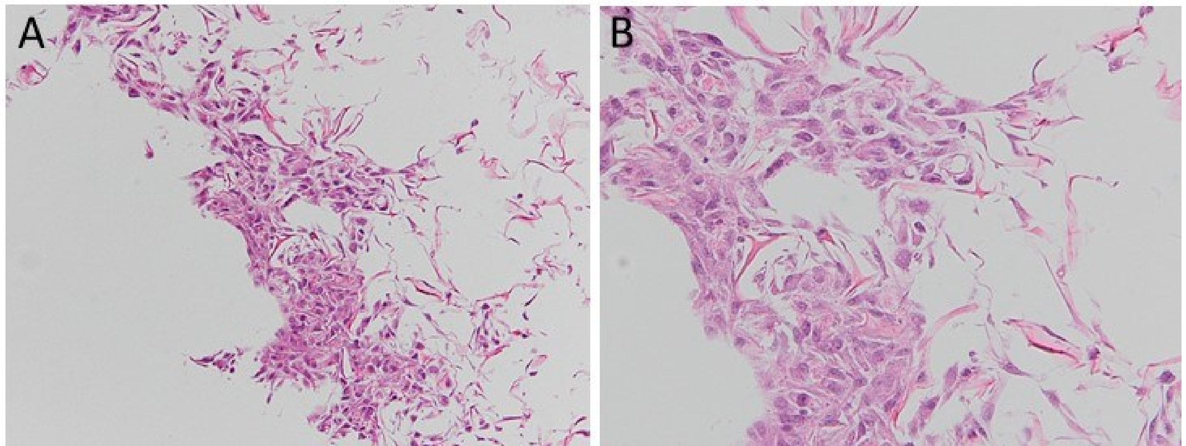

Fig. 11. *Integra scaffolds with 1x10^6 ADSCs at 6 h: A: HE, 10x; B: HE, 20x.*

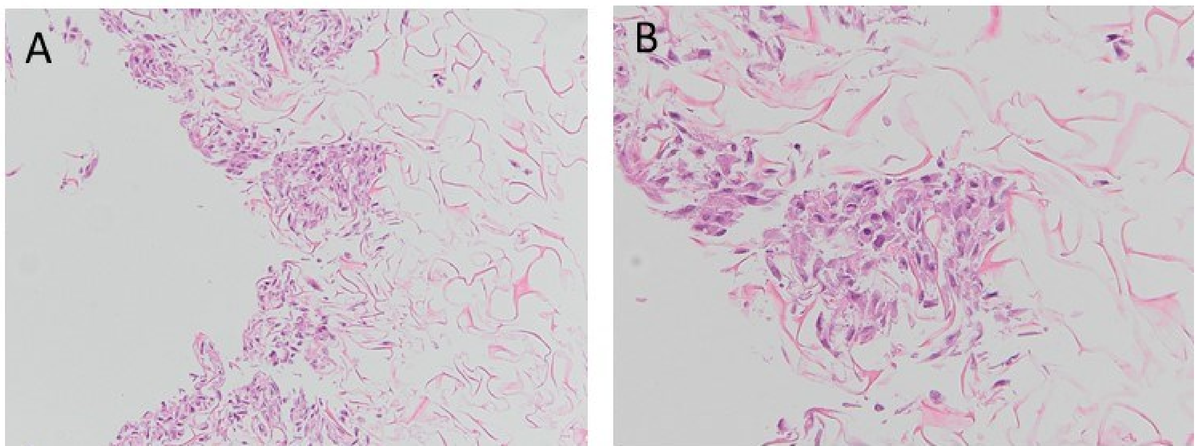

Fig. 12. *Integra scaffolds with 1x10^6 ADSCs at 24 h. A: HE, 10x; B: HE, 20x.*

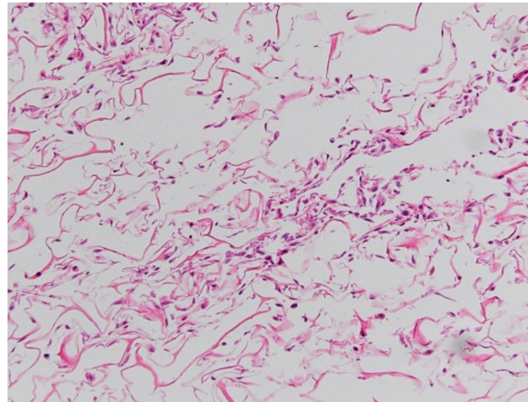

Fig. 13. *Integra scaffold with 1x10*^{6} *ADSCs at 48 h. HE, 10x.*

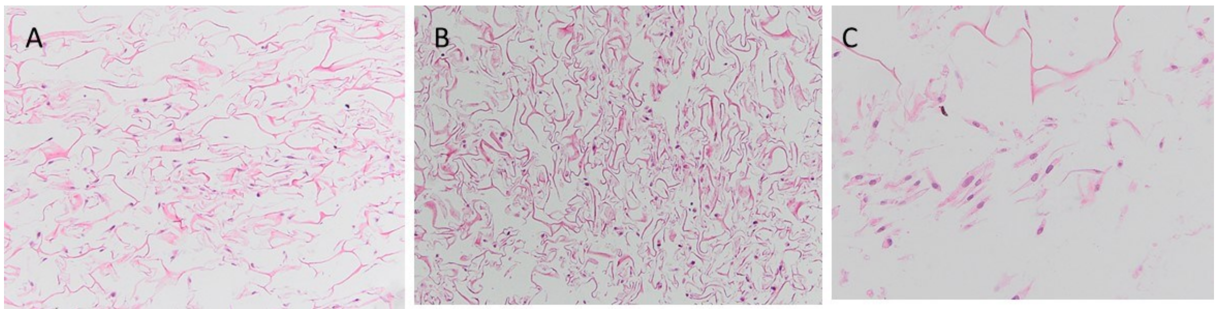

Fig. 14. *Integra scaffold with 1x10*^{6} *ADSCs at 6 days (A) and 10 days (B,C). A: HE, 10x; B: HE, 10x; C: HE, 20x.*

10.7. Survival of ADSCs within Integra® in the presence of PRP

The purpose of this part of the study was to create the conditions for reproduction of the method to be used eventually for in vivo implantation. For this reason, we repeated the experiment with Integra® by replacing the 10% of heat-inactivated fetal bovine serum (FBS) with the platelet reach plasma (PRP) of the same patient. PRP (8%) was added to the growth medium for ADSCs

instead of the FBS. After this first attempt, we observed a spread gelification of the PRP among the flask which inhibit cellular growth.
For this reason we decided to repeat the experiment reducing PRP percentage to 4%, and finally to 2% in order to find the right concentration allowing cellular survival. Indeed, while gelification was observed still with 4% PRP, we saw the presence of cells within the matrix with a PRP concentration of 2% and their easily growth, expressing mesenchymal lineage markers and keeping their original phenotype (not shown).

Thus, to mimic an in vivo approach, the adipose tissue was emulsified through few passages in syringe. This sample was subjected to the procedure for isolating ADSCs from the SVF as done before. Finally, ADSCs (1 x 10^6) were seeded onto each sheet of scaffold together with 2% PRP. Scaffolds were fixed at different times (1 hour, 24 hours, 48 hours, 7 days, 10 days and 15 days) and evaluated for histological assessment for histological analysis and immunohistochemical analysis.
As before, ADSCs colonized the scaffold as assessed by HE staining at 1 hour and 24 h (Figure 15A,B). and were found as aggregates. Scaffolds pictured at 48 hours showed that cell aggregates became bigger (Fig. 15 C).

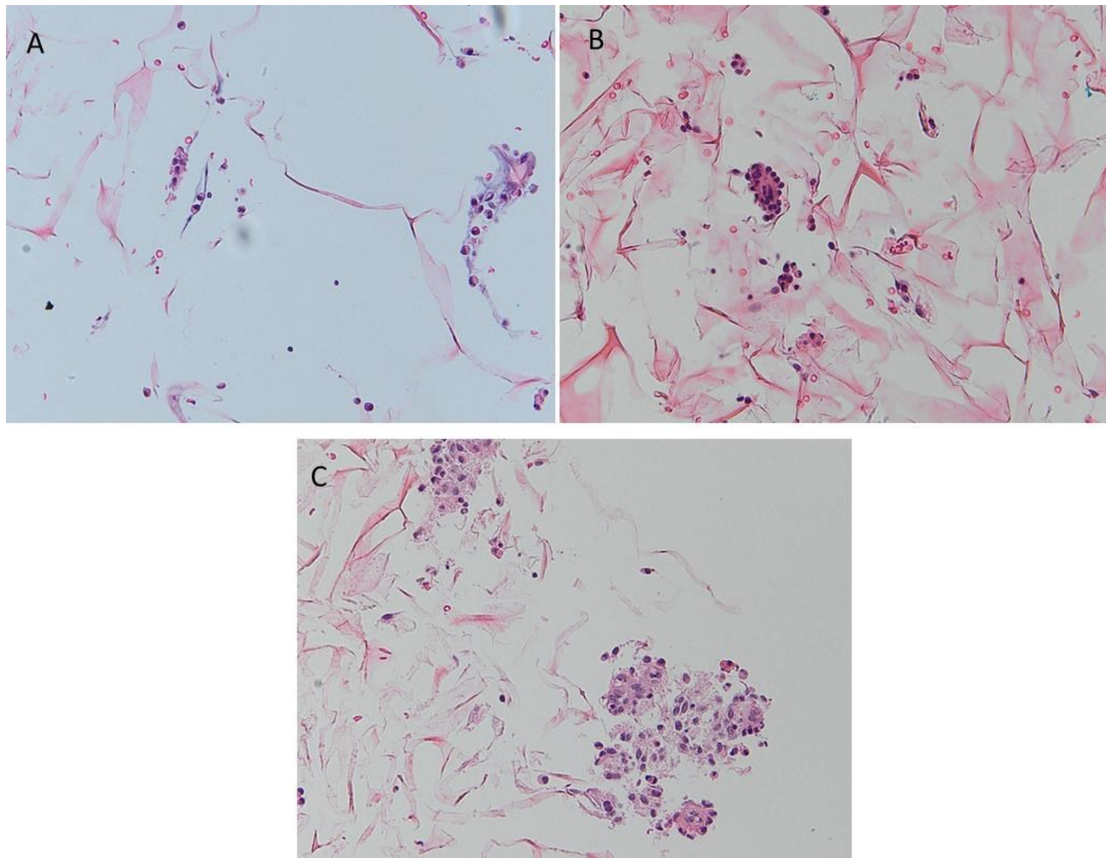

Fig. 15. *Integra scaffolds Integra scaffold with 1x10*^{6} *ADSCs and PRP at 1 h (A), 24 h (B), and 48 h (C). All panels: 20x. HE staining.*

At 7 and 10 days, aggregates became even bigger (Fig. 16 A-D). Interestingly, lacunar structures were noted (white arrows) where cells formed a continuous layer resembling endothelium (red arrow in D).

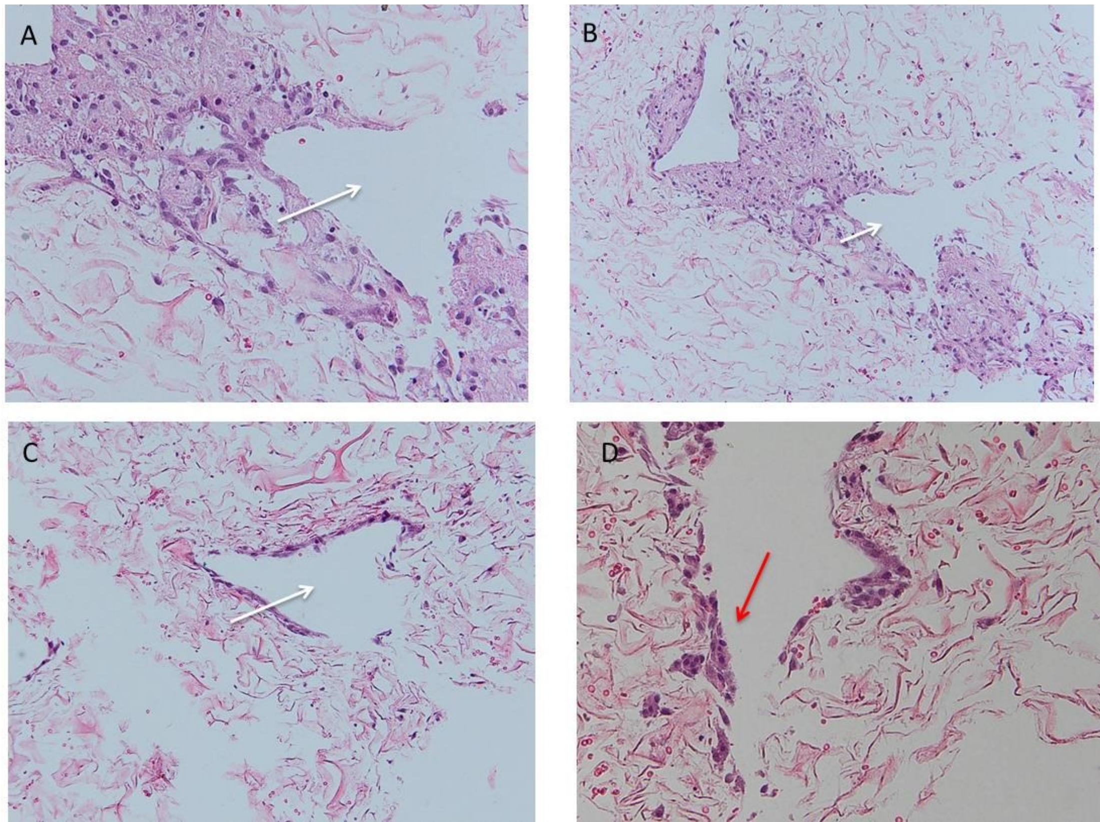

***Fig. 16**. Integra scaffolds Integra scaffold with 1x10^6 ADSCs and PRP at 7 days (A, B) and 10 days (C, D). B is a magnification of A. A, C, D : 20x; B: 10x. HE staining.*

In order to understand if these structures were vessel-like, a staining of CD31, a marker of endothelial cells was performed. Fig. 17 A-B clearly shows that ADSCs assumed a positive staining for this marker after 10 days of culture. Much alike, alpha actin, a marker of myofibroblasts, resulted positive in endothelial-like cells surrounding the lacunar structure as well as in cells scattered around these structures (Fig. 17 C, white arrows). S100beta, that is expressed by adipocytes and glial cells, was negative (fig. 18 D).

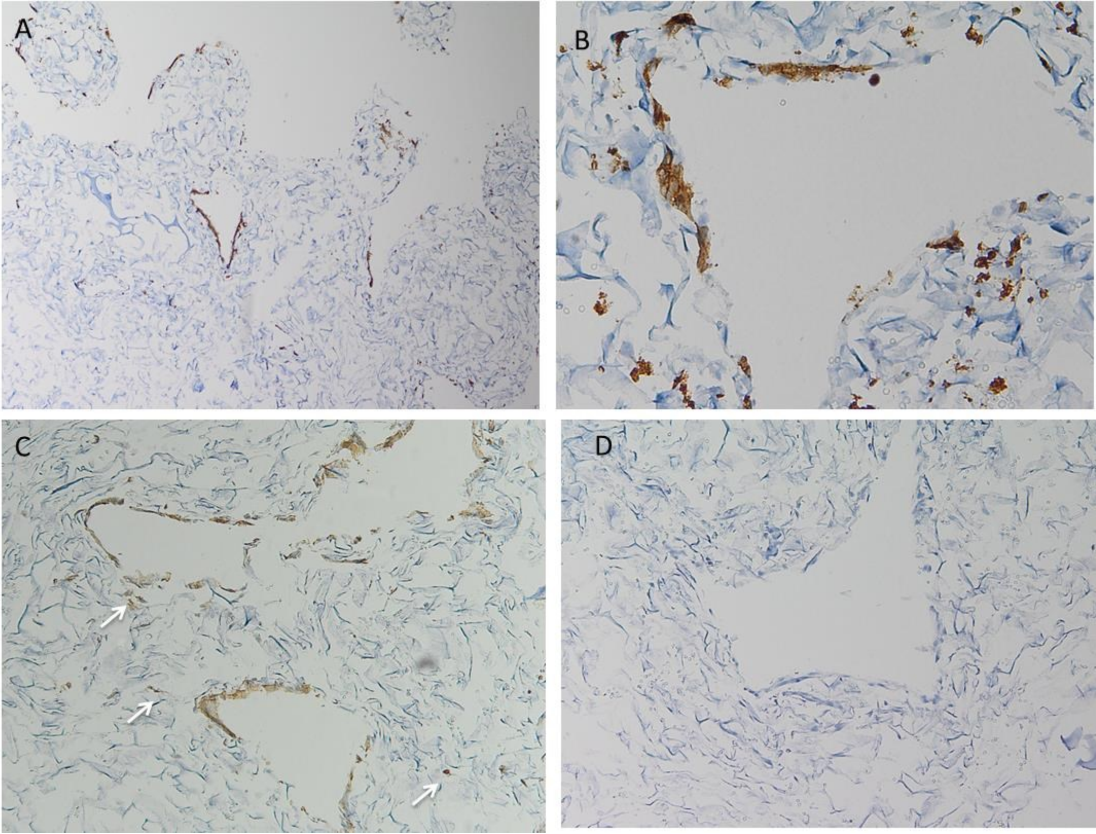

Fig. 18. *Integra scaffolds Integra scaffold with* $1x10^6$ *ADSCs and PRP at 10 days. A, B: CD31; C: alpha actin; D: S100beta. A: 4x; B-D: 10x.*

11. Discussion

ADSCs have a great potential for use in tissue repair and regeneration in the field of plastic and reconstructive surgery. ADSCs are isolated by enzymatic digestion, filtration, and centrifugation and are typically expanded in monolayer on standard tissue culture plastic with a basal medium containing 10% fetal bovine serum (FBS)[51]. FBS seems to be essential for cell culture, but it is not safe when the cultured cells are to be used in regenerative medicine. Animal-derived serum may contain xenoproteins that may cause rejection, and may contain transmissible infectious agents[220].

In order to eliminate the use of animal products in human ADSC cultures, platelet-rich plasma (PRP) has been recently proposed as a substitute of FBS, since PRP contains a wide range of growth factors, proteins, and enzymes supporting attachment, growth, and proliferation of cells. The advantages of using platelet concentrates in the clinical settings are many. When used as medium supplement, PRP has been shown to promote the growth of ADSCs and maintain their differentiation potential[221-222]. Furthermore, the antimicrobial and anti-inflammatory properties of PRP might represent a valuable adjunct to the enhancement of tissue regeneration. Wound healing and neovascularization in a porcine model were enhanced only when ADSCs were topically administered along with PRP[223], and these effects were largely attributed to the large amount of growth factors found in PRP.

The regenerative potential of platelet concentrates and ADSCs on hard and soft tissues has been explored considerably during the last decade. Preclinical studies in animal models have confirmed the synergistic effects of ADSCs and PRP in the healing process of wounds and in osteoarthritis[224-226].

A study by Cervelli and colleagues[227] reported the combination of PRP, autologous ADSCs and hyaluronic acid as a dressing in order to regenerate tissue and achieve epithelialization of wounds localized in the lower limbs, with a significant healing-time reduction in most of the 30 treated patients. Furthermore, the authors reported a fewer number of medications and subsequent improvement of the quality of life. Pak's group injected ADSCs and PRP in 91 patients with various orthopedic pathological conditions[228]. The follow-up conducted for up to 30 months showed that the treatment was safe, as tumors did not appear at the injection sites. In a study conducted by Bui et al.[229], 21 patients with osteoarthritis from cartilage injury at grade II to III have been enrolled. The goal of this group was to demonstrate the clinical safety and efficacy of autologous transplantation of ADSCs in combination with PRP. They obtained completely reduction of pain levels in 100% of patients without complications, such as tumor formation or microorganism infections.

More recently, the effect of a single injection of autologous ADSCs (as SVF) in combination with PRP administered intra-articularly in ten patients with symptomathic primary osteoarthritis of the knee has been studied[230]. The results demonstrated that, after 2 years from the treatment, ADSCs and PRP could reduce the pain levels in these patients and that the procedure was very safe because it did not caused any complications. Overall, these studies highlight the great potential of autologous ADSCs combined with PRP as a therapeutic agent in regenerative medicine especially in orthopedic conditions.

In our study we demonstrated that the procedure involving freshly isolated ADSCs following emulsification and the addition of PRP, leads to supposed differentiation of ADCs towards vessel-like structures, as identified by CD31 and alpha-actin. Future experiments will focus on longer time points to ascertain that these cells can form truly vascular organization and/or determine other fates, such as the adipogenic or neural ones. Other studies are needed to

comprehend which signals, coming either from the glycosaminoglycan of scaffold or PRP, are involved in this kind of differentiation. The potential capability of ADSCs to differentiate in vessel like –structure will improve the regenerative capacity of dermal substitute, decreasing healing time in patient with chronic lesion of the lower limbs.

12. Conclusions

The discovery of adult multipotent stem cells within the stroma of adipose tissue opened the door to a wide range of therapeutic avenues in regenerative medicine and tissue engineering. Growing autologous tissue from the patient's own stem cells in order to repair damaged tissue and restore tissue function is becoming a reality. A clear advantage of using ADSCs in regenerative medicine and tissue engineering resides in the possibility to harvest a great amount of subcutaneous adipose tissue. Currently, the use of autologous additives such as PRP is a promising approach in enhancing the applications of ADSCs. Preliminary results support the clinical application of PRP for ADSCs-based therapy in creating an innovative wound dressing. However, further studies are needed to better assess the proliferation of ADSCs within the dermal substitute, and the application of this original wound dressing in vivo and its effects on the healing of chronic lesions.

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