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USE OF ALLOGENIC MESENCHYMAL STROMAL CELLS FOR REPARATION OF TENDON AND LIGAMENT INJURIES IN EQUINE SPECIES

(SSD AGR/18)

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ABSTRACT

Mesenchymal stromal cells (MSCs) have different roles in cell therapy, particularly, in veterinary field, they are used for treatment of tendon and ligaments disorders, especially in horses. The major part of papers describes autologous implantation that presents a limit not negligible, in fact, isolation and amplification of cells require about 20 days, and for this reason, it is impossible to treat promptly the patient. Allogenic use has been suggested to overcome this problem. Cells can be prepared, characterized, controlled, and banked in advance, permitting an immediately available source of MSCs ready to be used at the proper time after tissue damage. However, the major concerns about use of allogenic cells refer to their safety. Indeed, risks can be associated with both origin and handling of cells, transformation and tumorigenic characteristics and the immune response in the patient. During the research, adipose-derived mesenchymal stromal cells (ASCs) were isolated, amplified and characterized for their typical features; finally, they were investigated for tumorigenic and transforming potentialities. Immunomodulation activity was evaluated monitoring their capacity to inhibit lymphocytes proliferation *in vitro*. All the considered parameters provided encouraging results supporting allogenic cell therapy purposes. At this regard a clinical study was performed. Horses with tendon/ligament damages were considered and animals treated with allogenic cells associated to platelet lysate, were compared to the control group, treated with pin fearing. All animals were subjected to a rehabilitation period and their ability to resume training was evaluated. In this study, implanted allogenic ASCs caused no adverse reactions or inflammation process, neither immediately after injection nor during observation period. Finally, all patients resumed their training activity already 6 months after cell therapy approach. On the contrary, no improvement was noticed in the animals of control group (pin firing treatment); in fact 66.6% of patients were retired from their sport activity.

The outcomes obtained during the study, prove the potential safety of allogenic ASCs for cell therapy purposes. Furthermore, ultrasound images evaluation and clinical observation of treated animals suggest the capacity of ASCs associated to platelet lysate to promote tissue healing.

Concluding, this research could give important information about a new strategy to overcome limitations associated to classical therapies for treatment of tendon and ligaments disorders in equine species and, in addition, related to use of autologous cells in regenerative medicine.

1. INTRODUCTION

Stem cells have evoked considerable excitement in the animal-owning public because of the promise that stem cell technology could deliver tissue regeneration for injuries, for which natural repair mechanisms do not deliver functional recovery and for which current therapeutic strategies have minimal effectiveness.

This research focuses on the use of stem cells within veterinary medicine, whose practitioners have used allogenic mesenchymal stromal cells (MSCs), recovered from adipose tissue, in clinical cases to treat tendon and ligament injuries in equine species.

1.1. Stem cells

Stem cells are undifferentiated cells characterized by self-renewal capacity, high potential for proliferation and differentiation capacity (Aranda et al. 2009).

They are classified by their development potential (figure 1) as:

- totipotent: capable of giving rise to all cell types, an example is represented by zygot;
- pluripotent: able to give rise to all embryonic cell types, these cells form the internal mass of blastocysts;
- multipotent: capable of giving rise to a great number of cellular lineages. This feature is typical of adult stem cells, such as hematopoietic stem cells (HSCs) and MSCs;
- oligopotent, capable of giving rise to a more limited number of cellular lineages than multipotential cells;
- unipotent, capable of giving rise to only one specific cellular lineage (Prindull 2005).

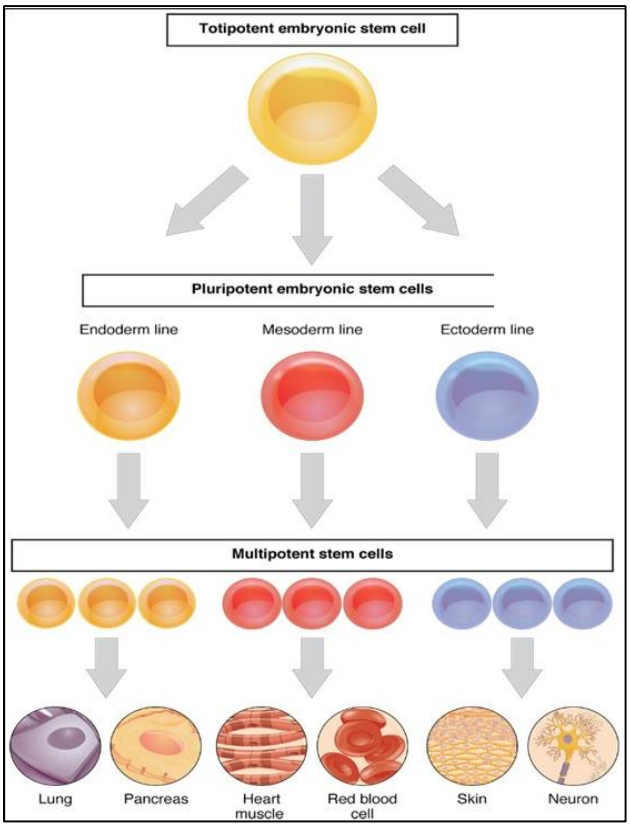


Figure 1. Classification of stem cells (from www.stemcellclinic.com).

1.2. Multipotent stem cells

In recent years, cell therapy has evolved quickly gaining great interest in both human and veterinary medicine. This approach regards the use of adult multipotent cells, which are capable of self-renewal, carry none of the ethical issues associated with embryonic stem cells because they are obtained from fully developed adult tissues. In fact, while application of embryonic stem cells is associated with immune rejection and teratoma formation, alternatively proposed MSCs have several advantages for the implementation in regenerative medicine (Bhartiya 2013; Ferro et al. 2012). Ethical and easy isolation of MSCs from adult tissues, their involvement in tissue regeneration due to differentiation capacity and immunosuppressive properties, are among the most important ones (Da Silva et al. 2006; Bhartiya 2013). In particular, some adult stem cells are considered to be more valuable than others, due to their accessibility, abundance, self-renewal properties, plasticity and ability to proliferate and differentiate. Two kind of multipotent stem cells are identified: hematopoietic stem cells and mesenchymal stromal cells.

HSCs are found in bone marrow in high concentration and in blood in low numbers in a mobilised cytokine-induced state. They give rise to platelets, red, and white blood cells and can form any cellular components of the blood (figure 2).

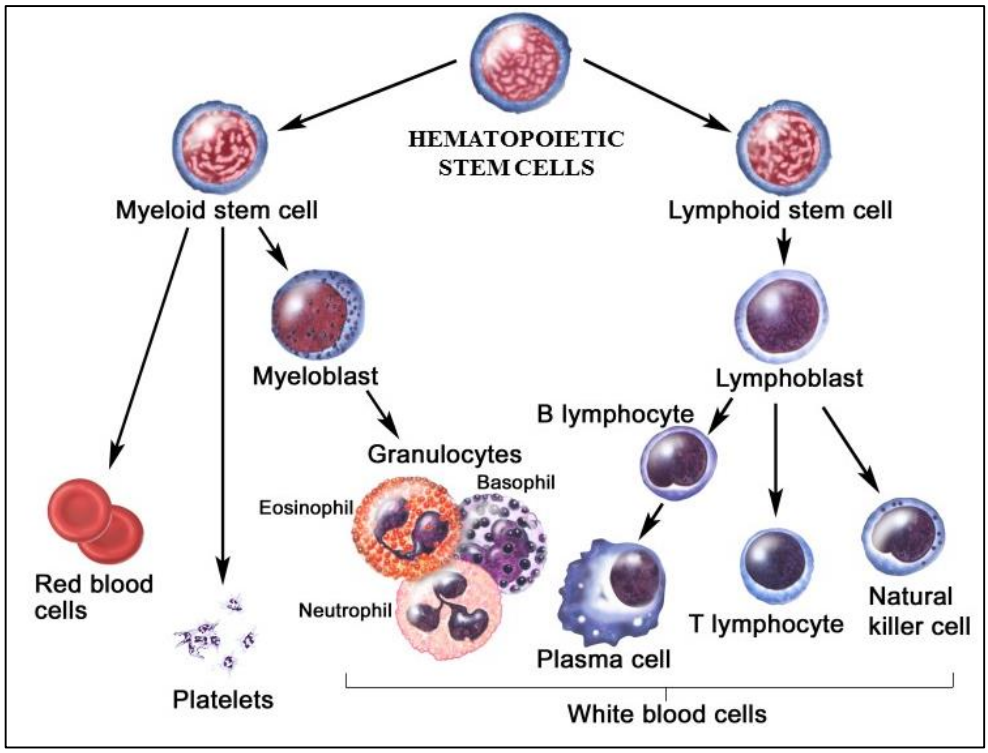


Figure 2. Differentiation potentialities of HSCs (from http://www.ncbi.nlm.nih.gov/).

MSCs can differentiate into many cell types (figure 3) including the chondrogenic, adipogenic, osteogenic, and myogenic lineages (Zuk et al. 2001). They are immuno-compatible and have an immuno-regulatory effect, both of which are advantageous when making therapeutic use of them (Augello et al. 2007).

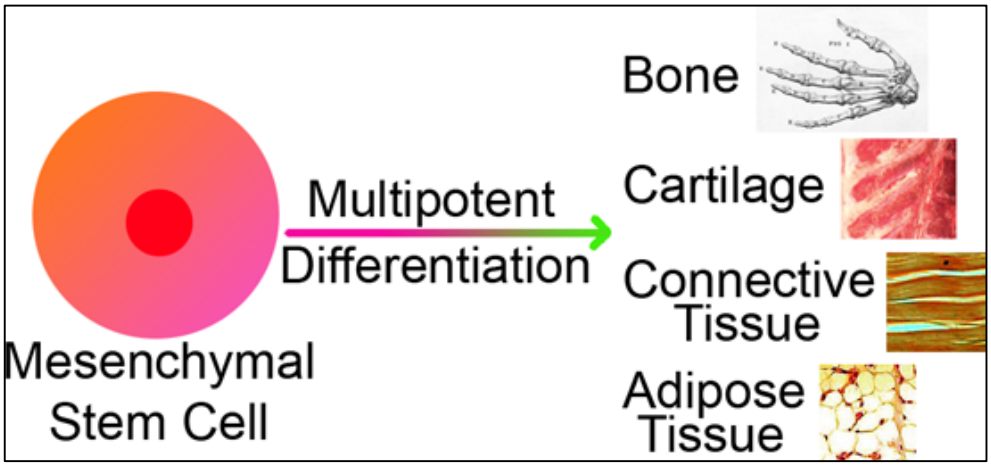


Figure 3. Differentiation potentialities of MSCs (from www.allthingsstemcell.com).

The main feature of MSCs is the ability to differentiate also *in vitro* along multiple pathways that include bone, cartilage, cardiac and skeletal muscle, tendon, connective and adipose tissue (Pittenger et al. 1999). For this reason, MSC-based cell therapies have been investigated for several years in human medicine and, more recently, the same approach has been considered in equine veterinary medicine, as a novel potential therapy for horse musculoskeletal diseases (Smith et al. 2003; Richardson et al. 2007).

1.3. Mesenchymal stromal cells

MSCs were first discovered in 1968 by Friedenstein and colleagues (Friedenstein et al. 1966), as adherent fibroblast-like cells in the bone marrow (BM), capable of differentiating into bone. These cells derive from the mesoderm during embryonic development and they have been described in several species and from different tissues, including bone marrow, peripheral blood, adult fat (Gronthos et al. 2001), umbilical cord blood (In't Anker et al. 2004), deciduous teeth (Potdar and Jethmalani 2015) and skeletal muscle (Jankowski et al. 2002).

They are identified for their growth characteristics, the subsequent differentiation, the expression of surface antigens, telomere length, and transcription factors expressed by genes. In particular, they are characterized by two fundamental features, that differentiate them from other types of cells, in fact they are able to divide and regenerate for long periods; furthermore, they are undifferentiated and can give rise to specialized cell types, when induced to differentiate, as a result of physiological or experimental conditions.

They appear morphologically *in vitro* as elongated cells, fibroblastic-like that replicate giving rise to groups of cells, which gradually reach confluence.

These cells express genes typical of mesenchymal cells and are negative for hematopoietic markers and endothelial adhesion molecules. MSCs were also characterized using molecular markers for "stemness" namely: Nanog, Oct4, Sox-2. Furthermore, they have a normal chromosomal content and the length of the chromosomes is not reduced in the early steps of differentiation (Barry and Murphy 2004). Finally, they are capable of multipotent differentiation, giving rise to adipocytes, osteocytes, and chondrocytes (Dominici et al. 2006).

The ability of mesenchymal cells to differentiate into adipocytes is witnessed by the fact that the bone marrow is partially replaced by adipose tissue with the passage of years.

1.4. Adipose tissue mesenchymal stromal cells

The isolation of stem cells from adult subject is usually performed from the BM. BM aspirate is collected from the sternum under sedation and local anesthesia. The site for BM injection is approximately, where the centre of a girth would be located on the sternum (figure 4). Ultrasonography is used to locate the sternebrae to avoid inadvertent penetration of the intersternebral space, which could lead to negative aspiration, or introduction of the needle caudal to the sternum, which could lead to puncture of the heart.

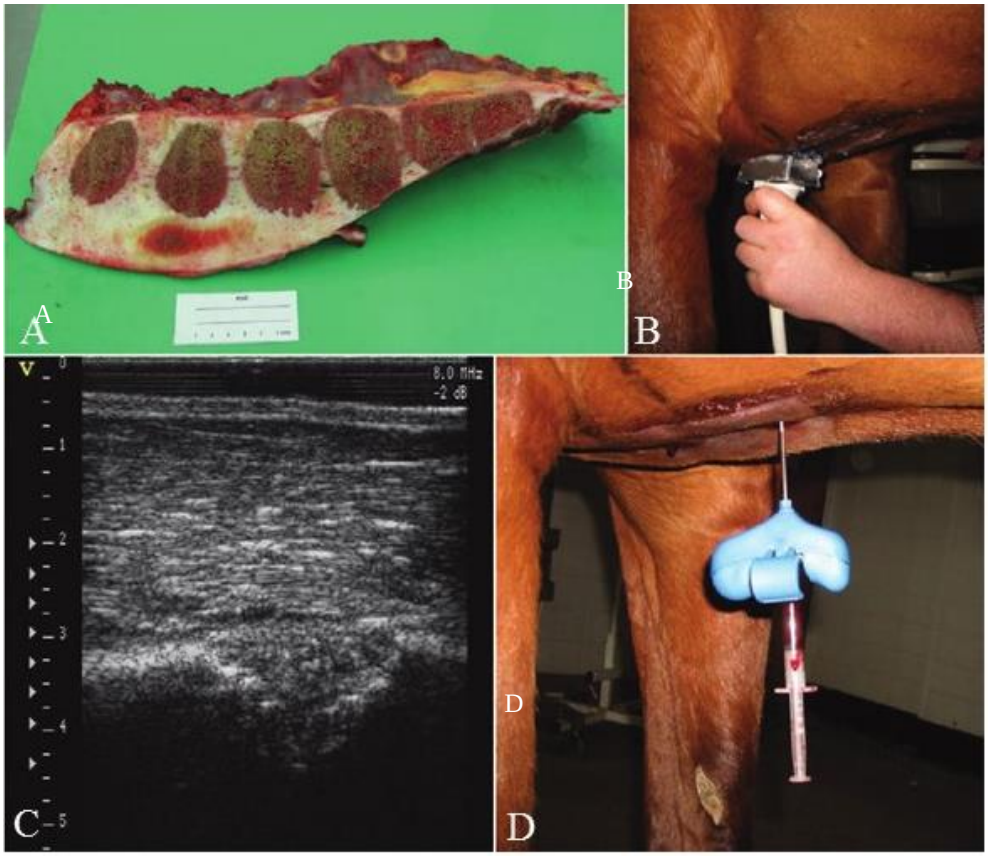


Figure 4. A: Equine sternebrae and intersternebral space section. B: Ultrasound guided location of sternebrae. C: Ultrasound evidence of intersternebral space. D: Bone marrow aspiration using a Jamshidi needle. (Avella and Smith 2009).

This kind of harvesting technique, presents several disadvantages: the primary disadvantage is the small number of stem cells that are contained in raw BM aspirates. In human beings, the number of stem cells in raw BM is reportedly 0.001% to 0.01% of the mononuclear cell population (Young et al. 1998). Furthermore, this technique is invasive and, in order to obviate this problem it is considered appropriate to isolate cells from biological material of different nature. Peripheral blood would represent an ideal alternative source, although it contains a small number of stem cell elements in natural conditions. In fact, the number of stem cells is lower than that found in the BM; however, the opportunity to make use of this donor site is motivated by the significant reduction of invasive procedures.

Recently adipose tissue has been identified as an alternative source; in fact, it is particularly rich in stem cells (2% of the cellular component). This tissue contains different cell types besides adipocytes, including adipose-derived mesenchymal stromal cells (ASCs). ASCs were first reported by Frohlich in 1972, as adherent, proliferating adipocyte precursors (Frohlich et al. 1972). Different names have been used to describe the adherent cell population that can be expanded from lipoaspirates, e.g. lipoblast, pericytes, preadipocytes, processed lipoaspirates cells, and many others. To clarify nomenclature, the International Fat Applied Technology Society. (IFATS) proposed to

name ASCs the isolated, plastic-adherent, multipotent cell population obtained from adipose tissue through different methods (Daher et al. 2008). Several works demonstrated ASCs capability of differentiating into cells of mesodermal origin, such as adipocyte, osteocyte, chondrocyte, and myocyte lineages (Zuk 2001).

Since these cells are readily accessible in large quantity in the body, they have received increasing attention for their possible application for the treatment of musculoskeletal diseases. In 2007 Vidal and colleagues (Vidal et al. 2007) have reported data on cell-doubling characteristics and differentiation ability of equine ASCs in comparison to bone marrow-derived mesenchymal stromal cells (BMSCs), showing that both cell types can be expanded and are able to differentiate into mesenchymal derivatives *in vitro*. The initial methods to isolate cells from adipose tissue were pioneered by Rodbell and colleagues in the 1960s. They minced rat fat pads, washed extensively to remove contaminating hematopoietic cells, incubated the tissue fragments with collagenase and centrifuged the digest, thereby separating the floating population of mature adipocytes from the pelleted stromal vascular fraction (SVF). This cell portion consisted of a heterogeneous cell population, including circulating blood cells, fibroblasts, pericytes and endothelial cells as well as "pre-adipocytes" or adipocyte progenitors (Rodbell 1966a; Rodbell 1966b; Rodbell and Jones 1966). The final isolation step selected for the plastic adherent population within the SVF cells, which enriched for the "pre-adipocytes". Nowadays, the collection of adipose tissue in equine species is performed in the region above the dorsal gluteal muscle (tail base), as described by Carvalho et al. in 2009 (figure 5).

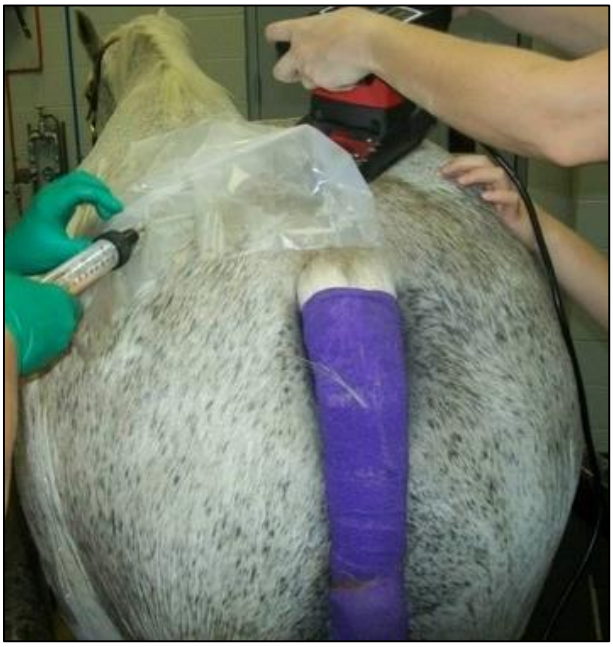


Figure 5. Collection of adipose tissue from the tail base in equine species.
(http://www.stilwellanimalhospital.com/images/stem-cell-collection-equine.jpg)

1.4.1. Immunophenotype

Multiple independent groups have examined the surface immunophenotype of ASCs, isolated from human and other species (Aust et al. 2004; Katz et al. 2005; Nakagami et al. 2005; Safford and Rice 2005; McIntosh et al. 2006; Mitchell et al. 2006; Yoshimura et al. 2006), considering expression of cluster of differentiation (CD). The CD system is commonly used as cell markers in immunophenotyping, allowing cells to be defined based on what molecules are present on their surface. While using one CD molecule to define populations is uncommon, combining markers has allowed for cell types with very specific definitions within the immune system.

The expression profile of ASCs changes as a function of time in passage and plastic adherence (McIntosh et al. 2006; Mitchell et al. 2006). After two or more successive passages in culture, the ASCs express characteristic adhesion and receptor molecules, surface enzymes, extracellular matrix and cytoskeletal proteins, and proteins associated with the stromal cell phenotype. Despite any differences in the isolation and culture procedures, the immunophenotype is relatively consistent between laboratories. Indeed, the surface immunophenotype of ASCs resembles that of BMSCs (Pittenger et al. 1999) and skeletal muscle-derived cells (Young et al. 1999). Direct comparisons between human ASC and BMSC immunophenotypes are 90% identical (Zuk et al. 2002).

Nevertheless, differences in surface protein expression have been noted between ASCs and BMSCs. For example, the glycoprotein CD34 is present on human ASCs early in passage but has not been found on BMSCs (Pittenger et al. 1999; McIntosh et al. 2006).

BMSCs express CD106 that is not or slightly expressed by ASCs; by contrast, BMSCs lack the expression of CD49d, which is expressed by ASCs (Strem et al. 2005). CD106 is the receptor of CD49d and both molecules are involved in haematopoietic stem and progenitor cell homing and mobilization from the BM (Lindroos et al. 2011).

Particular markers are normally expressed by ASCs and BMSCs, such as CD13, CD29, CD44, CD73, CD90, CD105 and MHC I (Zuk 2001; Dominici et al. 2006; Jones et al. 2006; McIntosh et al. 2006; Bühring et al. 2007; Gang et al. 2007; Zannettino et al. 2008; Bühring et al. 2009).

Other markers are only expressed by ASCs and not by BMSCs, such as CD34 and CD54 (Gronthos et al. 2001; McIntosh et al. 2006; Mitchell et al. 2006). By contrast, markers of the angiogenic and haematopoietic lineages, such as CD14, CD31, CD45, CD133 and MHC II are not or poorly expressed by ASCs.

The expression of STRO-1 by ASCs, as well as by BMSCs, is particularly controversial and strongly modulated during *ex vivo* expansion (Gronthos et al. 2001). The Stro-1 antigen, a classic BMSC-associated surface antigen (Simmons and Torok-Storb 1991; Zuk et al. 2001; Vogel et al. 2003), was observed to be expressed by a low fraction of cultured BMSCs, while ASCs do not.

Identification of the ASC surface immunophenotype has provided a mechanism to enrich or purify the stem cell population directly from the heterogeneous SVF cells (Hutley et al. 2001; Miranville et al. 2004; Sengenes et al. 2005). Investigators have used immunomagnetic beads or flow cytometry to both positively and negatively select for a subpopulation of cells within the SVF. For example, endothelial progenitors can be removed by negatively selecting for cells expressing CD31 or platelet endothelial cell adhesion molecule-1 (Hutley et al. 2001; Miranville et al. 2004; Nakagami et al. 2005; Sengenes et al. 2005; Nakagami et al. 2006). Likewise, positive selection has been performed using CD34 and other antigens. One group working with human adipose tissue has demonstrated that the CD34 positive SVF cell population contained the ASCs (Miranville et al. 2004; Sengenes et al. 2005).

1.4.2. Immunomodulative properties

In recent years, the capacity of human adult BMSCs to treat severe, steroid resistant graft-versus-host-disease (GvHD) has been set forth (Le Blanc et al. 2004). Following this observation, immunomodulatory properties of ASCs have been investigated *in vitro* and *in vivo* (García-Olmo et al. 2005; Puissant et al. 2005; Yañez et al. 2006; Fang et al. 2007; Constantin et al. 2009; Ra et al. 2011). Some comparative studies have been performed considering both BMSCs and ASCs (Bochev et al. 2008; Ivanova-Todorova et al. 2009) and they demonstrated the interaction of these cells with immuno system cells. MSCs do not express MHC class II molecules (Aust et al. 2004; Delarosa et al. 2012) and costimulatory molecules, such as CD80-B7 (Yañez et al. 2006) and CD40 (Gonzalez-Rey et al. 2009). Furthermore, when co-cultured with lymphocytes, ASCs are able to suppress peripheral blood mononuclear cell (PBMC) proliferation (Yañez et al. 2006), as well as the proliferation of purified T cells (McIntosh et al. 2006; Constantin et al. 2009). In addition, ASCs inhibit B cell proliferation, reduce immunoglobulin production and suppress B cell functions even more significantly than BMSCs (Bochev et al. 2008; Saka et al. 2011). Moreover, ASCs prevent natural killer (NK) cell activation in response to standard target cell lines, resulting in impaired cytotoxicity processes (Delarosa et al. 2012).

Recent studies have demonstrated the capacity of BMSCs to elaborate suppressive molecules, such as hepatocyte growth factor (HGF), transforming growth factor (TGF) β (Di Nicola et al. 2002), prostaglandins E2 (PGE-2) (Aggarwal and Pittenger 2005; Rasmusson et al. 2005), and indoleamine 2,3-dioxygenase (IDO) (Meisel et al. 2004). Furthermore, several mechanisms have been shown, such as division arrest of activated T cells and NK cells by inhibition of cyclin D2 expression (Glennie et al. 2005), induction of regulatory T cells (Tregs, CD4+/CD25+/FoxP3+)

(Aggarwal and Pittenger 2005), inhibition of antigen-presenting cells (Beyth et al 2005), dendritic cells (Ivanova–Todorova et al. 2009) and cytotoxic T cell maturation (Rasmusson et al. 2003).

Many of these molecular pathways involved in immunomodulation have been studied also in ASCs and it was observed that most of these immunosuppressive factors are shared by ASCs. Nevertheless, the main pathway involved is still unclear (McIntosh et al. 2006). In different culture settings, ASCs express IDO, cyclooxygenase-2 (COX-2), HGF, interleukin (IL)-10, histocompatibility antigen, class I, G (HLA-G), and are capable of inducing Tregs (Gonzalez-Rey et al. 2009). Moreover, after pro-inflammatory treatment, ASCs upregulate IDO expression and increase production of PGE-2 and HGF, thus resulting in stronger immunosuppression (Crop et al. 2010).

Another important aspect that has been noticed is represented by capacity of ASCs to exert immunomodulation without cell-to-cell contact (Yañez et al. 2006).

The capacity of ASCs to inhibit inflammation has been studied in animal models, in order to better understand whether *in vivo* ASC administration can block or reduce different inflammation-based diseases, such as rheumatoid arthritis, experimental autoimmune encephalomyelitis, Crohn's disease, ulcerous colitis and GvHD. The induction of IL-10-secreting T cells with antigen specific regulatory properties may significantly contribute to the suppressive activity of ASCs on T cells from rheumatoid arthritis patients (Gonzalez-Rey et al. 2009).

The capacity of human ASCs to regulate various inflammatory mediators and suppress T helper 1-type (Th1) responses, through the generation of Tregs, might offer a therapeutic advantage in rheumatoid arthritis over existing therapies directed against a single mediator (Gonzalez-Rey et al. 2009). *In vivo* rheumatoid arthritis models showed that systemic infusion of human ASCs significantly reduces the incidence and severity of experimental arthritis. This therapeutic effect is associated with the down-regulation of the Th1-driven autoimmune and inflammatory responses. Human ASCs decrease antigen-specific Th1/Th17 cell expansion and induce the production of anti-inflammatory IL-10 in lymph nodes and joints (González et al. 2009). Similarly, systemic infusion of ASCs significantly ameliorates the clinical and histopathological severity of experimental colitis, abrogating weight loss, diarrhea and inflammation, and increasing survival (Gonzalez-Rey et al. 2009; González et al. 2009). The therapeutic effect is associated with the down-regulation of Th1-driven inflammatory responses, by decreasing inflammatory cytokines and chemokines, and increasing IL-10 levels, thus affecting macrophage functions. In addition, human ASCs may impair Th1 responses in both colonic mucosa and draining lymph nodes, and the induction of IL-10-secreting Tregs is partially involved in this therapeutic effect. Finally, ASCs may protect from

severe sepsis by reducing the infiltration of inflammatory cells in various target organs and the production of various inflammatory mediators (Gonzalez-Rey et al. 2009).

ASCs have a significant beneficial effect also in chronic experimental autoimmune encephalomyelitis, not only when administered before the onset of symptoms, but also when injected in mice with consolidated disease, displaying a therapeutic effect by acting simultaneously in lymphoid organs and inflamed central nervous system. In fact, ASCs induce a Th2-type shift of antigen-specific CD4 T cells in lymph nodes, and display α4 integrin dependent migration inside inflamed CNS, by crossing the blood–brain barrier; thus, ASCs may participate to remyelination by promoting the survival and proliferation of endogenous precursor cells (Constantin et al. 2009).

Finally, the infusion of ASCs may control lethal acute GvHD in mice receiving haploidentical hematopoietic transplant (80% survival at day +70), but only when administered in the early phases (days 0, 7) and not later (days 14, 21, 28) (Yañez et al. 2006). These observations constitute the first experimental proof that ASCs can efficiently control GvHD, but their potential clinical application needs further investigation.

These findings suggest the potential ability of allogenic ASCs to inhibit immune response, when inoculated in a host, without activation of rejection process.

1.4.3. Mechanism of action

Several possible mechanisms of action of MSCs to enhance healing have been suggested in fact, there is *in vivo* and *in vitro* evidence supporting the possibility of these mechanisms. However now it is still uncertain which of these mechanisms are important in the *in vivo* situation, as well as their relative contributions to the overall healing process. MSCs may act in an autocrine and paracrine fashion to enhance tendon healing (Caplan 2007). Studies using MSCs to treat myocardial infarction support this mechanism of action: MSC conditioned *media* injected into infarct sites following myocardial infarction limit the number of apoptotic cells, reduce infracted area and improve left ventricular function (Gnecchi et al. 2005; Gnecchi et al. 2006). The ability of MSCs to differentiate into lineage-specific cells is clear, but it is uncertain, if this is the major pathway by which the stem cells produce their effects. Only small numbers of MSCs differentiate at all, and they may not fully integrate with the host tissue. Finally, it was observed that human BMSCs could dynamically transfer mitochondria or mitochondrial DNA (mtDNA) to cells with non-functional mitochondria to rescue aerobic respiration (Spees et al. 2006). Injuries to mitochondria are common early events in animal models, explaining why functional improvements may be seen despite lack of long-term incorporation of implanted cells.

Observation emerged during *in vivo* studies, have suggested an important role of paracrine action of MSCs. In fact, although MSCs exhibit multilineage differentiation potential and can migrate to injured sites after systemic administration, the differentiation of MSCs in cells of injured tissues contributed little to their therapeutic benefits. A growing number of evidence indicates that the *in vivo* effects of MSCs depend primarily on their capacity to secrete bioactive soluble factors that could inhibit fibrosis and apoptosis, enhance angiogenesis, stimulate mitosis and/or differentiation of tissue-intrinsic progenitor/stem cells (Caplan 2006) and modulate the immune response (Yagi 2010).

Soluble factors are not the only material released from cells, recent studies demonstrated that these cells are able also to release small vesicles, named membrane vesicles (MVs), and responsible of cell-to-cell communication (Ratajczak 2006; Camussi 2010). MVs are a heterogeneous population of small vesicles constituted by a circular fragment of membrane containing cytoplasm components which are released by different cell types. The two major classes of MVs released in the extracellular environment are the exosomes and shedding vesicles. Exosomes originate from inward of endosomal membrane, accumulate within multivesicular bodies, are secreted by a process of exocytosis and exhibit a 30-120 nm size. At variance, shedding vesicles take place from direct budding of plasma membrane surface and are more heterogeneous in size (80 nm-< 1mm), depending from the cell of origin and on stimuli (Cocucci 2009). The released MVs can be up-taken by neighbouring cells, either as result of surface receptor mediated interaction or by a process of membrane fusion. After interaction MVs can be internalized by the recipient cells and deliver their content (Ratajczak 2006; Camussi 2010). Regarding stem cell biology, it has been suggested that stem cells could communicate with injured cells by MVs system. In particular, this interaction is bi-directional (Ratajczak 2006): MVs derived from injured cells are able to induce tissue specific differentiation of BM cells and MVs derived from stem cells are capable to activate regenerative programs in cells survived to injury (Aliotta 2010).

1.4.4. Enzyme telomerase activity

Using somewhat different strategies, several laboratories have identified, isolated, and cultured MSCs with specific properties (Wagner et al. 2005; Kern et al. 2006; Bochev et al. 2008). The *in vitro* expansion permits to rapidly reach the desired cell counts for *in vivo* use, but it is well-known that life span of MSCs is limited, and that during *in vitro* culture they lose differentiation potential and progress to replicative senescence. Preserving their undifferentiated status and self-renewal capacity while simultaneously forcing their expansion to obtain sufficient number of cells presents a challenge for researchers (Trivanovic et al. 2015).

Mesenchymal cells elderly are subject to a shortening of telomeres following several divisions that have gone against. Telomeres as repeated DNA sequences are protective chromosome caps, which are essential for maintaining the genomic cell stability, within adult somatic tissues. Each cell division leads to telomere shortening to a critical length, triggering cell senescence and apoptosis. The maintenance of telomere length in stem cells is an important factor for their self-renewal and differentiation capacity (Serkinci et al. 2008).

Proliferative potential of stem, tumour and germ cells can be preserved by enzyme telomerase, whose role is to elongate or stabilize telomeres. Telomerase expression is involved in the regulation of MSCs proliferation and differentiation state and assaying for telomere status in MSCs might assist in their characterization prior to their use in cellular therapy (Trivanovic et al. 2015).

1.5. Use of allogeneic mesenchymal stromal cells

Much work has been done using autologous cells for tissue engineering applications. This is clearly advantageous from the point of histocompatibility. Transmission of infectious agents is also eliminated, although the risk of introduction of infectious agents during the *ex vivo* expansion step is still present. Finally, the regulatory hurdles required to bring this technology to clinical use is much less when compared to allogeneic cells.

In fact, autologous MSCs are certainly non-immunogenic and reduce the risks of transmission of infectious agents, but their application requires a delay in the therapy of about 15-20 days needed for patient-specific cell expansion *in vitro*.

In addition, there is individual variability in MSC harvest and detrimental changes to MSCs with age. The availability and the biological activity of cells isolated from older animal could be unsatisfactory (Baxter et al. 2004; Fortier and Smith 2008): with increasing age, the number, differentiation potential and lifespan of MSCs decrease (Nishida et al. 1999; Mueller and Glowacki 2001; Stenderup et al. 2003; Kern et al. 2006). This aspect has implications in the treatment of degenerative disorders common in the older age groups.

In contrast, allogenic MSCs can be isolated from young animals, prepared, characterized and banked in advance (Richardson et al. 2007; Fortier and Smith 2008; Watts et al. 2011) and hence provide a readily available source of stem cells to be used at the proper time after the lesion occurrence.

An example is represented by acute tendon injuries, whose repair or reconstruction needs to be within days to maximize outcome potential and the presence of “off-the-shelf” allogeneic cells would overcome this problem. Finally, the use of allogeneic cells will obviate the need for harvest, minimizing the risk of complications. Using MSCs as an allogeneic cell source, is particularly

advantageous as there is mounting convincing evidence to show that MSCs are able to circumvent the normal immune response associated with the mismatched allogeneic tissue. Several clinical trials utilizing allogeneic MSCs have shown benefit with the use of these cells. Gene-marked allogeneic BMSCs showed incorporation and increased growth velocity in 5 of 6 children undergoing standard bone marrow transplantation for severe osteogenesis imperfecta (Horwitz et al. 2002). This occurred despite the lack of pre- or post-procedure chemotherapy. The immunomodulatory effects of MSCs were used to treat severe steroid resistant acute GVHD (Ringden et al. 2006) and allogeneic BMSCs incorporated successfully in patients with metachromatic leukodystrophy and Hurler syndrome (Koç et al. 2002). The suggested mechanisms by which bone marrow mesenchymal stem cells avoid immune rejection have been reviewed recently (Ryan et al. 2005). These include hypoimmunogenicity of the MSCs themselves, the prevention of T cell responses by MSCs and the fact that the MSCs can induce a suppressive local environment.

1.6. Platelet lysate

Recently, MSCs has been implanted alone or in association with biological scaffolds, in particular in order to treat tendon disorders use of platelet rich plasma (PRP) or platelet lysate (PL) has become routine for many practitioners. PRP represents a further tool that has been recently introduced in the therapy of tendon lesions both in human and domestic animals (Fortier and Smith 2008; Torricelli et al. 2011). Manipulation of PRP permits to obtain PL that is nowadays widely applied in different clinical scenarios, such as orthopaedics, ophthalmology and healing therapies, as a growth factor pool for improving tissue regeneration.

PL corresponds to a turbid, light-yellow liquid that is obtained from PRP after freeze/thaw cycles. PRP is blood plasma with a concentrated platelet count, generally greater than two to four times normal. PRP is generated through a simple centrifugation or filtration process of venous blood to concentrate the platelets. A subsequent freeze/thaw cycle causes the lysis of platelets, releasing a large quantity of growth factors necessary for tissue regeneration. The role of platelets in homeostasis is well known. LP is an attractive biologic tool to enhance tendon and ligament regeneration, in fact platelets are a natural reservoir of a pool of growth factors, including PDGF, TGF β and VEGF. All of them have been shown *in vitro* and *in vivo* animal models to enhance tendon regeneration (Kang and Kang 1999; Dahlgren et al. 2002; Molloy et al. 2003; Tang et al. 2003; Zhang et al. 2003, Yoneno et al. 2005; Scala et al. 2014). Platelet degranulation is thought to release the growth factors and other substances that promote tissue repair and influence the reactivity of vascular and other blood cells in angiogenesis and inflammation. The advantages of

using LP include ease of use, administration of autologous peptides, and delivery of a combination of growth factors. In addition, LP clots after injection through exposure of platelet to the basement membrane of cells in damaged tissue, resulting in the formation of a fibrin scaffold to allow for cellular migration into the injury and as a mechanism to retain the growth factors at the site of injury. The primary disadvantage of LP is that it lacks a cell source and delivers a mix of growth factors associated naturally with scar healing. In an explant culture system, however, tendons cultured in pure LP showed enhanced gene expression of those matrix molecules characteristic of tendon, including cartilage oligomeric matrix protein (COMP) and an increased collagen type I/collagen type III ratio, with no concomitant increase in the catabolic molecule matrix metalloproteinase 3 or 13 (Schnabel et al. 2007). Suspensory ligament fibroblast matrix production is also stimulated by addition of LP but to a lesser extent than by application of BM supernatant (Smith et al. 2006; Schnabel et al. 2007). These findings support the *in vivo* investigation of LP as an autologous patient-side treatment for tendonitis and suspensory desmitis.

In the clinical arena, use of this biological scaffold is very promising, not only because of the release of growth factor but also for its ability to decrease significantly expression of pro-inflammatory gene markers and to increase expression of anti-inflammatory markers. Platelets have also been found to have anti-inflammatory activity via inhibition of the nuclear factor (NF)-kB pathway and limiting expression of IL-1 (Bendinelli et al. 2010; van Buul et al. 2011; Osterman et al. 2015).

Platelets are also a source of inflammatory mediators and modulators. In fact, they are able to release numerous anti-inflammatory cytokines, including IL-1 receptor antagonist (IL-1ra), inhibiting the bioactivity of IL-1 by blocking its receptors (Goldring 2001; Malemud 2010). Furthermore, platelets secrete sTNF-R that can bind to free TNFα.

Secretion of IL-4, IL-10 and IL-13 can increase IL-1ra production and reduce tumour necrosis factor alpha (TNFα)-induced prostaglandin E2 production (Alaaeddine 1999; Arend 2000). Although PRP also releases pro-inflammatory cytokines, such as IL-1α, IL-1β, TNFα, IL-6, IL-8, IL-17 and IL-18, their concentrations are much lower than those of the anti-inflammatory counterparts (Woodell-May 2011). The significant difference between the concentrations of anti-inflammatory cytokines and those of the pro-inflammatory factors in PRP suggests that PRP may suppress inflammation protecting tissues and reducing pain.

1.7. Stem cell therapies

1.7.1. Tendon and tendonitis

Tendons join muscle to bone and are usually considered to act by allowing the forces generated by the musculature to act upon the skeleton and initiate movement (locomotion). In large quadrupeds such as the horse, the bulk of the musculature is located proximally to reduce weight in the distal limb, thereby making limb protraction, and therefore locomotion, more efficient. This leads to long tendons, such as the superficial digital-flexor tendon (SDFT), deep digital flexor tendon (DDFT) and the suspensory ligament (SL). In addition, the horse has a hyper-extended metacarpophalangeal joint, which means that these long tendons, located on the palmar aspect of the limb, encounter large weight-bearing loads. There are many similarities between the weight-bearing tendons of the horse and those of the human athlete (e.g. Achilles tendon) in their hierarchical structure and matrix composition (figure 6), their function and the nature of the injuries sustained (Richardson et al. 2007).

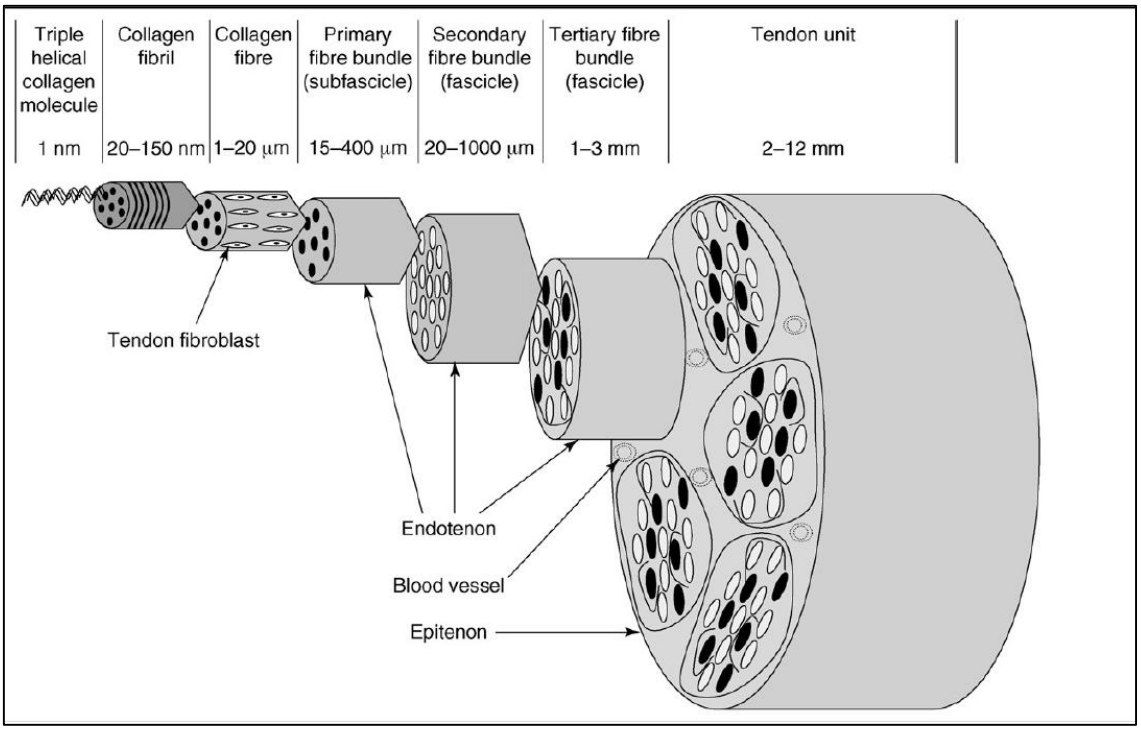


Figure 6. Tendon structure (Richardson et al. 2007).

In contrast to positional tendons, such as the hand tendons, all weight-bearing tendons also function as springs, absorbing and releasing elastic energy during the different phases of the stride. By doing this, they contribute to the high efficiency of locomotion and also act as a shock absorber for the limb (Wilson et al. 2001). This is true for both the human and the horse, but the horse has maximized this potential, so that the efficiency of locomotion is in excess of 100% at a gallop (Minetti et al. 1999). For a spring to function at maximum efficiency, it must be proportionate to the

weight applied to it, and it should stretch close to its maximum. Maximal strains in the SDFT of the horse have been recorded at 16% during galloping in thoroughbreds (Stephens et al. 1989). The strains measured *in vitro*, however, indicate that the failure limit in the SDFT is somewhere between 10% and 20% (Goodship et al. 1994), indicating that the SDFT is operating close to its functional limit. With a tendon designed to operate close to its functional limit, together with the gradual acquisition of cumulative degenerative damage characteristics in adult horses subjected to high-intensity exercise, it is not surprising that these weight-bearing tendons commonly fail, in both horses and humans. The racehorse is essentially a professional athlete, and therefore it is no great surprise to discover that injuries to the palmarly situated tendons, in particular the SDFT, are very common. All horses used for competitive sport, which includes racing on flat-surfaces or over hurdles (National Hunt), eventing, show-jumping, endurance riding and dressage, can be affected.

In response to acute injury, there is an initial pronounced inflammatory reaction. Injury-related intra-tendinous haemorrhage is quickly accompanied by oedema and the infiltration of macrophages, which remove necrotic tissue (Williams et al. 1980; Woo et al. 2000; McIlwraith 2002). This inflammatory response is short lived (usually only a few days) and clinically evident inflammation (and pain) is not a feature of chronic injury in the horse.

Growth factors and cytokines (PDGF, TGF-β, TGF-α, βFGF and EGF) are released by the invading macrophages and platelets, and these elicit a chemotactic and proliferative response in fibroblasts and encourage the synthesis of collagen types I, III and V, which form scar tissue. The tendon cellularity is initially increased when compared with normal tendon (McIlwraith 2002). The morphology of the invading scar tissue fibroblasts differs from that of the normal tenocytes; they are larger and more basophilic and have large vesicular nuclei, and they are thus more similar to myofibroblasts than tenocytes (Williams et al. 1980). The origin of these fibroblasts is not known, but possible sources include the resident tendon-derived cells, probably from a stem cell-like pool within the tendon or surrounding the tendo (known as intrinsic repair) or from the systemic circulation (probably derived from the MSC population within the BM). Recent data from green fluorescent protein (GFP)-labelled chimaeric rats subjected to BM transplantation and tendon injury enable the relative contributions of BM-derived and tendon-derived cells to the repair to be evaluated (Zantop et al. 2006; Kajikawa et al. 2007). This has suggested that the initial cellular infiltrate was systemically derived but is likely to be largely white blood cell in nature and associated with the inflammatory response and debridement, whereas, in the later phases, cells associated with tissue repair were derived locally. However, it cannot be ruled out that a small number of systemically derived cells do enter the tendon after injury and exert a paracrine effect on the locally invading cells to initiate a healing response (Zantop et al. 2006). The newly formed

collagen in the scar is less highly cross-linked than that in the normal tendon, and there is much more type III collagen present (<1% type III in normal tendon compared to 20%–30% in tendon scar tissue) (Williams et al. 1980). Type III collagen differs from type I because of its smaller fibre diameter, which might provide greater elasticity but confers reduced strength. As the scar increases in size and matures, more-stable cross-links are formed, and the proportion of type I collagen increases, as does fibril diameter. This is associated with an increase in strength and stiffness. The collagen concentration thus returns toward normal; however, the mechanical properties of the tendon are still inferior as a result of a persistently deficient structural organization and composition of the matrix (Wang 2006). So that sufficient structural strength can be achieved, larger amounts of fibrous tissue are laid down within and around the tendon, thus giving rise to a tendon that is persistently enlarged but that also has greater structural stiffness. Consequently, this increased stiffness reduces the efficiency of the tendon as a spring and compromises the performance of the horse (or human). Completion of fibrous healing takes a long time (1–2 years), but the tendon is never restored to its previous mechanical properties (Richardson et al. 2007).

1.7.2. Tendon and ligament treatment

Tendon repairs are often weak and susceptible to re-injury. Given the frequency and increasing cost of these injuries, mainly in sport horse, as well as the relatively poor result of surgical intervention, it is not surprising that new and innovative strategies like tissue engineering have become more appealing. In fact, traditional therapeutic approaches to tendon healing do not always result in a satisfactory anatomical and functional repair, and healed tendon is often characterized by functional impairment and high risk of reinjury.

Recently, MSCs and LP have been proposed as novel therapeutic treatment to improve the tendon repair process.

The preparation of cell sample is commonly performed as following indicated: cell isolation can be effected by a portion of adipose tissue in the area in the vicinity of the head of the tail. The sample, preserved in ice, is sent to a laboratory where it is manipulated in order to isolate ASCs. The obtained cell suspension is concentrated in syringes, which can then be sent back to the veterinarian and used for the therapy. The theory is based on the ability to directly repair the injured area through a multiplication and cell differentiation, that give rise to a tendon elastic or osteoblasts capable of regenerating structures irreversibly compromised.

In order to obtain satisfactory results, it is essential that the grafting takes place within about 30 days subsequent to injury, when the body is in the active phase in an attempt to regeneration of new tissue. The *optimum* time to implant MSCs is after the initial inflammatory phase and before fibrous

tissue has formed. Even chronic wounds ligament can benefit from these cells because they are able to remodel the scar tissue.

The use of stem cells does not reduce the duration of the period of convalescence but only the risk of eventual relapse. Patients undergoing this therapy are more likely to regain full functionality of the damaged tissue.

Although growth factors seem to enhance tendon and ligament regeneration and repair, efforts to improve healing further are currently centred on the delivery of stem cells to the site of injury. In horses, the greatest clinical interests are in the application of ASCs or BMSCs for tendon healing, and variations of both cell types are being used clinically with reported success.

Arguments can be made for a stem cell source being optimal for applications in regenerative therapies, and, importantly, studies are needed to define the necessity of stem cells in such endeavours. There are more data available regarding cartilage tissue engineering, and those data support the need for the presence of cells (chondrocytes or stem cells) in a graft composite, but similar data are less abundant for tendon regenerative studies (Oreffo et al. 2005; Cohen et al. 2010).

In the horse, the therapeutic use of adult MSCs has been reported (Smith et al. 2003; Carstanjen et al. 2006; Crovace et al. 2007; Pacini et al. 2007; Wilke et al. 2007; Guest et al. 2008; Riccò et al. 2013). The efficacy of these treatments is difficult to determine, since the use of control animals is rarely reported and often the stem cell treatment is combined with other biological factors, such as bone marrow supernatant, autologous *serum*, or platelet-rich plasma (PRP). In 2003, Smith et al. (2003) were the first to report on the reimplantation of culture-expanded autologous BMSCs into a spontaneously occurring core lesion of the superficial digital flexor tendon. This case demonstrated the feasibility of using culture-expanded MSCs therapeutically, but, more importantly, no adverse reactions were noted after injection. Autologous BMSCs are now offered commercially for treatment of ligament and tendon injuries, but data from controlled clinical trials on the efficacy of this treatment modality has lagged behind clinical use, due to technical difficulties in developing a good injury-induced model of core tendon lesions and the reluctance of horse owners to enrol valuable horses in a controlled study with placebo treatment groups. In 2007, Pacini et al. (2007) reported that 9 out of 11 Italian racehorses with spontaneously occurring incomplete lesions of the superficial digital flexor tendon, returned to racing and were still racing 2 years after treatment with culture-expanded BMSCs. All 15 control horses reinjuried the tendon within 4 to 12 months. Ultrasonographic evaluation revealed superior fibre alignment in the MSC-treated tendons compared with that in the tendons of the control horses. The significance of these observations is difficult to determine for a number of reasons: 1) the MSCs were resuspended in autologous *serum*

for injection. 2) The horses were not randomly allocated to treatment and control groups. Instead, horses were included in the treatment group based on availability of the MSCs procedure and owner consent to the use of MSCs. The allocation of horses based on owner preference introduces the risk of owner-induced placebo effect, since owners of MSC-treated horses may adhere more stringently to the rehabilitation program. 3) The number of MSCs injected varied widely between horses (range $0.6x10^6$ to $31x10^6$ cells) and the age of the horses also varied, with the MSC-treated horses being 2 to 15 years of age and the control horses being 4 to 8 years of age. 4) Histological examination and molecular analysis, which would have helped determine the quality of the repair tissue, were not possible due to the continuing performance of the horses.

Crovace et al. (2007) created core lesions in the superficial digital flexor tendon by injecting collagenase into 3 of 4 tendons in 3 horses. The lesions were then treated with either culture expanded BMSCs suspended in fibrinogen, freshly isolated mononuclear cells from BM aspirates suspended in fibrinogen, or a placebo treatment with an unknown substance. The purpose of this study was to evaluate whether the culture expansion of BMSCs provides an advantage over the use of freshly isolated mononuclear cells from BM aspirates, since avoidance of cellular culture expansion would allow for a 1-step procedure. However, the tendon lesions created by collagenase injection varied, the number of cells varied, and the volume of fibrinogen in which the cells were resuspended varied. Thus, the number of variables used makes comparisons between the treatment groups exceedingly difficult.

Recently, autologous and allogenic BMSCs were transfected with GFP prior to injection into surgically induced lesions of the superficial digital flexor tendon in 2 horses. This pilot study was not designed to evaluate treatment efficacy, but it warrants attention because it involved the injection BMSCs of allogenic origin, as well as the use of a cellular marker. Significant adverse reactions were not observed clinically during the 30-day study and on histological examination of the lesions; there was no increase in the number of inflammatory cells surrounding the GFP-marked MSCs of allogenic *versus* autologous origin (Guest et al. 2008).

Another study regarding use of allogenic MSCs, demonstrated absence of chronic or acute clinical adverse reaction in the short or long time. In fact, tissue swelling, temperature raising and lameness at walk were not reported in any animal following allogenic cells implantation, nor was the formation of abnormal tissue reported by ultrasonography or palpation after the 24 months follow-up (Riccò et al. 2013)

These encouraging data should be improved by other studies, in order to evaluate whether the allogenic use of MSCs in the horse is safe and efficacious.

1.7.3. Treatment of other disorders

Based on both *in vivo* and *in vitro* studies, MSCs have been applied to various clinical field. One of the most studied application regards equine cartilage repair, in fact it is increasingly being considered.

However, most work has been restricted to *in vitro* studies (Fortier et al. 1998; Koerner et al. 2006; Koch et al. 2007; Stewart et al. 2007; Giovannini et al. 2008; Kisiday et al. 2008; Reed and Johnson 2008), and there are no reports on the feasibility, safety, and efficacy of MSCs in horses suffering from spontaneously occurring cartilage injuries. Wilke et al. (2007) induced cartilage lesions in the femoropatellar joint of 6 horses and treated the defects with autologous fibrin alone or in combination with culture-expanded BMSCs. They noted improved healing characteristics in the fibrin/BMSC-treated group, compared with the group treated with fibrin alone, at follow-up arthroscopic examination 30 days after treatment. This difference in treatment outcome was not sustained 8 months after treatment, when no difference was observed between the 2 groups. More studies are needed to determine if the initial difference in treatment outcome was a genuine MSC-mediated effect. Under *in vitro* culture conditions, MSCs do not divide indefinitely and it is possible that the lack of sustained superior treatment effect in the fibrin/BMSC group was due to the death of injected MSCs or their becoming metabolically inactive with time (Bonyadi et al. 2003; Kern et al. 2006; Sethe et al. 2006; Stolzing et al. 2008). Autologous cancellous bone is often used in equine patients with substantial bone loss in order to enhance bone repair by providing a scaffold of osteoprogenitor cells (some of which are MSCs) and various growth factors (Koch et al. 2009). However, the number of osteoprogenitor cells in equine cancellous bone has been shown to vary between donor sites (McDuffee and Anderson 2003; Harriss et al. 2004; McDuffee et al. 2006) and due to decreased potency of the MSCs, because of either an age-related decline in MSC number with increasing age or reduced metabolic function of MSC from aged individuals, as has been reported in human studies (Bonyadi et al. 2003; Sethe et al. 2006; Stolzing et al. 2008). The use of culture-expanded or otherwise purified and concentrated equine MSCs may therefore be desirable in selected cases, in order to obtain a sufficient number of cells with appropriate osteogenic potential. A number of reports have evaluated the *in vitro* potential of MSCs from various equine tissue sources, to differentiate towards the osteogenic cell lineages (Koerner et al. 2006; Vidal et al. 2006; Arnhold et al. 2007; Hoynowski et al. 2007; Koch et al. 2007; Vidal et al. 2007; Giovannini et al. 2008); but the *in vivo* use of culture expanded or purified MSCs or of fractions of mononuclear cells in equine experimental or clinical cases for enhanced bone repair have not been reported. MSCs and bone regeneration in veterinary medicine has been reviewed in detail elsewhere (Kraus and Kirker-Head 2006). The retention of culture-expanded BM cells in repair tissue of the

soft palate in a horse 14 days after surgical repair of a naturally occurring, cleft palate has been reported (Carstanjen et al. 2006). The cells were injected directly into the repaired tissue at the end of the surgical repair procedure. The regenerative or reparative significance of the injected cells is unknown, due to the short follow up period and the lack of a control case.

1.8. Classical therpy

There are many treatment options available to equine practitioners dealing with injuries to tendons and ligaments and an equally large number of techniques and modalities that can be used to help manage joint problems in the athletic horse. Today these traditional therapeutic methods are considered out-of-date and for this reason regenerative medicine could be considered an intriguing alternative to them.

1.8.1. Pin firing

One of the most used technique, especially in the last decades, is represented by pin firing or thermocautery (figure 7). Pin firing is a therapy that uses a small, red-hot probe to cause cauterization (burning) of tissue in horses with chronic injuries to produce an abundant, serous inflammatory process. As opposed to other inflammation processes such as infections or bruising, *serum* has little or no fibrin (clotting material) or cellular content and does not coagulate. Firing causes maximal exudation, or oozing, and minimal tissue degeneration. The flooding of *serum* seems to flush out any chronic irritation, and it does not displace old scar tissue.

Firing is done more often in racehorses than in other performance horses, and has been used for more than a century in conditions of recurring injuries such as a splint, curb, or chronic bowed tendon. The process is performed under sedation and local anaesthesia, and the pain inflicted is fairly short-lived and usually well-tolerated by the patient.

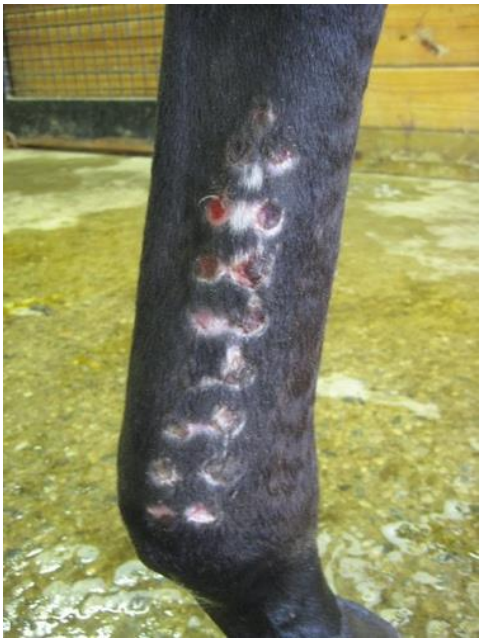


Figure 7. Equine limb treated with pin firing.

The driving idea behind firing is that it makes chronic inflammations acute and allows them to heal. When the body responds to the new injury of firing, which is performed over the old injury, it responds in a different way than the initial injury.

This approach requires special attention to the opposite leg, in fact after pin firing treatment the horse will place excessive weight on the unaffected leg straining it. For this reason, many times, both of the front or hind legs are treated at the same time.

Following firing, specific nursing care is necessary. This involves a strict regimen of ointments and keeping the area clean. The horse must have time off, from six months to a year, depending on the condition.

Pin firing is common with some practitioners, but it is not generally taught today in veterinary schools. This technique is not an aesthetically pleasing process and today it is considered very archaic. A reason that discourage pin firing is that this process does not allow circulation between the areas of the cautery, causing skin death. White hairs might appear at the points of firing, and in show horses, this would not be desirable. In addition, the practitioner must be very careful not to cauterize either over or through superficial blood vessels, or close to a joint capsule.

1.8.2. Magnetic therapy

Magnetic therapy has been used for thousands of years to promote healing, remove toxins, and decrease pain by increasing circulation to affected areas. This technique is performed specifically to act upon the bi-polar nature of all living cells to break up calcifications and stimulate tissues. All cells are electrical in nature, with a positively-charged nucleus and a negatively-charged outer membrane. When alternating polarity is applied through magnetic therapy, these cells are

stimulated, circulation is improved, and toxins are forced out to be carried away by the bloodstream. This is beneficial in reducing inflammation, prohibiting the calcification of joints, easing navicular symptoms, and promoting healing of damaged tissues in a non-invasive, time and cost-efficient manner.

1.8.3. Tendon splitting

Tendon splitting was initially advocated for the treatment of chronic SDF tendinitis to improve blood flow, but subsequent research demonstrated excess granulation tissue formation, trauma to the tendon tissue and persistent lameness following treatment (Stromberg et al. 1974).

Tendon splitting involves inserting a scalpel or tendon knife directly into the core lesion and moving it longitudinally through the entire lesion. It can promote getting rid of accumulated fluid while at the same time stimulating the arrival of an increased blood supply, both of which aid in the healing process.

Splitting the tendon decompresses the lesion and causes early reduction of tendon and core lesion size. The rapid reduction of the core lesion hypothetically prevents the development of excessive scar as the tendon heals.

This technique was first reported in Sweden, where it was successfully used in Standardbred racehorses and it was put into use on Thoroughbreds in the United States in the 1970s.

Often, the response to tendon splitting appeared excellent within several months after surgery so that horses were placed back to work without adequate time for tendon healing. However, with the arrival of the ultrasound in the 1980s, the rate of healing could be monitored.

Core lesions are observed to disappear within 8 to 30 days after splitting, whereas non-split lesions can persist from 3 to 4 weeks to several months. In experimentally created tendinitis, microangiographs suggest that blood flow improves after tendon splitting. Vascular ingrowth also appears more rapid and is more mature in the split tendon in both collagenase-induced tendinitis and clinical cases.

Although tendon healing appears to progress more rapidly after tendon splitting, clinical results suggest that 4 to 5 months of rest is warranted before initiating training, and a minimum of 8 months is required prior to testing the strength of the healed tendon in a race or other similar strenuous event. In some cases, turn out or light work for a full year is recommended prior to racing.

Reports in racehorses suggest tendon splitting is equivalent to treatment with intra-tendon hyaluronic acid or polysulfated glycosaminoglycans, with 50%-60% of horses returning to successful racing.

1.8.4. Desmotomy

Another surgical treatment is proximal ligament desmotomy. It has been reported to help horses return to racing without re-injury. The surgery's goal is to release the SDFT by severing its attachment to bone behind the knee.

This allows the tendon to stretch more during loading, hypothetically improving its blood supply and elasticity.

It was observed no difference in the rate of re-injury when horses treated with the check ligament surgery were compared to those healed by conservative treatment and rehabilitation only. Furthermore, horses treated with proximal check ligament desmotomy were more likely to develop suspensory desmitis.

2. AIM

Aim of this research is the evaluation of allogenic adipose tissue mesenchymal stromal cells (ASCs) associated with PL for reparation of tendon lesions in equine species.

Nowadays, Italian Veterinary Regulatory System lacks of guidelines regarding use of allogenic cells for treatment of animal diseases.

In light of this fact, the safety of allogenic ASCs associated to biological scaffold has been considered during this study.

Initially, several methodologies were set up, in order to investigate ASCs *in vitro* abilities and their typical features. For this reason, cell isolation procedure, *in vitro* amplification and microbiological controls were standardized. Furthermore, cells has been characterized for self-renewal capacity and multipotentiality. Samples were tested by colony forming unit test, differentiation in three mesodermal lineages (adipogenic, osteogenic and chondrogenic), expression of specific markers and evaluation of different parameters, indicating safety of cells (tumorigenic/transforming potentialities, telomerase activity, telomeric length and P53 mutation).

Another aspect that it has been considered, was the possibility to bank cell samples for future *in vivo* employment, for this reason a cryostore solution was investigate, able to improve cell viability after freezing/thawing process.

Furthermore, in order to understand what happen to ASCs when they are amplified for many passages, several parameters were investigated. In fact, *in vitro* expansion conditions could compromise the stemness and differentiation capacity of MSCs, while not limiting the sufficient cell number required for cell therapy.

In particular, 5 samples were amplified until 10^{th} passage and analysed at 4^{th}, 6^{th} and 10^{th} passages. These samples were inoculated into nude mice and soft agar, in order to evaluate their ability to promote development of neoformation *in vivo* of *foci in vitro*, respectively. Cell samples were incubated in differentiation *media* and tested by flow cytometry for expression of specific mesenchymal markers, in order to evaluate MSCs ability to preserve multipotential features during amplification.

Finally, cells at the 3 considered serial passages were investigated for telomerase activity, telomeric length and mutation of P53 gene, considering variation of these 3 parameters during *in vitro* amplification.

Regarding use of allogenic cells, another parameter has been tested and it was represented by the capacity of ASCs to inhibit PBMCs proliferation, witnessing the immune-modulatory activity of ASCs.

The encouraging results obtained by *in vitro* characterization tests, suggest the potential safety of ASCs for cell therapy purposes. At this regard, it was decided to collaborate with a veterinary practitioner in order to set up a clinical study.

In particular, ASCs were associated with PL, as vehicle for *in vivo* administration, and they were employed for cell therapy purposes in horses affected by tendon and ligament lesions.

In particular, 18 animals characterized by the same kind of lesions, were randomly subdivided in two groups. Group 1 was composed by 12 animals treated with ASCs and PL, whereas group 2 was represented by horses treated with a traditional clinical approach: pin firing.

Cell suspension was prepared by the laboratory and it was provided to the practitioner, who performed the clinical therapy in both group.

Treated animals were subjected to the same rehabilitation protocols and they were observed during follow up period. Finally, practitioner evaluated the quality of regenerated tendon considering ultrasound images and clinical behaviour of animals.

The capacity of ASCs associated to PL was not the unique parameter that was considered. In fact, in the scenario of allogenic stem cell use, it was of paramount importance to evaluate development of adverse reaction in the recipient host, treated with allogenic ASCs. This aspect was observed immediately after *inoculum* and during the follow up progress.

3. MATERIALS AND METHODS

3.1. Sample collection

Thirty samples of equine adipose tissue were analysed. Approximately 50 grams of equine subcutaneous adipose tissue was collected from abdominal fat deposit under sterile conditions immediately after slaughtering. Animals were aged between 1 and 20 years. All samples were collected in PBS containing penicillin 5000 IU/ml (Sigma Aldrich), streptomycin 625 µg/ml (Sigma Aldrich), amphotericin B 51 µg/ml (Bristol-Myers Squibb) and carried to the laboratory.

3.2. Mesenchymal stromal cell isolation

Samples were processed within 1 hour from the isolation. Tissue fragments were washed with sterile PBS added with antibiotics, minced in small pieces and enzymatically digested with type I collagenase (7.5 mg/ml; Sigma Aldrich) under mild shaking for 1 hour at 37°C. Cell suspension was filtered through a 50 µm nylon mesh, in order to remove tissue fragments, and cell pellet was washed three times by centrifugation (250 g, 10 minutes, 20°C). Finally, ASCs were resuspended in complete culture *medium* (NH Expansion Medium; Miltenyi Biotec) added with 1000 IU/ml penicillin, and 100 µg/ml streptomycin, automatically counted with Cellometer Automated Cell Counters with Trypan Blue staining (Abcr GmbH), seeded (50000 cells/cm^2) in tissue culture flasks and incubated at 37°C in 5% CO_2.The *medium* was changed after 48 hours and then every third day.

3.3. Cell amplification

Subcultures were performed when ASCs reached 70-80% confluence. In particular, cell monolayer was rinsed with PBS for 10 minutes at 37°C, the PBS was discarded and finally, in order to detach the cell culture it was treated with a trypsin-EDTA solution for 5 minutes at 37°C; cells were splitted at 1:3 ratio in complete culture medium and a maximum of 3 serial passages was carried out.

Five samples (ID11-15) were amplified until 10^{th} passage and cryopreserved at 4^{th}, 6^{th} and 10^{th} passages, in order to permit development of tests able to define features of amplified cells.

3.4. Quality controls

All cell batches were tested for microbial contamination. Mycoplasma infection was evaluated using the commercial MycoSensor PCR Assay Kit (M-Medical S.r.l.). Bacteria, fungi and yeast contaminations were investigated following inoculation of 2 ml of each cell suspension in microbiological media (Agar Sabouraud, Triptic Soy Agar and/or Brain Heart Infusion, incubated at 30°C and 37°C, respectively). In order to detect possible viral contamination, all cell samples were

co-cultured with susceptible cell lines. They were incubated at 37°C in 5% CO_2 for 5 days, in order to observe cytopathic effects. These cell lines are stored in IZSLER Cell Culture Bank (www.ibvr.org). In particular, equine *Herpesvirus*, *Arterivirus* and *Flavivirus* infections were detected following cell sample inoculation in equine dermis (Ede, Cod. BSCL40) and rabbit kidney (RK13, Cod. BSCL74). Furthermore, *Flavivirus* and *Pestivirus* (no-cytopathic Bovine Viral Diarrhoea Virus), after cultivation in EDe cells, were evaluated by Realtime PCR, by Virology Laboratory of IZSLER.

3.5. Evaluation of self-renewal ability of cells

Capacity of ASCs to form clone colonies was investigated immediately after cell isolation by colony forming unit (CFU) assay. In particular, $2x10^5$, $1x10^5$, $0.5x10^5$ cells were inoculated in three different wells (Ø 35 mm) with specific cultural *medium* (NH CFU-F Medium; Miltenyi Biotec) and incubated at 37°C, 5% CO_2. On day 15, the *medium* was removed from the cell culture vessels and cell culture layer was washed twice with 5 ml PBS. Cell cultures were fixed adding in each well 3 ml of 10% formalin at 20-25°C for 5 minutes. Subsequently, cultures were washed three times with 5 ml PBS, in order to discard formalin. Prior to use the Giemsa solution (Fluka) has been filtered and diluted 1:20 in PBS. Three ml of the staining solution was added to each well for 5 minutes at 20-25°C. After this period, the staining solution was removed and the cell culture was washed twice with deionized water. Culture vessels were air-dried until deionized water has evaporated and finally, colonies were counted at optical microscope.

3.6. Evaluation of multipotential ability of adipose-derived stromal cells

3.6.1. Multilineage differentiation

In order to control *in vitro* multilineage differentiation capacity of ASCs, cell cultures at serial passage 3 were incubated in three specific *media* and a cell control (not treated cells) was included in each test.

The same test was performed on five samples at 4^{th}, 6^{th} and 10^{th} serial passage.

Adipogenic differentiation: ASCs were diluted to a final concentration of $5x10^4$ cells/ml in NH AdipoDiff *medium* (Miltenyi Biotec). In particular, 1.5 ml of cell suspension was transferred to a cell culture dish (Ø 35 mm), incubated at 37°C in 5% CO_2 and every third day *medium* was changed. Detection of adipocytes was verified on day 21. Cells were washed twice with 5 ml PBS, 3 ml of methanol (SIGMA Aldrich) were added and plates were incubated at 20-25°C for 5 minutes. Methanol was discarded and cell culture dish was washed twice with deionized water. Finally, 3 ml of Oil Red O (SIGMA Aldrich) staining reagent was added to each well at 20-25°C.

After 20 minutes incubation staining was removed and culture dish was washed twice with 5 ml deionized water. Immediately after staining, cells were examined under microscope.

Osteogenic differentiation: cells were resuspended to a final concentration of $3x10^4$ cells/ml in NH OsteoDiff *medium* (Miltenyi Biotec) and incubated in 1.5 ml in 6-well plates at 37°C in 5% CO_2. *Medium* was changed every three days and at day 10 osteoblasts were detected. *Medium* was removed and cells were washed twice with 5 ml PBS. Cells were fixed by incubation in methanol at -20°C for 5 minutes, solution was discarded and cell culture was washed with 5 ml deionized water. Finally, water was removed and 1 ml of 5% silver nitrate (Sigma Aldrich) solution was added. Cells were incubated under ultraviolet light for 1 hour, they were washed twice with deionized water and un-reacted silver was removed with 5% sodium thiosulfate (Sigma Aldrich) for 5 minutes. Finally, cell culture were washed 3 times with deionized water and immediately after this step, cells were examined under microscope.

Chondrogenic differentiation: $2.5x10^5$ cells were centrifuged (150 g, 5 minutes, 20°C) in a 15 ml conical tube and 1 ml NH ChondroDiff *medium* (Miltenyi Biotec) was added to the cell pellet. Every three days *medium* was changed and on day 24 the single chondrocyte nodule was immersed overnight in neutral buffered formalin at 20-25°C with agitation. Subsequently, the nodule was dehydrated by ethanol and stained with Alcian Blue (Sigma Aldrich) for 12 hours; finally, the it was washed twice with deionized water.

Alternatively, after overnight incubation, the nodule was stored in neutral buffered formalin at 20-25°C and it was sent to the IZSLER Histology Laboratory, where it was embedded in paraffin. Finally, 5 µm tissue sections were generated using a microtome and they were stained Alcian Blue.

3.6.2. Specific surface marker expression

At 80% of confluence, five batches of adipose tissue MSCs (ID11-15) were evaluated by flow cytometry at passages 4^{th}, 6^{th} and 10^{th}.

In particular, 4 specific mesenchymal markers were investigated:

- Mouse anti rat CD90 Fitc: 1:25;
- Mouse anti horse CD44: 5 µl;
- Mouse anti human CD29 Fitc: 5 µl;
- Mouse anti horse 4E1 (developed at IZSLER Laboratory of Monoclonal Antibodies and Recombinant Antigens): 1:16.

Briefly, cells were mechanically harvested by a cell scraper, counted and resuspended at a concentration of 1×10^6 cells/100 µl in FACS buffer (i.e. PBS + 2% heat-inactivated FBS (Gibco) + 0.1% sodium azide (Sigma Aldrich)). For each sample, $5x10^5$ cells were diluted in 50 µl and

distributed in 6 wells of a 96-well microtitre plate for testing mAb to CD44, CD90, 4E1, CD29 and two negative controls. The primary antibodies CD90 (1:25) and 4E1 (1:16) and the secondary antibody Fitc-conjugated anti-mouse IgM (1:200) were diluted in FACS buffer, according to the manufacturers' directions. Cells were incubated with the primary antibodies for 30 minutes at 4°C, in the dark. Then, the plates were washed three times with 100 µl/well of FACS buffer at 500 g, for 3 minutes, at 5°C. Finally, cells marked with CD44 and 4E1 were incubated at 4°C for 30 minutes in the dark, with the secondary antibody. Three washes were performed as described above. Finally, 10000 events were analysed for each well. The following negative controls were performed: unstained cells to detect autofluorescence and secondary antibody control omitting the primary antibody. Data were acquired by GUAVA® easyCyte™ HT equipped with a laser BLUE 488 nm and analysed by InCyte software version 2.2.2 (Merck Millipore).

3.7. Evaluation of cryopreservation solution and procedure

In order to identify a cryostore solution, able to increase cell viability during freezing/thawing process and to reduce DMSO (Sigma Aldrich) concentration, 15 cryogenic solutions were considered (table 1).

CRYOGENIC MEDIA	DESCRIPTION
CM1	Culture *medium* + 20% (v/v) FBS + 10% (v/v) DMSO
CM2	Commercial *medium* containing 2% (v/v) DMSO
CM3	Commercial *medium* containing 5% (v/v) DMSO
CM4	Commercial *medium* containing 10% (v/v) DMSO
CM5	Culture *medium* + 20% (v/v) FBS + 5% (v/v) DMSO + 60mM Trehalose
CM6	Culture *medium* + 20% (v/v) FBS + 2,5% (v/v) DMSO + 30mM Trehalose
CM7	Culture *medium* + 20% (v/v) FBS + 264mM Trehalose
CM8	Culture *medium* + 5% (v/v) DMSO + 20% (v/v) FBS + 500mM Trehalose
CM9	Culture *medium* + 7% (v/v) DMSO + 5% (v/v) HES + 2% (v/v) BSA
CM10	Culture *medium* + 5% (v/v) DMSO + 4% (v/v) HES +4% (v/v) FBS
CM11	Culture *medium* + 10% (v/v) DMSO + 12% (v/v) HES + 8% (v/v) FBS
CM12	Culture *medium* + 30 mM Caspase Inhibitor z-VADfmk + 10% (v/v) DMSO + 20% (v/v) FBS
CM13	Vitrification *medium* (1 M DMSO, 1 M acetamide) 3 M propylene glycol
CM14	Vitrification *medium* added with 20% (v/v) DMSO
CM15	FBS + 10% (v/v) DMSO

Table 1. Cryogenic *media* (CM) considered in the study.

In particular, the traditional freezing *medium*, represented by cultural *medium* added with 20% (v/v) FBS and 10% (v/v) DMSO, was compared with the other 14 freezing solutions. Five batches of MSCs (samples ID6-10) were frozen in the above mentioned CM and a total of 75 vials were frozen. Equine ASCs, enzymatically disaggregated, were centrifuged at 125 g for 10 minutes at 4°C and cell pellets were diluted in culture *medium* devoid of *serum* and re-centrifuged in the same conditions. Each cell pellet was cryopreserved in the cryogenic *media*, except for CM13 and CM14. Cryovials were stored at 4°C for 60 minutes and frozen with a gradual reduction of temperature at -1°C/minute to -40°C. From -40°C to -70°C the decrease rate was -10°C/minute faster. The process was performed in a controlled rate freezer (CryoMed, Forma Scientific), which was programmed to the parameters for the temperature diminution.

When the cryovials reached -70°C, they were moved to the liquid nitrogen vapor phase of a storage tank.

Regarding CM13 and CM 14, they were employed for vitrification procedure. In particular, 200 µl of vitrification solution were added to each cell suspension sample. This procedure was performed in direct contact with liquid nitrogen. Special care was taken to ensure that the temperature of vitrified samples did not exceed -130°C to prevent recrystallization or devitrification. Sample cryovials were thawed 5 days after freezing. They were immediately immersed in water at 37°C and replaced with appropriate *medium*, with a substitution step for CM12. Thawed cell suspensions were diluted in culture *medium* and centrifuged (125 g, 10 minutes, 4°C) twice in order to eliminate cryoprotective agents. Finally, cells were seeded in 6-well plates and incubated in culture *medium* at 37°C in 5% CO_2. Cells cryopreserved with CM12 were seeded in culture *medium* containing 30 mM caspase inhibitor z-VAD-fmk and incubated at 37°C in 5% CO_2. Cell viability and death were evaluated after thawing each vial at different time points: 0 (T0), 24 (T24), and 48 (T48) hours after seeding, respectively. MSCs were counted with Cellometer Automated Cell Counters with Trypan Blue staining. Finally, data were analysed and the mean value for samples, cryopreserved in the same *medium*, was calculated. Differences among cryogenic media at three time points were checked by two-way ANOVA. The significance threshold was set at $p < 0.01$ (Prism 2.01, GraphPadSoftware).

3.8. Transformation/tumorigenic evaluation

In order to verify the potential transformation and tumorigenic evolution of ASCs during *in vitro* amplification, an *in vitro* (soft agar) test and *in vivo* (nude mice) inoculation were perform. In particular, ASCs at 4^{th}, 6^{th} and 10^{th} passages were included in the assays, furthermore a positive

(human larynx epidermal carcinoma, Hep2 at passage 446, Cod. BSTCL23) and a negative control (human fetal lung, MRC 5 passage 144, Cod. BSCL68) were considered.

3.8.1. In vitro transformation assay

The *in vitro* procedure was performed by seeding ASCs in solid agar *medium* (Macpherson and Montagnier 1964). Briefly, a layer of 1% agar noble (Becton Dickinson) was mixed with 50% of culture *medium* free of antibiotics and phenol red, supplemented with 20% (v/v) FBS and stratified in 6 wells of 35 mm diameter, plastic plates (3 ml/well). After solidification, this layer was overlaid with 0.8 ml of $6x10^5$ cell suspension diluted in a mixture composed of MEM enriched with 10% FBS and 0.6% noble agar. Plates were incubated first at room temperature to allow solidification and then at 37°C in 5% CO_2 for 3 weeks. Cell cultures were examined weekly to detect the appearance of transformed colonies.

3.8.2. In vivo tumorigenicity test

The *in vivo* test was performed on groups of 20-day-old male athymic mice (Nu/Nu genotype), which were received from Harlan Laboratories. The animal treatment was performed in accordance with local animal welfare guidelines. Groups of ten mice for each cell culture were used. They were sub-divided into groups of 5 mice/cage, maintained at the IZSLER Division of Laboratory Animal on sterile bedding and given water and feed *ad libitum*. Animals were injected subcutaneously into the abdominal wall with 0.2 ml of 10^7 cell suspension. After the injection, they were observed for 12 weeks. Finally, animals were sacrificed humanely (CO_2 inhalation), both in the event of tumour formation and of its absence.

A necropsy was performed on each mouse with the aim of detecting tumours at the injection site and in other organs and tissues (regional lymph nodes, lung, brain, spleen, kidney and liver). The local area of injection, together with all organs and tissues, was collected for histological examination, finally they were stained by haematoxylin-eosin (Sigma), as described in Ferrari et al. 2003, and examined by IZSLER Histology Laboratory.

The test was considered invalid if less than 9 of the 10 animals injected with the Hep2 cells, used as the positive control, show progressively growing tumours (according to European Pharmacopoeia Ed. 8 Ch.5).

3.8.3. Telomerase activity evaluation

The five samples of ASCs at 4^{th}, 6^{th} and 10^{th} passages were analysed with "Quantitative Telomerase Detection Kit™ (Allied Biotech, Inc.)", able to reveal by Real-Time PCR telomerase enzyme activity.

3.8.4. Telomere length evaluation

The same samples were tested by Southern Blotting technique at the Biotechnology Research Centre of University of Sacro Cuore (Cremona); in particular, TRF analysis was performed using TeloTAGGG Telomere Length Assay kit (Roche Diagnostics), following manufacturer's instructions.

The DNA was treated with restriction enzymes HinfI e RsaI, in order to permit chromosomes fragmentation in short segments; on the other hand, repeated sequences of chromosomal termination do not contain restriction sites recognized by these endonucleases.

Digested DNA samples were separated by electrophoresis, transferred on a membrane and hybridized with oligonucleotide probe, able to recognized repeated sequences typical of telomeres.

The probe signal was highlighted by a chemiluminescent substrate and autoradiography; thanks to this approach, it was possible to compare the revealed track of the sample with a known electrophoresis marker, in order to define the length of telomeric ends. In addition, a specific software called "Telometric" was used for an automatic analysis of the image (http://bioinformatics.fccc.edu/software/OpenSource/telometric/telometric.html).

When autoradiography sheet background prevented automatic analysis of the image, the operator compared the major intensity area of the signal with bands of known molecular weight markers.

3.8.5. P53 gene sequencing

The five ASCs samples at 4^{th}, 6^{th} and 10^{th} passages were tested for mutations of p53 gene.

Total RNA was extracted from samples using the automated extraction system QIAcube® (QIAGEN). In particular, the protocol RNeasy Mini Kit was performed following manufacturer's instructions.

The assay was performed considering equine P53 gene sequence reported in *NCBIGenBank*, in order to select the following specific primers encoding for p53 protein:

- horseP53f1 5'-TGGAGGAGACGCAGACAG-3'
- horseP53r1 5'-GTACAGTCAGAGCCAACCT-3'
- horseP53f2 5'-CCTCCTCAGCATCTCATCC-3'
- horseP53r2 5'-TTTTTATGGGAGGAGGTAGAC-3'

RT reaction developed in one cycle and the protocol is described in table 2, furthermore, concentration of reagents is described in table 3.

RT reaction	Temperature	Duration
Phase 1	42°C	60 minutes
Phase 2	95°C	5 minutes

Table 2. RT reaction.

Mix final volume was 17 µl and it was added with 3 µl of RNA of each sample.

Reagent	Initial concentration	Final concentration
$MgCl_2$	25 mM	5 mM
PCR 10X Buffer	10X	1X
dNTPs	2.5 mM	1 mM
RNase inhibitor	20 U/µl	1 U/µl
MuLV Reverse Transcriptase	50 U/µl	2.5 U/µl
Random Hexamers	50 µM	2.5 µM

Table 3. RT reaction reagents.

PCR reaction cycles are described in table 4.

RT reaction	Temperature	Duration	Cycles number
Initial denaturation	94°C	2 minutes	1
Denaturation	94°C	30 seconds	35
Annealing and elongation	63°C	1 minute	
Final elongation	72°C	10 minutes	1

Table 4. PCR reaction conditions.

Concentration of PCR reagents is represented in table 5.

Reagent	Initial concentration	Final concentration
Go Taq® Long PCR Master Mix	2X	1X
Sterile water	/	/
Primer forward	50 μM	3 μM
Primer reverse	50 μM	3 μM

Table 5. PCR reaction reagents.

Amplicons obtained by PCR reaction were purified using *QIAquick® PCR Purification Kit* (QIAGEN) employing the automated extraction system *QIAcube®*, in order to obtain 25 μl of purified samples.

Finally, purified amplicons were analysed for sequencing reaction by Biotechnology Laboratory of IZSLER. Reactions were performed using Prism BigDye® Terminator v1.1 Cycle Sequencing Kit, on the automated sequencer ABI 3130 DNA (Applied Biosystems).

3.9. Immunomodulation evaluation

In order to consider allogenic *in vivo* implantation of ASCs, their immunomodulation activity was verified.

Eight samples of equine peripheral whole blood were collected, in order to isolate PBMCs, necessary for evaluation of immunomodulatory properties of ASCs. In table 6 quantity of collected blood and PBMCs recovery are reported.

	Samples identification							
	ID1	**ID2**	**ID3**	**ID4**	**ID5**	**ID6**	**ID7**	**ID8**
Quantity of blood (ml)	200	220	190	450	490	425	400	325
Recovery of PBMCs ($x\ 10^7$)	1	2.5	1.2	20	25	9.9	15	17

Table 6. Samples of PBMCs, obtained from equine whole blood.

PBMCs and three samples of ASCs were transferred to CREM (Centro di Ricerche E. Menni,) laboratory of Poliambulanza Foundation Hospital (Brescia), where they were employed to evaluate ASCs immunomodulatory activity.

In particular, lymphocyte proliferation was induced by stimulating horse PBMCs ($2x10^5$ cells/well) in a 96-well, flat-bottomed plate (Corning), with PHA (Sigma-Aldrich), at a final concentration of 2 mg/ml in a final volume of 200 µl/well of complete culture *medium* (RPMI (Euroclone) +10% v/v FBS).

To study the effects of ASC on T lymphocyte proliferation two assays were performed. The first one considered co-culture of ASCs and PBMCs, whereas the second test was performed in transwell conditions.

To study immunomodulation property in a cell-cell contact setting, different concentrations of cells ($2x10^5$, $1x10^5$, $0.5x10^5$) were plated in complete culture *medium* and they were left to adhere overnight. The next day, ASCs were gamma irradiated (3,000 cGy), in order to ensure that any proliferation observed could be attributed completely to the proliferation of responder lymphocytes and $2x10^5$ activated PBMC were added to each well, in order to have PBMC:MSC ratios of 1:1, 1:0.5, 1:0.25.

Simultaneously, in order to study the effects of ASC on T lymphocyte proliferation in non-contact settings, transwell chambers with 0,4-mm pore size membranes (Corning, Milan, Italy) were used to physically separate the lymphocytes from the ASCs. Different amounts of cultured ASC ($2x10^5$, $1x10^5$, $0.5x10^5$) were plated in RPMI complete *medium* in the upper chambers of the transwell inserts. The next day, $2x10^5$ activated PBMC were added to each lower chamber in order to have PBMC:ASC ratios of 1:1, 1:0.5, 1:0.25.

Lymphocyte proliferation was assessed 3 days after culture by adding 0.67 mCi per well (96-well tissue culture plates) of [3H]-thymidine (INC Biomedicals) for 16-18 hours. Cells were then harvested with a Filtermate Harvester (PerkinElmer), and thymidine incorporation was measured using a microplate scintillation and luminescence counter (TopCountNXT; PerkinElmer). The proliferation value was expressed in cpm.

All cultures were carried out in triplicate and PBMC activated with PHA were used as controls.

Data obtained by the analysis of the three ASCs samples were used to calculate the average value and the standard deviation.

In order to evaluate significant differences between proliferation values of activated PBMCs (control) and PBMCs in presence of ASCs, T test student “unpaired” was performed. The statistical analysis was performed with by GraphPad v6 and the statistically significant threshold was set up as $p < 0.001$.

3.10. Platelet Lysate preparation

Whole venous blood of horses (200-250 ml), waiting for cell therapy treatment, was collected into a transfusion bag containing CPDA-1.

At the laboratory, blood was transferred under sterile conditions in 50 ml tubes that were centrifuged at 360 g for 10 minutes at 18°C, without brake. Plasma enriched with platelets was collected in other 50 ml tubes and it was centrifuged at 2200 g for 15 minutes at 18°C. Platelet pellet was collected and diluted in 5 ml plasma, cells were counted by the hematology analyser CELL-DYN 3500R (Abbott) and diluted in plasma at the final concentration of 10^9 platelets/ml.

The platelet suspension was cryopreserved for at least 12 hours at -80°C; subsequently the sample was rapidly thawed in water bath at 37°C, in order to permit platelets lyses and release of growth factors and anti-inflammatory molecules. The plasma enriched with platelet components was centrifuged at 2200 g for 15 minutes at 20°C. The pellets, composed by cell debris, was discarded and the suspension was centrifuged twice. Each sample has been controlled for mycoplasma, virological and bacterial contamination. In particular, mycoplasma infection was evaluated using the commercial MycoSensor PCR Assay Kit. Bacteria, fungi and yeast contaminations were investigated following inoculation of 2 ml of each suspension in microbiological media (Agar Sabouraud, Triptic Soy Agar and/or Brain Heart Infusion, incubated at 30°C and 37°C, respectively). In order to detect possible viral contamination, all samples were co-cultured with susceptible cell lines. They were incubated at 37°C in 5% CO_2 for 5 days, in order to observe cytopathic effects. These cell lines are stored in IZSLER Cell Culture Bank (www.ibvr.org). Equine *Herpesvirus*, *Arterivirus* and *Flavivirus* infections were detected following cell sample inoculation in EDe (Cod. BSCL40) and RK13 (Cod. BSCL74) cell cultures. Furthermore, *Flavivirus* and *Pestivirus* (no-cytopathic Bovine Viral Diarrhoea Virus), after cultivation in EDe cells, were evaluated by Realtime PCR, by by Virology Laboratory of IZSLER.

Finally, only PL samples free from any contamination were banked and subsequently used as diluent for allogenic ASCs ($2.5x10^6$ cells/ml) employed for cell therapy purposes.

3.11. Cell sample preparation for implantation

In order to treat horses, equine ASCs, cryopreserved at IZSLER biobank, were rapidly thawed at 37°C in water bath; cell suspension was diluted in 9 ml of PBS and centrifuged at 360 g for 10 minutes at 18°C. Pellets was washed twice more and finally cells were counted with the automated cell counter after Trypan Blue staining. Cells were diluted in autologous PL to a volume related to lesion size (1 ml/cm^2). Cell suspension was delivered to the practitioner and the sample was stored at 4°C until use up to 12 hours.

3.12. Clinical cases

The clinical study was performed in collaboration with a veterinary practitioner, who was responsible of evaluation of lesions, ultrasound examination, animal behavior observation and finally clinical therapy.

Eighteen, from 3 to 13-year old, male or female, race horses, affected by tendon or ligament lesions, were included in the clinical case. Twelve horses were affected by superficial tendonitis, 7 on the frontal right, 4 on the frontal left and 1 on the left hind limb. Furthermore, 6 animals showed desmitis, 3 of the frontal right, 1 of the frontal left and in 2 cases the lesion of the suspensory ligament affected the hind left limb. In particular, tendons involved in the injury were SDFT and in one case the DDFT; otherwise, the desmitis affected the fetlock suspensory ligament (FLSL).

All the animals showed oedema in the metacarpal and fetlock area, but no lameness. Patients were subjected to clinical observation (orthopedic inspection) and ultrasound evaluation. Prior to treat animals, lesions were assessed by clinical and ultrasound examination. Only animals with lesions in the range of 15–30% of the transversal tendon surface and ligaments were included in the clinical case. All horses were found to contain a hot and painful swelling of the structure in question, which occurred more than 48 hours before the clinic visit, not associated with lameness. All lesions were of acute type and the subjects, starting from the time of the lesion diagnoses, were withdrawn from training and left to rest in a box until the time of transfer to rehabilitation facilities. The treatment was performed around 20 days after the traumatic event.

Patients were not treated with any drug medication (anti-inflammatory) for the duration of the therapy, but they were treated with cold showers.

Horses were divided in two groups: group 1 represented by 12 animals treated with allogenic ASC, group 2 represented by 6 control animals, which received traditional therapy (pin firing). Horses, belonging to both groups, were characterized by the same lesions, the rehabilitation program was set up in the same conditions and for the same period. Finally, ultrasonographic examinations were performed on all animals at fixed intervals and by the same operator.

3.13. Rehabilitation program

After treatment, animal rested in its stables for 7 days. From the second week, horse was submitted to a controlled workload: walking in hand twice a day, the duration increased gradually from 10 to 40 minutes till the 16^{th} week; later on, 5 minutes of trotting was added to walking and duration increased every week until 30 minutes at the 25^{th} week. During each ultrasound control, the rehabilitative protocol was re-evaluated in accordance with the evolution of the repair process.

Depending on the severity of the initial injury and its evolution after treatment, horse gradually resumed training for competitions in about 6 months.

3.14. Adipose-derived stromal cell implantation

Group 1 included 12 horses, from 5 to 13-year old, 7 animals were male and the other 5 were female. In particular, the involved limb was in 3 cases the hind left, in 3 animals the fore left, and in 6 cases the fore right limb. Seven animals were affected by SDFT lesions, in one case the involved structure was the DDFT and finally, 4 animals were affected by lesions of the FLSL.

All animals belonging to this group were considered for cell therapy approach.

All material used in this phase was disposable and sterile (needles, gloves, syringes). Horse was sedated with detomidine chlorohydrate 10 μg/kg (Detogesic, Fort Dodge Animal Health S.p.A.) and xylazine 0.3 μg/kg (Megaxilor 20%, Bio98 S.r.l.) by the i.v. route, the site of the lesion was shaved and subsequently disinfected with povidone-iodine (Betadine, Meda Pharma S.p.A.) and denatured alcohol.

Allogenic ASCs implantation into the core lesion was carried out under ultrasound guidance, in order to verify the correct position of the *inoculum*, using a 22 gauge needle. Finally, the area was disinfected and sterile bandages were applied. After *inoculum* the horse was examined by an ultrasound technique using a 7.5/10 MHz linear T-shape probe at 4, 8, 12, 16 and 24 weeks after ASCs implantation. During this period, animals were not treated with any other drug (anti-inflammatory).

Furthermore, clinical evaluation of the ASCs administration was evaluated: onset of acute or chronic clinical adverse reactions in the short or long time was delved. Tissue swelling, temperature raising and lameness at walk were considered parameters; finally, development of abnormal tissue was evaluated by either ultrasonography or palpation until the end of the follow up period (24 weeks).

3.15. Control group

Six horses were randomly allocated to the control group (group 2). They were 3 male and 3 female and their age ranged from 3 to 8 year-old. The selected animals had the same lesions of the ASCs treated group. In particular, 4 animals were affected by tendonitis and 2 by desmitis. In this case, it was decided to operate using a "classical" therapy: pin firing. For treatment, animals were sedated and the site of the lesion was tricotomised and disinfected, as above mentioned. Perineural anesthesia of the medial and lateral palmar metacarpal nerves was performed by lidocaine 2% 3 ml/area (Fort Dodge Animal Health S.p.A.). Subsequently, a pin firing treatment was set up, as following described: a thermocautery was applied on the metacarpal flexor area for few seconds,

the area was treated with proximodistal direction and a distance of 2 cm among each application was kept. No anti-inflammatory drug was given to the animals of control group.

Animals were checked by ultrasound examination, in order to verify treatment efficacy, at the same time intervals (4, 8, 12, 16 and 24 weeks after pin firing) as the animals treated with ASCs.

4. RESULTS

4.1. Sample collection and mesenchymal stromal cell isolation

A total of 30 adipose tissue samples were collected. In particular, about 50 grams of tissue were isolated from abdominal fat deposit; the age of the animals ranged between 1 and 20 years and finally, the mean number of obtained cells was about 1.5 x 10^7 cells/sample (Table 7).

	Grams	**Age**	**N° of cells (x10^7)**
1	55	14	1.2
2	49	13	1.0
3	50	15	1.3
4	51	20	1.2
5	49	16	1.8
6	47	11	1.3
7	47	4	1.8
8	54	1	1.0
9	52	4	1.3
10	56	12	1.6
11	52	15	1.5
12	49	7	1.7
13	51	14	1.2
14	49	9	1.4
15	55	4	1.8
16	54	12	2.0

	Grams	Age	N° of cells (x10^7)
17	49	9	1.6
18	49	8	1.0
19	47	16	1.6
20	55	20	1.8
21	51	14	1.6
21	42	9	1.4
22	42	9	1.4
23	48	6	1.8
24	51	10	1.8
25	49	7	1.4
26	47	8	1.2
27	46	15	1.0
28	55	18	2.0
29	52	14	1.8
30	49	6	1.3

Table 7. Adipose tissue samples (grams of isolated tissue, age of animals and number of collected cells).

4.2. Cell amplification

ASCs began to adhere to the surface of the flask 24 hours after seeding. Following replication, cells showed a typical fibroblastic-like shape 48 hours after seeding and reached sub-confluence in about 10 days (figure 8). A maximum of 3 serial passages was performed, in order to prevent any specific tissue cell differentiation. At passage 3, about 40x10^6 cells were obtained.

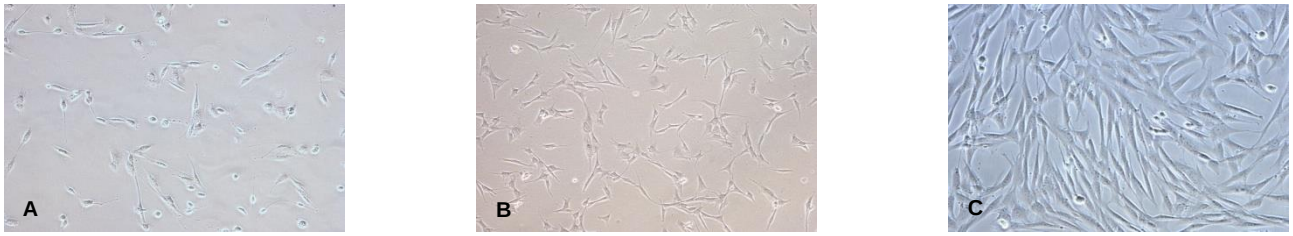


Figure 8. MSCs isolated from adipose tissue (100x): A) 5 days after seeding. B) 8 days after seeding. C) 10 days after seeding, subconfluence.

4.3. Quality controls

Only one ASC batch was found to be contaminated by bacteria and mycoplasma. No viral infection was detected. The contaminated cell batch was discarded and only those free from any contamination were used for analyses or banked for clinical therapy.

4.4. Evaluation of self-renewal ability of cells

Self renewal ability of multipotent elements was verified by CFU assay. Individual colonies were generated when ASCs were plated and they were scored macroscopically, showing a 1–5 mm diameter. Aggregates of more than 20 stained cells were considered as CFUs and counted. In particular, 33, 12 and 5 colonies were obtained after seeding of $2x10^5$, $1x10^5$, $0.5x10^5$ ASCs (figure 9), respectively, indicating cells with *in vitro* self-renewing capacity.

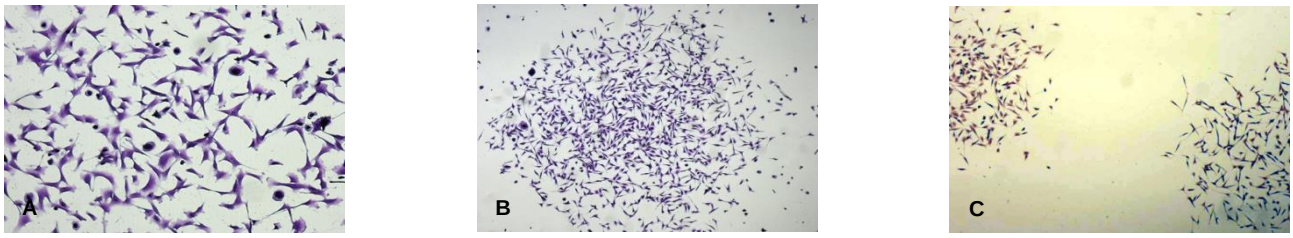


Figure 9. ASCs colony forming unit: A) 100x. B) 40x. C) 4x.

4.5. Evaluation of multipotential ability of adipose-derived stromal cells

4.5.1. Multilineage differentiation

In order to determine whether MSCs are able to differentiate into multiple cell types, they were placed in specific induction *media*. All analysed samples at 3rd passage and at the other 3 considered serial passages (4th, 6th and 10th), showed the ability to differentiate into the 3 mesodermal lineages (adipogenic, osteogenic and chondrogenic lineages). Results demonstrated that all cell batches incubated in NH Adipodiff *medium* acquired an adipocytic phenotype; in fact, 2-3 weeks after seeding large vacuoles start to appear, as detected by the presence of red lipid droplets in the cell cytoplasma (Oil Red O staining. figure 10).

Von Kossa staining, a marker for the osteocytic phenotype, evidenced calcium formation in cells, treated with NH Osteodiff *medium*. Cells acquired an osteoblastic morphology with the deposition of calcium rich mineralized extracellular matrix (figure 11).

Chondrogenic differentiation ability was demonstrated by the development of nodules in all cell cultures. The whole nodule and a pellet section of each batch was stained with Alcian Blue to highlight the differentiation into a chondrogenic lineage, witnessed by the presence of chartilage matrix (figure 12). Finally, untreated cells, used as control, did not present any reaction.

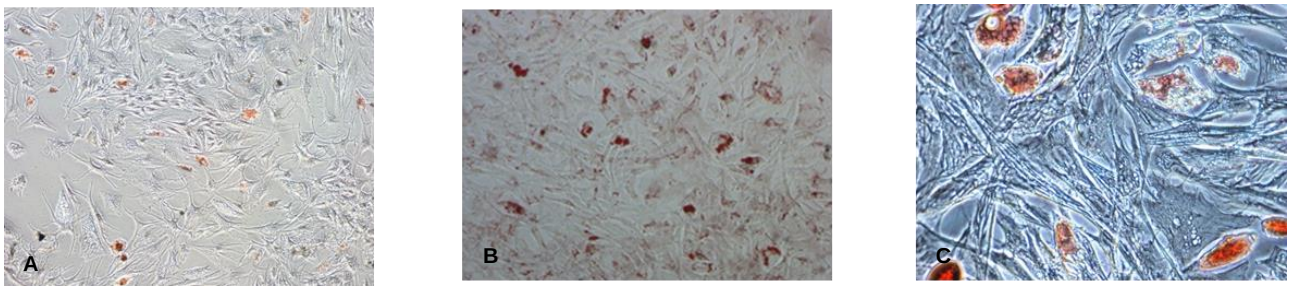


Figure 10. Oil Red O staining, ASCs differentiated into adipocytes: A) 100x. B) 100x. C) 200x.

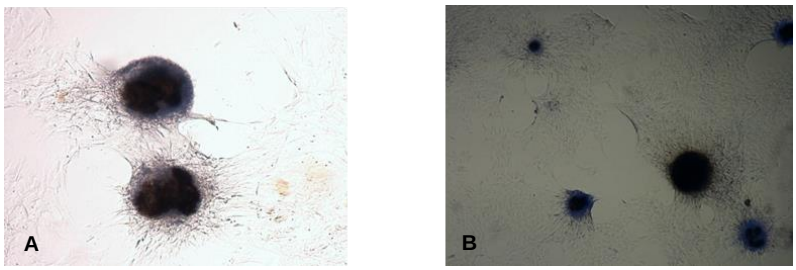


Figure 11. Von Kossa staining, ASCs differentiated into osteocytes: A) 100x. B) 40x.

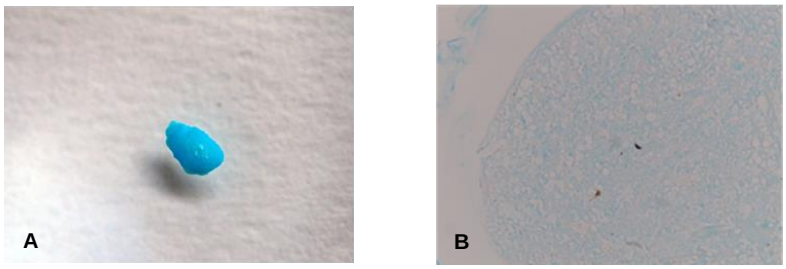
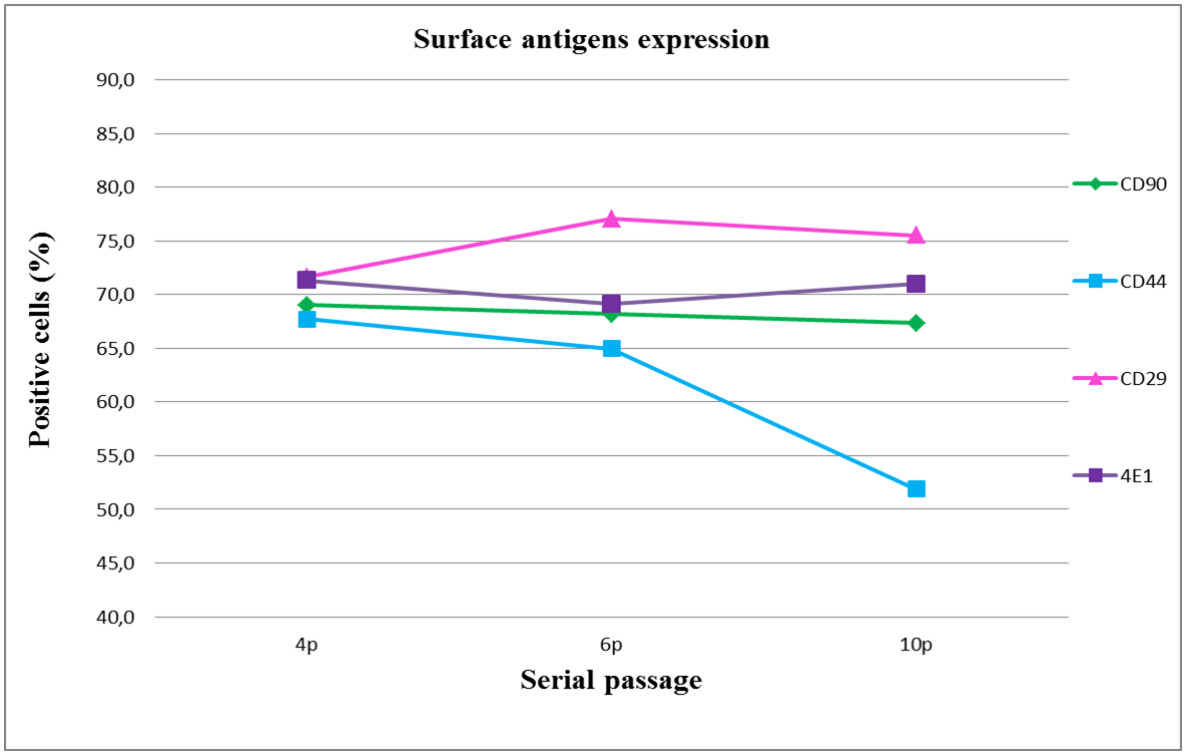


Figure 12. Alcian blue staining, ASCs differentiated into chondrocytes: A) 3D nodule formation. B) pellet section 100x.

4.5.2. Specific surface markers expression

Flow cytometry was performed in order to evaluate the expression of specific markers underlying the multipotential ability of ASCs during amplification (at 4^{th}, 6^{th} and 10^{th} passages).

Data obtained by flow cytometry analysis were normalized considering 10.000 events. All the analysed markers were expressed in all the samples. In particular, 4E1 and CD90 showed a stable level of expression; in fact, the data did not show significant differences among passages. On the contrary, CD44 expression decreased from passage 4 to 10, with a significant difference at the 10^{th} passage, only. CD29 expression initially seems to increase (passage 6^{th}), but at passage 10^{th} it decreases, as other surface antigens (graph 1).



Graph 1. Expression of CD90, CD44, CD29 and 4E1 surface antigens in ASCs at 4th, 6th and 10th serial passages.

4.6. Evaluation of cryopreservation solution and procedure.

Viability of cells, frozen in *media* either containing low concentrations or completely deprived of DMSO (CM2, CM3, CM5, CM6, and CM7), appeared unsatisfactory. In fact, cell survival and replication capacity decreased as soon as 24 hours post-thaw.

ASCs frozen with CM1 (culture *medium* with 20% (v/v) FBS and 10% (v/v) DMSO), CM4 (CS10, with 10% (v/v) DMSO), and CM8 (*medium* containing 5% (v/v) DMSO, 20% (v/v) FBS, and 500 mM trehalose), showed a high percentage of viability and replication capacity.

The overall results indicated that the best CPAs were CM1 and CM4, both containing 10% (v/v) DMSO. Cells frozen in CM1 were compared to those frozen in CM9, CM10, CM11, CM12, CM13, CM14, and CM15. ASCs showed better viability with CM1, CM9, and CM11 than CM10 ($p < 0.0001$) on T0. This parameter decreased after thawing, until a complete loss of viability at T48. When ASCs, cryopreserved with caspase inhibitor z-VAD-fmk (CM12), were thawed they showed 98% viability, and 48 hours later it decreased to 76% ($p < 0.0001$). The vitrification method appeared completely unable to preserve viability of cells. Finally, samples cryopreserved with FBS added with 10% (v/v) DMSO (CM15) reached confluence at T48 (table 8).

	Cell Viability at Thawing	Cell Viability after Thawing	
Cryogenic *Medium*	**T0**	**T24**	**T48**
CM1	98±0.77%	90±0.63%	100±0%
CM4	80±6.4%	82±5.9%	85±4%
CM8	70±2.6%	73±3.3%	75±3.4%
CM9	89±2.21%	45±2.93%	0%
CM10	78±3.15%	38±5.18%	0%
CM11	95±5.61%	80±6.39%	0%
CM12	98±0.2%	79±0.54%	76±0.68%
CM13	72±3.07%	0%	0%
CM14	58±3.35%	0%	0%
CM15	98±0.44%	90±0.7%	100±0.13%

Table 8. Percentage of cell viability after freezing with 15 cryostore solutions at different time points.

4.7. Transformation/tumorigenic evaluation

Amplified ASCs were inoculated *in vitro* in a semi solid *medium* and *in vivo* in athymic nude mice, in order to evaluate transforming/tumorigenic potentialities of cells at 4^{th}, 6^{th} and 10^{th} passages.

4.7.1. In vitro transformation assay

ASCs were not able to induce *in vitro* transformed colonies development (figure 13). No difference was observed among the considered passages tested: 4^{th} (figure 13A), 6^{th} (figure 13B) and 10^{th} (figure 13C).

This behavior was similar to that observed for MRC 5 negative control, in which single cells remained unaltered until the end of the observation period (figure 14A). In contrast, Hep2 cell line induced transformed colonies in semi-solid *medium*. These colonies began to appear 7 days after seeding and then increased gradually in number and size (figure 14B).

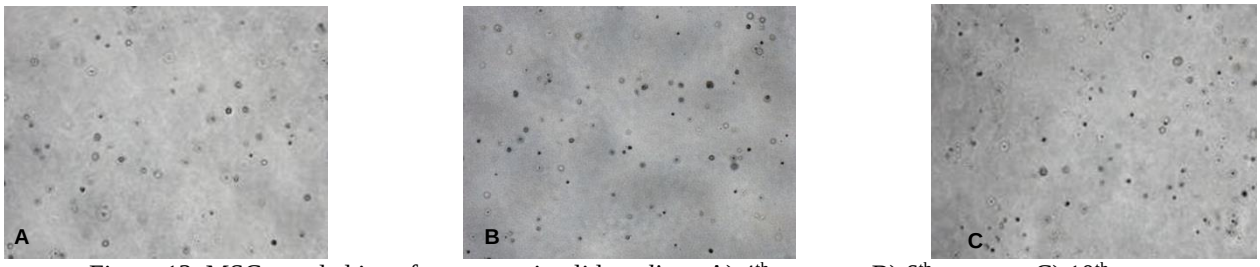


Figure 13. MSCs seeded in soft agar, semi-solid *medium*. A) 4th passage. B) 6th passage. C) 10th passage.

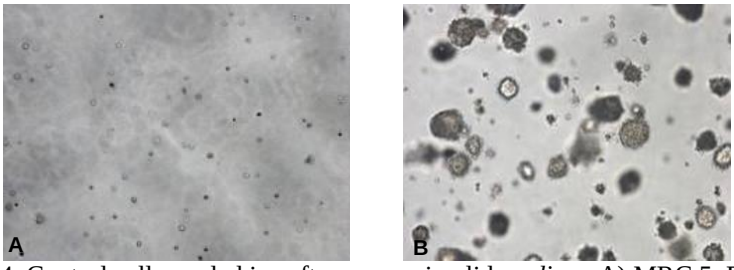


Figure 14. Control cells seeded in soft agar, semi-solid *medium*. A) MRC 5. B) Hep2.

4.7.2. In vivo tumorigenicity test

The same passages of ASCs used for the *in vitro* assay and inoculated into nude mice did not induce any tumor formation during the observation period. This trend was also observed in the mouse group that was injected with MRC 5 cells, used as negative control. The animals did not show any macroscopic lesion, no inflammatory process was observed and the *inoculum* was re-absorbed completely within a few days (an average of seven days).

In contrast, tumors were detected in all mice receiving Hep2 cells. In particular, nodules were observed at the inoculation site about 10 days after the injection (figure 15). They appeared smooth, uniform and lobular (10 mm Ø); later, they developed a multi-lobular shape and increased in size (20 mm Ø). At about 30 days after the injection, the mice were humanely sacrificed to avoid animal pain and suffering.

In the animals inoculated with ASCs at different passages and the animals inoculated with the MRC 5 negative control lineage, the necropsy detected no tumor formation at the site of inoculation or in other organs and tissues. These observations were confirmed by histological examination (figure 16A, 16B).

In contrast, tumors were detected at the site of inoculation of mice injected with Hep2 cells, but no other macroscopic alterations in organs and tissues were detected. The histological examination of the mice injected with Hep2 cells showed the presence of polygonal cells in subcutaneous and dermal tissues; the *nuclei* of such cells were irregular in shape, with evident nucleoli (atypical mitosis); moreover, neoplastic cells were observed in the vessels (figure 16C). These alterations were restricted to the *inoculum* site in all the animals and metastases were not found.

Figure 15. *In vivo* neoformation induced by Hep2 inoculation.

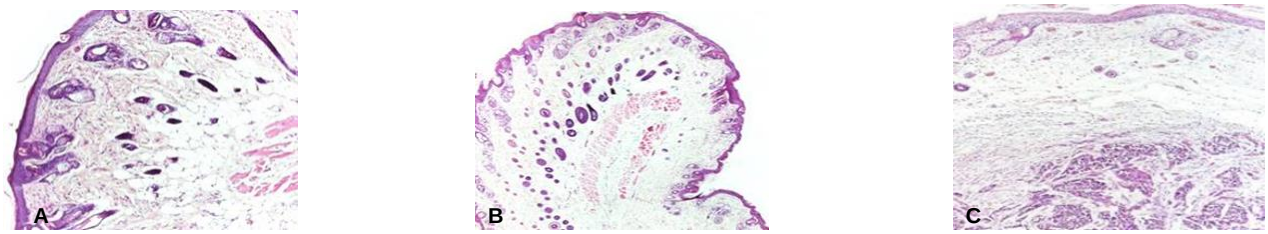


Figure 16. Control cells inoculated in athymic nude mice, histological exam of *inoculum* site. A) MSCs. B) MRC 5 (negative control). C) Hep2 (positive control).

4.7.3. Telomerase activity evaluation

Results obtained during analysis of telomerase activity using "Quantitative Telomerase Detection Kit™", were adapted for protein content (3 mg/ml). Nevertheless, samples did not demonstrated any homogeneous trend during expansion process.

Telomerase activity is expressed by Ct values that are not reproducible. During *in vitro* expansion, activity of the enzyme in some sample increased and in others decreased, as described in table 9.

Sample ID	**Serial passages**		
	4th	**6th**	**10th**
11	25.22	23.94	23.55
12	25.44	23.99	26.54
13	23.46	26.07	25.41
14	24.67	25.72	26.54
15	24.60	24.01	27.21

Table 9. Telomerase activity (expressed as Ct) among ASCs serial passages.

4.7.4. Telomere length evaluation

Southern Blotting tests carried out on the five (ID11-ID15) samples of ASCs at the 3 selected serial passages showed a variability of telomeres length. This trend is not reproducible among the five samples (table 10).

Sample ID	Serial passages		
	4th	**6th**	**10th**
11	15.63	16.14	10.87
12	9.29	8.12	7.36
13	8.24	7.75	7.48
14	15.22	15.84	15.06
15	10.69	9.33	8.71

Table 10. Telomeres length of equine MSCs (in Kbp).

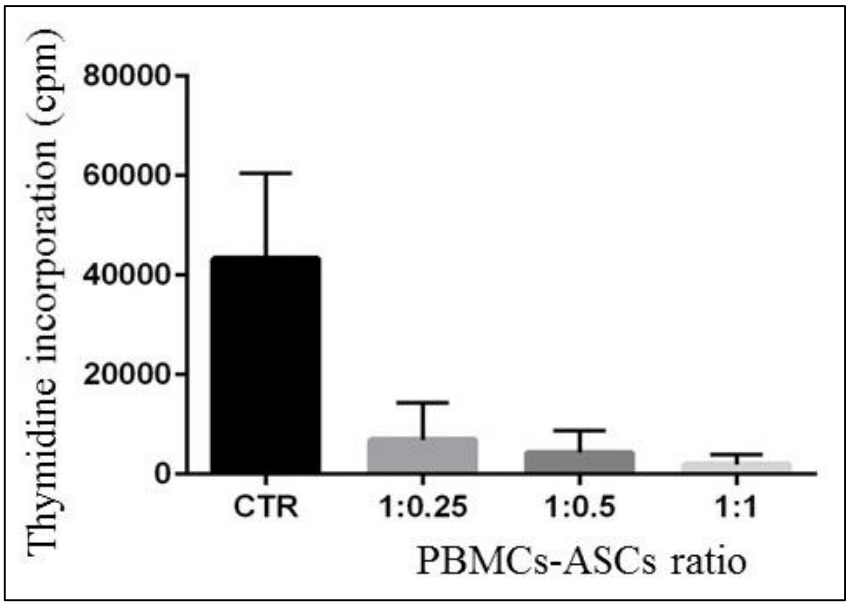
4.7.5. P53 gene sequencing

P53 gene encoding for P53 protein, has been sequenced for all considered samples. Sequences have been assembled, aligned and compared using *MegAlign*® software (10.1 version). Comparison of the same sample at the three considered serial passages (4^{th}, 6^{th}, 10^{th}) did not demonstrated presence of mutations in the gene sequence of p53 protein. This situation was observed for the five ASCs samples amplified *in vitro* (ID11-ID15).

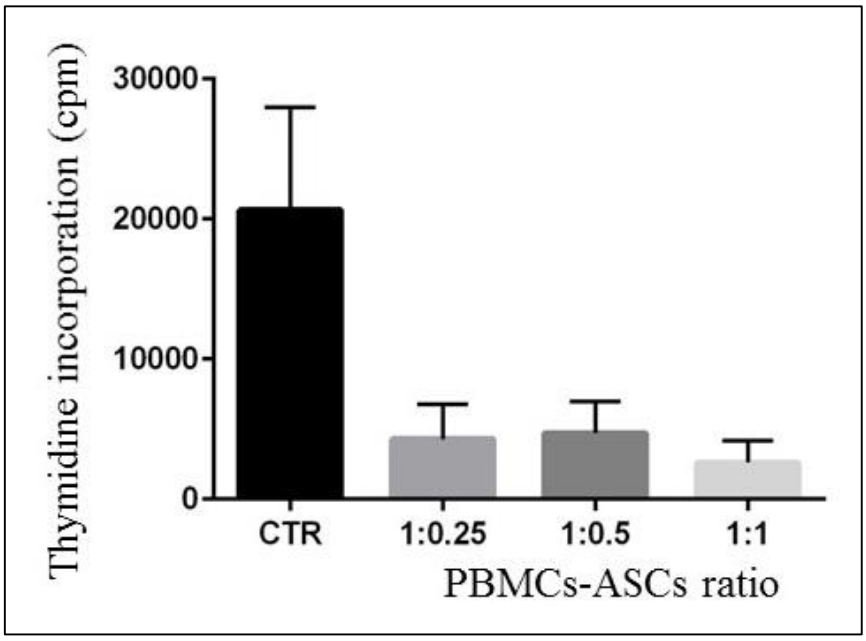
4.8. Adipose-derived stromal cell immunomodulation evaluation

Immunomodulatory property of ASCs was evaluated considering proliferation of PBMC in presence of decreasing concentration of ASCs. In order to test this feature, two assays were carried out, in particular during the first method ASCs were co-cultured with PBMCs and in the second assay, their interaction was evaluated in a transwell system.

In both cases, it was verified that ASCs are able to inhibit PBMC proliferation *in vitro*. In fact, PBMCs activity is modulated either when the two populations are cultivated in direct contact, or when they are in separated conditions by transwell system. The higher anti-proliferative effect is observed when ASCs and PBMCs ratio is 1:1, this situation is reproducible either in co-culture conditions (graph 2) or in transwell system (graph 3).



Graph 2. Proliferation of PBMCs when co-cultured with ASCs. Cpm = counts per minute. CTR = PBMCs activated with PHA.



Graph 3. Proliferation of PBMCs when cultivated with ASCs in transwell system. Cpm = counts per minute. CTR = PBMCs activated with PHA.

4.9. Platelet Lysate preparation

PL was prepared from 200-250 ml whole blood, the manipulation process permitted to obtain about 25-30 ml of the 10^9 platelet suspension.

The lysate solution washed three times by centrifugation procedure, demonstrated a turbid, light-yellow liquid aspect.

4.10. Adipose-derived stromal cell preparation for implantation

Equine ASCs, cryopreserved at IZSLER biobank, were rapidly thawed at 37°C in water bath, counted cells showed a viability of 98% $\pm$ 0,44%.

The obtained cell suspension was diluted in autologous PL to a volume related to lesion size ($2.5x10^6$ cells/ml/cm^2). The cell suspension demonstrated absence of flocks, cells resulted homogenously distributed and the aspect of the solution was clear.

4.11. Clinical outcome after adipose-derived stromal cell treatment

Implantation of ASCs, resuspended in PL, did not induce any deleterious effect on the treated tissue, as reported by the practitioner. ASCs did not hasten any acute, clinically valuable tendon reaction, and no signs of lameness, local swelling heat or pain on palpation were reported after cell administration. Furthermore, nor lameness nor other adverse events were clinically detected during follow up. Ultrasound examination of the tendon (figure 10) did not give evidence of abnormal tissue within or around the implantation site in the long term. Furthermore, ultrasonographic exams were performed at different intervals: 4, 8, 12, 16 and 24 weeks after treatment. The evaluation of ultrasound images permitted to have an indication on development of tendon/ligament abnormalities. In fact, this technique allow appreciating structural orientation of repaired fibres and absence of scar tissue.

In particular, 4 weeks after implantation, tendon appeared homogeneous in both transversal and longitudinal section and fibres began their lining up.

Furthermore, 8–16 weeks after implantation, in longitudinal section fibres were progressively and correctly directed. Finally, 24 weeks after implantation tendon appeared anatomically restored, in fact, fibres demonstrated a correct orientation, and this tissue improvement was also witnessed by capacity of all animals to resume their training activity (table 11).

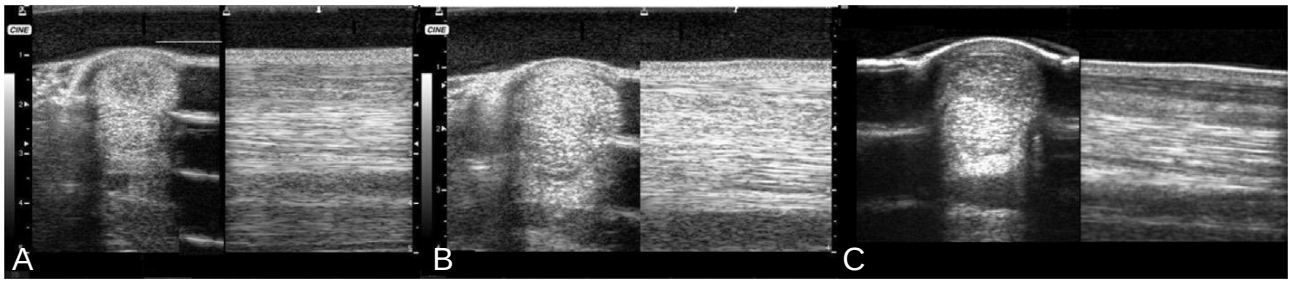


Figure 10. Ultrasound image of an injured tendon treated with ASCs, trasversal and longitudinal sections: (A) Lesion of frontal left limb SDFT. (B) 4 weeks after implantation fibres begin to align. (C) 24 weeks after implantation there is a completer alignment of the fibres.

ID	Gender	Age (years)	Site lesion	Limb	Outcome
H1	M	5	SDFT	HL	Training
H2	M	8	SDFT	FL	Training
H3	M	7	SDFT	FR	Training
H4	M	8	SDFT	FL	Training
H5	F	10	SDFT	FR	Training
H6	F	5	SDFT	FR	Training
H7	M	13	DDFT	FR	Training
H8	M	11	SDFT	FL	Training
H9	F	6	FLSL	HL	Training
H10	F	11	FLSL	FR	Training
H11	M	5	FLSL	FR	Training
H12	F	9	FLSL	HL	Training

Table 11. Animals of group 1 treated with adipose mesenchymal stromal cells (ASCs). H: horse; M: male; F: female; FL: frontal left; FR: frontal right; HL: hind left.

4.12. Clinical outcome after pin firing procedure

Animals were checked by ultrasound examination, in order to consider the same parameters evaluated in group of animals treated with ASCS. In particular, ultrasound images, performed at the same time intervals as the animals treated with ASCs (4, 8, 12, 16 and 24 weeks after pin firing), were evaluated by practitioner for fibres orientation and absence of scar tissue development. These aspects gave information about treatment efficacy. Unfortunately, due to the scar presence, it was difficult to observe the real tissue restore and improvement. In transversal section, an anechogenic area was observed in the middle part of the tissue and hyperechogenic areas in longitudinal section, due to scar development (figure 11B). Furthermore, there was no fibre alignment pattern, as suggested by the veterinary practitioner; this aspect could witness the absence of an improvement in tendon structure.

Six months after pin firing treatment, 2 horses (33.3%) were still in training and they seem able to return to their previous activity; while in the remaining 4 horses (66.6%), the persistence of lameness and local swelling suggested an incomplete healing of the injured tissue, this was confirmed by ultrasound image evaluation (figure 11).

This feature made the return of the animals to their sport activity impossible (table 12).

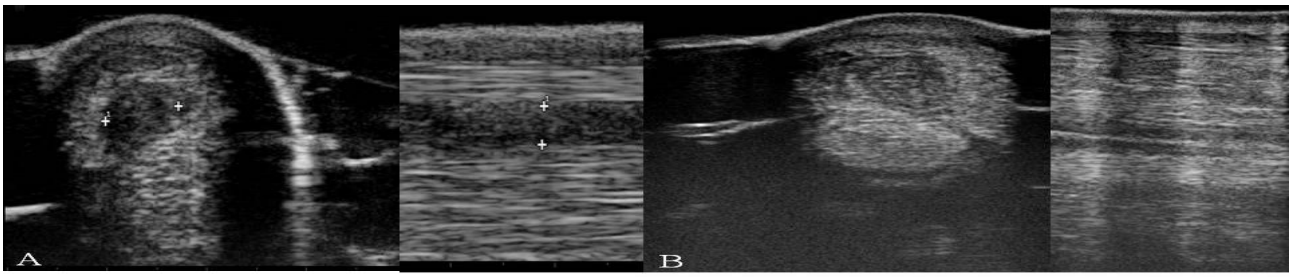


Figure 11. Ultrasound image of an injured tendon treated with pin firing, transversal and longitudinal sections: (A) Lesion of frontal right limb SDFT. (B) 24 weeks after treatment, scar presence and disorganization of fibres was noticed in both sections.

ID	Gender	Age (years)	Site lesion	Limb	Outcome
H13	F	6	SDFT	FR	Retired
H14	F	3	SDFT	FR	Retired
H15	M	5	FLSL	FL	Retired
H16	F	4	SDFT	FL	Retired
H17	M	4	SDFT	FR	Training
H18	M	8	FLSL	FR	Training

Table 12. Animals of group 2 treated with pin firing. H: horse; M: male; F: female; FL: frontal left; FR: frontal right.

5. CONCLUSIONS

MSCs are considered the survival kit of the organism; in fact, they are responsible either for reparation of damaged tissues or for renovation of health structures. The MSCs field in veterinary medicine continues to evolve rapidly as demonstrated by numerous applications reported in literature (Smith et al. 2013) and in addition, animals could serve as models for preclinical evaluation of their applications in human beings. The most commonly used sources of MSCs in the veterinary clinical setting are bone marrow and adipose tissue. However, the sternal bone marrow collection technique commonly used exposes animals to potential complications like pneumothorax and pneumopericardium (Ackerman and Alden 1958; Durando et al. 2006).

The significantly greater number of available primary cells makes the MSCs isolated from adipose tissue an interesting prospect or achieving the large cell numbers deemed necessary for tissue engineering applications (Muschler and Midura 2002). Nowadays, practical approach in veterinary field focuses on equine (Radtke et al. 2015) and canine species (Zeira et al. 2015). Cells were employed in musculoskeletal disorders, wound repair and, as an adjunct, therapy for spinal cord disease.

The traditional approaches (surgery and no invasive techniques), commonly used over the years, in order to heal traumatic lesions, have not been completely effective and often are unable to prevent reinjures, with negative consequences on the animal health. Because the improvement in regenerative medicine techniques, MSCs have gained great interest in reparation of musculoskeletal disorders, thanks to their own features. In particular, these cells are used for treatment of tendon and ligament disorders in equine species; in fact, sport horses are often involved in damaged of these structures.

The clinical treatments in which autologous MSCs can be used are limited because patient-specific tissue collection and cell expansion require time. For clinical applications in which MSCs should be used right away, it would be more practical to use cells collected from a donor, expanded *in vitro* and banked to be readily available when needed (Al-Daccak and Charron 2015).

This approach seems to be possible thanks to the immunomodulatory properties demonstrated by MSCs. In fact, literature suggests these cells as able to regulate immune cells proliferation, differentiation and phenotype in different animal species, including equine species (Salem and Thiemermann 2010). For this reason, during this project the attention was focused on the possibility to employ allogenic MSCs for tendon and ligament reparation in equine species.

In particular, MSCs were collected from equine adipose tissue immediately after slaughtering, the tissue samples were transferred to the laboratory where they were manipulated in order to isolate

ASCs. Initially, several *in vitro* methodologies (isolation, amplification, characterization) were set up and typical features of ASCs were investigated.

All the considered techniques have been well standardized and they have demonstrated their feasibility during the routinely approach. All the defined protocols permitted to limit cell contaminations, enabling the success of the project.

All cell samples showed their capacity to grow *in vitro*, giving rise to clones, and to differentiate into three mesodermal lineages (adipogenic, osteogenic and chondrogenic). These two features demonstrated *in vitro* self-renewal and multipotential ability of these cells, witnessing ASCs as promising tool for regenerative medicine.

Furthermore, in order to create an ASCs biobank, it was performed a study considering several cryostore solutions, this strategy permitted to identify a cryogenic *medium* able to increase viability of banked cells. The results of this test set forth the importance of DMSO, although it has cytotoxic properties, in fact only solutions containing 10% DMSO (cultural *medium* + 20% FBS or only FBS) guarantee high percentage of cell viability and achievement of confluence already 48 hours after thawing.

Regarding cell amplification, it was important to verify that this process do not compromise MSCs features. For this reason, ASCs were amplified *in vitro* until the 10^{th} passage and they were analysed, in order to evaluate several parameters. First of all, capacity of cell samples to reach 10^{th} passage was tested and all samples satisfied this parameter, which is an indicator of absence of senescence evolution.

Safety of cells was tested considering others parameters; in particular, cells inoculated *in vivo* in nude mice and *in vitro* in soft agar *medium* did not give rise neither to neoformation nor to *foci*, respectively. As expected, these methods demonstrated that cells did not induce tumour development *in vivo* or the acquisition of transforming feature *in vitro*.

Furthermore, the outcomes obtained by differentiation into adiopogenic, osteogenic and chondrogenic lineage and flow cytometry demonstrating ASCs expression of specific mesenchymal markers (CD90, CD44, CD29 and 4E1) until 10^{th} passage, suggested the maintenance of multipotentiality.

During following steps of the project, other features have been investigated, in order to understand if ASCs, during long lasting amplification process, could undergo the immortalization process. This aspect has been delved by evaluation of telomerase activity, telomeric length and mutation of p53 gene, that could give information also regarding potential transformation evolution. Results obtained by these methodologies were not reproducible among cell samples, this behavior could be related to individual features of the subject from which cells have been collected. Because of the

different trends demonstrated by samples during the tests, it was decided to focus the attention on samples until the 3rd passage, in order to avoid use of compromised cells for the *in vivo* application.
Moreover, in order to permit allogenic cell therapy, *in vitro* capacity of ASCs to inhibit lymphocyte T proliferation has been investigated. The test has provided *data* regarding capacity of ASCs to inhibit stimulated PBMCs proliferation *in vitro*, when co-cultured in contact with ASCs or in a transwell system.
This immunomodulatory capacity of ASCs has been considered an indicator for the possibility to employ allogenic ASCs for cell therapy purposes, without development of immune reaction in host. This aspect and *data* obtained by the other *in vitro* studies, permitted to consider allogenic ASCs for cell therapy in an experimental trial, as the initial goal intended.
At this regard, a group of 18 horses affected by tendonitis or desmitis was included in a field clinical case. They were subdivided in two groups, in order to compare animals treated with allogenic ASCs associated with platelet lysate (12 horses) with patients subjected to pin firing (6 animals), one of the most popular methods used in equine practice. Clinical controls, rehabilitation program and follow up were the same for both groups, this approach permitted to compare outcome of the two therapies. In particular, evolution of tendon reparation was verified by ultrasonography that provided information about the quality of repaired tissue. This aspect was improved by observation of horse behavior during rehabilitation and follow up; in addition, clinical signs gave indications about health status of the animal.
Ultrasound images performed on animals treated with ASCs showed absence of scar tissue development and correct parallel orientation of the fibers, already 4 weeks after cell administration, suggesting initial tendon/ligament tissue healing.
Regarding pin firing treatment, used in animals of group two, it seems to indicate the poorness of this therapy; in fact, presence of scar limited ultrasound observation of the involved structure, furthermore, no improvement of the clinical outcome was observed and most of animals (66.6%) were unable to return to racing.
Unfortunately, the reduced number of considered animals did not permit to include the statistical analysis in the data elaboration. In regards to this aspect, it will be useful to improve these data considering a major number of subjects, in order to test statistical differences between the two treatments.
Irrespective of quality of repaired tissue, implantation of allogenic cells seems to be promising in terms of safety; in fact, no adverse reaction were set forth in the recipient host, neither immediately after administration nor during observation period. In fact, no clinical sign of acute inflammation were highlighted treated limb did not showed redness, increased heat, swelling, pain or loss of

function. This outcome, together with *in vitro* immunomodulatory results demonstrated ASCs capacity to trigger the cytokine loop and the consequent anti-inflammatory response, promoting use of MSCs not only for autologous but also for allogenic treatment (Wang et al. 2014).

This preliminary study indicates that allogenic ASCs are potentially safe and together with platelet lysate seems to be able to support treated tissue restoration and to enhance functional recovery in equine traumatic injuries of tendon and ligament.

Unfortunately, this research could not suggest if the reparation process is induced by platelet lysate or ASCs. In fact, literature data witness anti-inflammatory effects of platelet lysate and its capacity to secrete growth factors that induce tissue healing. On the other hand, it is not yet clear how ASCs induce tissue reparation; in fact, recent studies have suggested that the beneficial effect of MSCs in cells of injured tissues is not attributed to their differentiation, but rather to the activation of a protective mechanism and stimulation of endogenous regeneration. This contention is supported by the production of bioactive soluble factors known to inhibit apoptosis and fibrosis, enhance angiogenesis, stimulate mitosis and/or differentiation of tissue-intrinsic progenitor cells and modulate the immune response. MSC-secreted bioactive molecules may act as paracrine or endocrine mediators that directly activate target cells and/or cause neighboring cells to secrete functionally active agents. It has been recently demonstrated that extracellular vesicles or microvesicles released from cells are an integral component of the cell-to-cell communication network involved in tissue regeneration and therefore may contribute to the paracrine action of MSCs (Biancone et al. 2012).

In order to improve both theoretic and practical knowledge about the quality of repaired tissue, it will be necessary to consider a higher number of clinical cases in a long-term follow-up period and to evaluate the re-injury percentages. These data, although obtained from a small number of animals and without any histological or bio-mechanical analysis of the healed tissues, suggest that ASC therapy is more effective than pin firing. In particular, absence of adverse reaction potentially suggests allogenic cell therapy protocol development. This strategy could be of paramount importance in order to overcome limits typical of autologous cell implantation. Finally, it is clear that it is also necessary to understand if the tissue reparation process is promoted by ASCs or platelets action. Probably, the tissue regeneration is enhanced by the *in vivo* co-operation of these two biologicals, but in the future this aspect should be deeply investigated.

6. ABBREVIATIONS

MSCs: mesenchymal stromal cells.

ASCs: adipose derived-stromal cells.

HSCs: hematopoietic stem cells.

BM: bone marrow.

IFATS: International Fat Applied Technology Society.

BMSCs: bone marrow-derived mesenchymal stromal cells.

SVF: stromal vascular fraction.

CD: cluster of differentiation.

GvHD: graft-versus-host-disease.

PBMC: peripheral blood mononuclear cell.

NK: natural killer.

HGF: hepatocyte growth factor.

TGF: transforming growth factor.

PGE-2: prostaglandins E2.

IDO: indoleamine 2,3-dioxygenase.

Tregs: regulatory T cells.

COX-2: cyclooxygenase-2.

IL: interleukin.

HLA-G: histocompatibility antigen, class I, G.

CNS: central nervous system.

Th: T helper.

mtDNA: mitochondrial DNA.

MVs: membrane vesicles.

PRP: platelet rich plasma.

PL: platelet lysate.

COMP: cartilage oligomeric matrix protein.

NF-kB: nuclear factor–kB.

IL-1ra: interleukin-1 receptor antagonist.

sTNF-R: tumour necrosis factor receptor.

TNFα.: tumour necrosis factor alpha.

SDFT: superficial digital-flexor tendon.

DDFT: deep digital flexor tendon.

SL: suspensory ligament.

PDGF: platelet-derived growth factor.

FGF: fibroblast growth factor

EGF: epidermal growth factor.

GFP: green fluorescent protein.

PBS: phosphate buffer saline.

EDTA: ethylenediaminetetraacetic acid.

Ede: equine dermis.

RK13. rabbit kidney.

PCR: polymerase chain reaction.

IZSLER: Istituto Zooprofilattico della Lombardia e dell'Emilia Romagna.

CFU: colony forming unit.

FBS: fetal bovine serum.

DMSO: dimethyl sulfoxide.

HES: hydroxy ethyl starch.

BSA: bovine serum albumin.

Hep2: human larynx epidermal carcinoma.

MRC 5: human fetal lung.

TRF: Telomerase Restriction Fragment.

PHA: phytohemagglutinin.

CPM: counts per minute.

CPDA-1: citrate–phosphate–dextrose.

FLSL: the fetlock suspensory ligament.

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