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A COMPREHENSIVE STUDY OF ADULT STROMAL CELLS DERIVED FROM MESENCHYMAL TISSUES AND THEIR APPLICATION IN TENDON REGENERATION

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ABSTRACT

This research project focused on study of adult stromal cells derived from equine and canine mesenchymal tissue and the application of these cell sources in musculoskeletal injuries, particularly in tendon repair. Adult MSCs gained general attention in both human and veterinary medicine, however despite the advances in this field, much studies are needed in order to better understand MSCs behavior. With this goal the following research project focused on:

- A comprehensive characterization and study *in vitro* of mesenchymal stromal cells derived from peripheral blood of horses (ePB-MSCs) and from adipose tissue of dog (cA-MSCs). Moreover the possibility to cryopreserve these cells in the long-term period and the delivery methods of both ePB-MSCs and cA-MSCs in the short-term period was verified.

- The use of MSCs from peripheral blood and from adipose tissue in the tendon regeneration process was investigated in two different work: the use of adipose tissue derived MSCs in the re-cellularization of a human biocompatible scaffold, and the use of MSCs derived from ovine peripheral blood injected in experimental tendon lesions of the deep digital flexor tendon (DDFT) in a ovine model.

The full characterization of both ePB-MSCs and cA-MSCs was achieved using FACS sorting in order to identify the Cluster of Differentiation (CD) expression on the surface of these cells, by the study of population doubling time (PDT), the analysis of telomerase enzyme presence by Real Time PCR, and the alkaline phosphatase positivity. The differentiative potential of both ePB-MSCs and cA-MSCs was assessed by the *in vitro* induction of these cells into osteo, muscular and adipogenic lineages and verified by expression of tissue specific gene, like PPAR-γ, Desmin, and Osteopontin (SPP-1). The same characterization and differentiative potential were then verified on ePB-MSCs and cA-MSCs after 1 year. Results obtained from this study furnished novel information on adult MSCs and confirmed the possibility to cryopreserve these cells in the long-term period for their potential applications in clinical field. The study on delivery of both ePB-MSCs and cA-MSCs in the short-term period, performed by exposing cells to different media, time and temperature, lead to the conclusion that cells could be delivered in PBS at room temperature no longer than 9-12 hrs; cells were also monitored for CD expression, for apoptotic resistance and β-galactosidase activity during different time intervals. The second challenge of this research project was on study of regeneration capability of adult MSCs derived from peripheral blood and from adipose tissue in

tendon pathologies. A first work was performed using MSCs derived from human lipoaspirate in order to recellularize a human tendon scaffold to be used as insert in full-thickness lesions of flexor hand tendons. A previous re-cellularization was successfully obtained using Tris solution and enzymatic digestion, and the absence of native genonic DNA were assessed by standard PCR. Moreover a good protocol for re-cellularization was obtained, using a collagen matrix gel, which helped a good penetration of stromal cells inside the biocompatible scaffold. Secondly a comparation study among the effect of PB-MSCs, platelet rich plasma (PRP) or combination of both on tendon healing was performed using ovine model. Experimental lesions were made using collagenase 1A injection in the left deep digital flexor tendon (DDFT) of 18 Bergamasca sheep, using the right DDFT as internal control (lesioned, not treated). Sheep were further divided in two groups, the first euthanized after 1 month and the second after 4 months, and tendon were then analyzed by histological and immunohistochemical assays. Results obtained after 4 months showed a significant difference between the treated tendon of all group respect to the relative internal control (lesioned, not treated); notably after 4 months the group that received the treatment with MSCs alone or in combination with PRP, showed significant improvement of the healing process respect to PRP treated group, becoming similar to the normal healthy tendons.

RIASSUNTO

Il seguente progetto di ricerca ha avuto l'obiettivo di studiare in modo approfondito le cellule stromali adulte derivate da sangue periferico di cavallo e da tessuto adiposo di cane e successivamente verificare le loro potenzialità applicative nelle patologie muscolo-scheletriche, in modo particolare nella rigenerazione tendinea. Negli ultimi anni le cellule stromali adulte di derivazione mesenchimale hanno sollevato l'attenzione della comunità scientifica sia in medicina umana che veterinaria, per la potenzialità che possono rivestire nel trattamento di patologie che a tutt'oggi non trovano completa risoluzione clinica. Tuttavia nonostante i notevoli progressi fatti in questo campo di ricerca, ulteriori approfondimenti sono necessari per comprendere del tutto i diversi meccanismi delle cellule stromali adulte sia *in vivo* che *in vitro*. Questo studio ha avuto la finalità di:

- Effettuare una completa ed esaustiva caratterizzazione delle cellule staminali adulte isolate a partire da sangue periferico di cavallo (ePB-MSCs) e da tessuto adiposo di cane (cA-MSCs). È stata poi indagata la possibilità di criopreservare entrambi i tipi di cellule per un anno, ed infine è stato studiato l'effetto di parametri ritenuti importanti nell'influenzare la vitalità cellulare durante la spedizione delle cellule nel breve periodo evitando il congelamento.

- Indagare l'uso di cellule stromali isolate da sangue periferico e da tessuto adiposo nella rigenerazione tendinea in due diversi studi: il primo con l'obiettivo di ottenere la ricellularizzazione di uno scaffold tendineo precedentemente decellularizzato, il secondo con l'obiettivo di verificare il contributo alla rigenerazione tendinea *in vivo* di cellule stromali isolate da sangue periferico di pecora, immesse in lesioni sperimentali indotte con collagenasi 1A.

Per l'ottenimento di una completa caratterizzazione di entrambe le ePB-MSCs e le cA-MSC è stato utilizzato il FACS sorting per analizzare l'espressione dei cluster di differenziamento (CD) presenti sulla membrana cellulare di queste cellule, inoltre è stato effettuato lo studio del tempo di duplicazione cellulare (PDT) e la positività alla fosfatasi alcalina. La potenzialità differenziativa delle ePB-MSCs e delle cA-MSCs è stata verificata mediante l'induzione al differenziamento *in vitro* di queste cellule verso tessuto osseo, muscolare e adiposo, e successiva verifica dell'espressione di specifici geni, quali la PPAR-γ, la Desmina e l'ostopontina (SPP-1) mediante PCR. Gli stessi esperimenti effettuati per definire la caratterizzazione di entrambe le ePB-MSCs e le cA-MSCs sono stati effettuati dopo un anno di criopreservazione delle stesse, concludendo che le

caratteristiche di staminalità non cambiano nelle cellule stromali adulte di entrambe le specie dopo congelamento. É stata effettuata un'indagine sull'effetto che diversi mezzi di coltura, tempo e temperatura hanno sulla sopravvivenza di entrambe le ePB-MSCs e le cA-MSCs durante la spedizione nel breve periodo. Le cellule stromali di entrambe le specie sono state analizzate per l'espressione dei cluster di differenziamento, per la presenza di resistenza all'attività apoptotica e per la positività alla beta- galattosidasi. I risultati ottenuti hanno portato alla conclusione che le cellule stromali adulte possono essere spedite in soluzione salina (PBS) a temperatura ambiente per non più di 9-12 ore.

Il secondo obiettivo di questo progetto di ricerca è stato finalizzato all'applicazione delle cellule stromali isolate da sangue periferico e da tessuto adiposo nella rigenerazione tendinea. Il primo lavoro effettuato ha previsto l'utilizzo di MSCs derivate da lipoaspirato umano per la ricellularizzazione di uno scaffold tendineo umano con la finalità di utilizzarlo nelle lesioni totali dei tendini flessori della mano. Lo scaffold biocompatibile è stato in precedenza decellularizzato grazie all'utilizzo di soluzioni detergenti ed enzimatiche. L'assenza di residuale materiale genomico è stata verificata tramite PCR. Questo studio ha permesso di mettere appunto una tecnica di ricellularizzazione efficiente che, avvalendosi dell'ausilio di una matrice gelificante di collagene, ha garantito una buona penetrazione cellulare all'interno della matrice dello scaffold. Il secondo lavoro effettuato sulla rigenerazione tendinea, ha avuto la finalità di paragonare l'effetto di tre diversi trattamenti (cellule stromali derivate da sangue periferico di pecora (sPB-MSCs), sPB-MSCs + platelet rich plasma (PRP), e PRP) sulla rigenerazione tendinea a 1 mese e a 4 mesi dopo lesione sperimentalmente indotta sul tendine flessore profondo delle falangi di pecora (DDFT). Dopo eutanasia delle pecore a 1 e 4 mesi i tendini sono stati analizzati tramite analisi istologica e immunoistochimica; i risultati ottenuti dopo 4 mesi hanno evidenziato una differenza significativa nel grado di riparazione tissutale tra tutti i gruppi di pecore trattate e i loro rispettivi controlli interni (lesionati, non trattati). Inoltre le pecore che hanno ricevuto il trattamento con sPB-MSCs hanno dimostrato avere il migliore processo riparativo tendineo rispetto al gruppo di pecore trattato solo con PRP per tutti i parametri istologici valutati, risultando molto più simili al tendine sano usato come controllo positivo.

GENERAL INTRODUCTION

Chapter 1: Adult stromal cells derived from mesenchymal tissue:

Adult stromal cells:

The subject of stem cells received considerable attention in the last two decades in human and veterinary medicine, as demonstrated by the increasing number of researcher team studying these cells. Indeed this raising interest in the field of stem cells is ascribed to the great promise that adult stomal cells offer in treating previous incurable disease and because of the lack of ethical controversies like the one associated with embryonic stem cells (ES) (Fortier LA, 2005).

Adult stromal cells are broadly defined by three main characteristics: the ability to undergoes self-renewing cells divisions and give rise at least to one identical daughter cell, maintaining the stem cells pool; the capacity to go through lineage commitment and differentiation resulting in terminally differentiated cells. ES cells are defined totipotent, because they are able to create an entire organism, a property retained by early progeny of the zigote up to the 8-cell stage of the morula; most of the adult stem cells are multipotent since are able to differentiate into multiple cells type that are, however, restricted to a given tissue. Third, stromal cells have the potential to robustly restore a give tissue *in vivo*, which implies they are able to respond to specific needs to differentiate into cells type of that particular tissue.

The presence of stromal cells in the postnatal tissue has been recognized since the 1960s with the discover that blood and bone marrow contains cells able to restore the bone marrow function, and since then a large number of studies were focused on characterization of hematopoietic stem cells (HSCs), which nowadays remains the best defined class of adult stem cells population and that were firstly identified by Till and McCulloch in 1961.

Hence several other tissue-specific stem cells have been defined, supporting the main hypothesis that each different tissue has a stem cells reservoir to guarantee the replenish of the tissue composed of mature cells with finite half-life; example of identification of somatic stromal cells in adult tissue include hematopoietic system, skin, hair follicle, gut, muscle, liver, testes, breast, central as well as

peripheral nervous system. Indeed adult stromal cells are divided by the tissue from which they originate into stromal cells derived from ectoderm, endoderm and mesoderm layer. Mesenchymal stromal cells are characterized by their common derivation from mesodermal layer.

Mesenchymal stromal cells:

Mesenchymal stromal cells (MSCs) are a class of adult stem cells, which have been firstly identified in bone marrow by Till and McCulloch in 1961. MSCs are described as adherent, clonogenic, non phagocytic cells capable to form fibroblast-like colonies (CFU-Fs) and to differentiate into osteocytes, adipocytes and chondrocytes pathways *in vitro* and when implanted *in vivo* (da Silva Meirelles L, et al., 2009). Adult mesenchymal tissue has a unique turnover dynamics: some relatively rapid, like bone, and others slow like cartilage. In the early '80 several investigators proposed that ostogenic progenitors reside in the bone marrow for the reason that all bone is in contact with marrow elements, and then it was speculated that adipose progenitor were also in the marrow; since then the presence of a stromal system, with a stromal cell (MSC) at the top of the hierarchy (Fig. 1), was firstly proposed by Owen (1988), based on the analogy with the hierarchical system previously described for HSCs. This mesengenic process would require for some researchers that MSCs fit the definition of the HSCs, in that the "stem cells" must rescue lethally irradiated animals. This property of stem cells is apple and orange, since the HSCs is a life or death progenitor and the MSCs are used for natural turnover and tissue repair (Caplan AI., 2006).
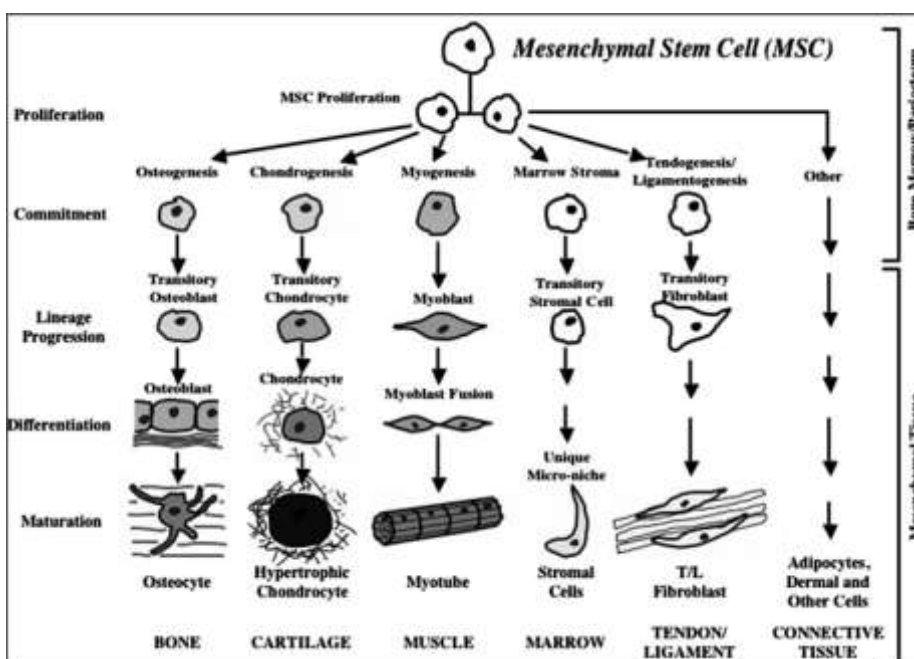

Fig.1: The mesengenic process describing mesenchymal progenitor cells entering different lineage pathways to contribute to mature tissue formation.

MSCs were originally and foremost isolated from bone marrow, but then other tissue were found to host a population of stromal cells with analogous characteristic of bone marrow MSCs. It then became clear that post-natal tissues have reservoir of specific stem cells, which contribute to maintenance and regeneration; examples include epithelial stem cells in the epidermis (Chunmeng S et al., 2004), in the intestinal crypts (Slack J.M, 2000), neural stem cells in the central nervous system (McKay R, 1997), satellite cells in muscle (Chargé SB and Rudnicki MA, 2004); indeed MSCs were further isolated from other mesenchymal sources like adipose tissue (Zuk PA et al., 2002), tendon (Salingcarnboriboon R. et al., 2003), synovial membrane (De Bari C et al., 2003), synovial liquid (Jones E et al., 2008), periodontal ligament (da Silva Meirelles L et al., 2006) and lung (Sabatini F et al., 2005). Moreover mesenchymal stem cells populations were found also in blood and umbilical cord blood (Erices A et al., 2000), placentar villi (Igura K et al., 2004) and amniotic liquid (In't Anker PS et al., 2003). Indeed due to the etherology of these sources, different methodologies are required in order to isolate, cultivate and characterize mesenchymal stem cells related cells type. Consequently there is still a lack of consensus on hierarchy intrinsic to the MSCs compartment and this is highlighted by one of the first studies aimed to characterize MSCs both histologically and phenotipically (Bianco P et al., 2008); interestingly it has been found that a wide range of non-hematopoietic stem cells exist in the bone marrow and that MSCs are merely a subset of this population: this include the existence of similar cells such as multipotent adult progenitor cells (MAPCs) (Reyers M et al., 2001), marrow-isolated adult multilineare inducible cells (MIAMI) (D'Ippolito G et al., 2004), recycling stem cells (RS-1, RS-2) (Colter DC et al., 2000), endothelial progenitor cells (EPCs), and the very small embryonic-like stem cells (VSELs).

On the other hand, even there are evidences that suggest that MSCs exist not only in the bone marrow but virtually in all organs, the exact localization of the MSCs *in vivo* remains poorly understood and there are currently growing evidence that indicates a relationship with pericytes (Doherty MJ et al., 1998; Farrington-Rock C et al., 1998). Pericytes are located at the abluminal site of the blood vessels in close contact with endothelial cells, and there are evidence suggesting their similarity with MSCs *in vitro*; this theory speculate that they may behave as tissue-specific stem cells *in vivo* (Fleming JE et al., 1998; Brighton CT, et al., 1992). The finding that the frequencies of fibroblastic colonies strongly correlate with vascular density, results in a natural association of MSCs with blood vessels (da Silva Meirelles L et al., 2008). This perspective is that some pericytes are stem cells in the tissue from that they originate and correspond to MSCs in connettive tissue. However this theory does not imply that pericytes/MSCs from different organs are equivalent, since MSCs isolated from some different sources show dishomogeneus differentiation potential and gene

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expression profiles. Even if there is evidence that the best *in vivo* candidate for the role of MSCs in connettive tissue is pericyte, definitive proof that this cells can self-renew for a lifetime is still lacking.

Mesenchymal stromal cells niche:

The ability of stem cells to both self renew and produce daughters cells able to initiate the process of differentiation, is the key for tissue homeostasis, providing a continuous supply of new cells to replace short-lived but highly differentiated cells type. The decision between stem cells self-renew or differentiation must be tightly controlled, and now is known that the stem cells niche provide the integration of intrinsic factor and extrinsic cues to regulate the stem cells number, division, self-renew, and differentiation. The concept of "niches" was introduced in 1978 by Schofield, who defined the niche a "stable micro-environment that might control hematopoietic stem cells behavior" (Schofield R., 1978). The precise spatial organization of the stem cells respect to surrounding support cells plays an important role in the ability of the niche to adequately provide proliferative and anti-apoptotic signals and to exclude factors that promote differentiation. The intimate contact between stem cells and surrounding support cells serve a source of critical signals controlling stem cells behavior (Jones DL and Fuller MT, 2006). Adult and tissue specific stem cells are found in specialized niches in their corresponding tissues of origin. Specialized niches for different types of adult stem cells are characterized by the complex interactions between surrounding cells, extracellular matrix molecules, and soluble factors. In the bone marrow MSCs are an important component of the HSCs niche. Although HSCs are the best-characterized adult stem cells, their niche is still poor understood. Recent studies have shown the existence of two types of niches in the bone marrow compartment: an "endosteal" niche and a "perivascular" niche, which may be closed to each other or may be interdigitated. MSCs-derived cells, such osteoblast and fibroblast, are mainly involved with the endosteal niche, located at the endosteum of the trabecular bone. Besides producing mature, specialized cells that interact with the HSCs, mesenchymal stem cells are also directly involved with regulation of the hematopoietic process. The main differentiated cells types that originate from MSCs, osteoblast, chondrocytes, and adipocytes, are present throughout the entire organism. The location of these stem cells must allow their progeny and therapeutic effects to reach most or all tissues and organs. Considering this, three possibilities could be envisioned. In the first, MSCs are located in only one specific tissue or organ, from which they exit and circulate to other sites to replenish cells population that undergo apoptosis through physiological turnover process. The considerable difficulty to establish conventional MSC cultures

from peripheral blood under physiological conditions or stimulation by cytokines argues against this possibility. Experiments using rats exposed to low-oxygen condition suggest that MSCs may be specifically mobilized into peripheral blood as a consequence of hypoxia. However the origin of mobilized cells remain unclear. The second possibility speculate that postnatal MSCs have been isolated from different tissues, in addiction to bone marrow; MSCs cultures with very similar morphologic, immunophenotypic, and functional properties have been established from brain, spleen, liver, kidney, lungs, bone marrow, muscle, thymus, and pancreas of mice, even when the animals had their blood washed out from their vessels by perfusion prior to cells isolation. These findings suggest that cultured MSC-like cells can be derived from a variety of tissue locations, arguing that different tissue intrinsic stem cells might behave as MSCs when characterized *in vitro*. The third possibility has been suggested previously and involves a relationship between MSCs and perivascular cells. (da Silva Meirelles et al., 2008).

Mesenchymal stromal cells plasticity:

Adult stem cells are undifferentiated cells which are able at the single cell level to both self renew, and to go toward differentiation into terminally effectors cells. Stem cells have been classified for their developmental potential as totipotent (able to give rise to all embryonic and extra-embryonic cells type), pluripotent (able to give rise to all cells of the embryonic proper), multipotent (able to give rise to a subset to cells lineages) oligopotent (able to give rise to a more restricted cells subset respect to the multipotent cells) and unipotent (able to give rise to one mature cells type) (Wagner W et al., 2010). Traditionally adult mesenchymal stem cells are defined as multipotent cells committed to a particular cells fate to produce cells from the tissue of origin and not cells of non-related tissue, currently there are increasing evidences that suggest their ability to differentiate into ectodermal and endodermal lineages under certain microenvironment conditions (Lakshmipathy U and Verfaillie C, 2005; da Silva Meirelles L et al., 2006). Examples include the findings that HSCs have been reported to give rise to liver cells (Petersen BE et al., 1999) and neural stem cells (NSCs) give rise also to hematopoietic precursor (Bjornson CRR et al., 1999). These findings lead to the concept of "stem cells plasticity" and this issue has generated both enthusiasm as well as skepticism in the field of stem cells research, as the ability of adult stem cells to change fate holds great therapeutic potential. It is crucial that the concept of stem cells plasticity be rigorously defined and experimentally proven. In fact till now most studies has not shown that multi lineage differentiation is derived from the single cells that differentiate into the expected cells type, and even when this happens it is a very low frequency (Lakshmipathy U and Verfaillie C, 2005). Much of the problem

regarding the plasticity of stem cells is derived from the lack of established parameters that help to uniformly define plasticity. Lakshimpaty et al. suggested three main criteria based on which stem cells plasticity had to be examined:

- a single cell differentiate into multiple cell lineages;
- differentiated cells are functional *in vitro* and *in vivo*;
- engraftment is robust and persistent

The paperwork of Wagers and Weissman (2004) suggest some possible explanations of the mechanisms that underlie the stem cells plasticity. One of the mechanisms that could be involved is trans-differentiation, which is the ability of adult stem cells to contribute to cells type of different lineages. This theory emerged when first papers were published suggesting evidence of HSCs or BM-MSCs contribution to non-hematopoietic tissue (Ferrari G et al., 1998) or of neural stem cells to blood lineages (Bjornsonn CRR et al., 1999). This lineage conversion was proposed to occur directly, by activation of an otherwise silent differentiation program to change the commitment of the cells (figure 2A). Brockes and Kumar in 2002 suggested that lineage differentiation could also theoretically occur via dedifferentiation of a tissue specific cells into a more primitive, multipotent cells, and subsequent redifferentiation along new lineage pathway (Fig 2B). Even if this dedifferentiation mechanism has been described in amphibians, in adult mammals has not be clearly and unequivocally documented, and at the present, there is no proof that support the trans-differentiation or de-differentiation events as an explanation for stem cells plasticity *in vivo*. A third possible explanation for adult MSCs plasticity relates to the purity and homogeneity of the population. In fact in order to demonstrate complete differentiation of a particular lineage-specific stem cells, it is essential to exclude the possibility that multiple, distinct stem cells could be contributing to the observed outcomes. Many demonstration of stem cells plasticity deduced such activity after transplant of large number of heterogeneous population of cells. Included in above mentioned population there are un-fractionated BM cell which likely contains multiple stem cells or progenitor cells population (like HSCs), non-HSCs stem cells, endothelial precursor or muscle progenitor, suggesting the possibility that different stem cells could contributing, consisting with their fate commitment, to each of the different lineage outcomes observed (Fig 2C). Another explanation for contribution to tissue type, arise through the action of a single, rare pluripotent cells present in BM and/or other tissue. The existence of pluripotent stem cells in the BM of mice and human has previously been proposed, and this idea has given support after the isolation of multipotent adult progenitor cells (MAPC) which give rise to tissue of different germ layers following intravenous trasplant and contribute to all following tissue injection into blastocyst (Jiang et al. 2002) (Fig 2D). Finally the last mechanism to explain stem cells plasticity is cells-cells fusion,

which is a mechanism that occurs naturally, for examples in the generation of multinucleated skeletal myofibers from myoblast or osteoclast from monocytes/macrophages cells lineages (Anderson JM, 2000; Vignery A, 2000).
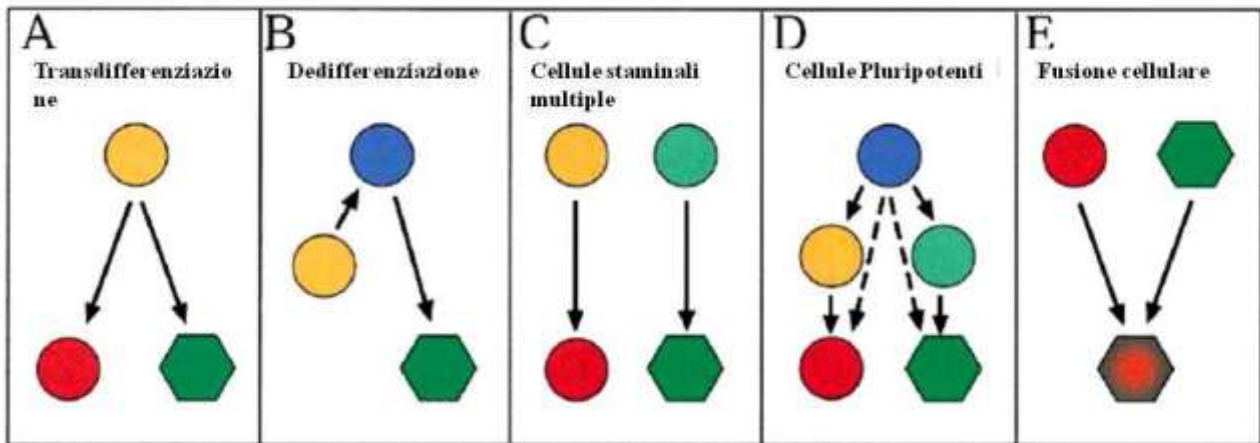

Fig.2: Schematic diagram depicting potential mechanisms and explanations for observations of adult stem cell plasticity. Tissue-specific stem cells are represented by orange or green ovals, pluripotent stem cells by blue ovals, and differentiated cells of the "orange" lineage by red ovals and of the "green" lineage by green hexagons.

The plasticity of mesenchymal stem cells *in vitro* is very well known if compared to the *in vivo* one. Methods have been developed for the culture of mesenchymal stem cells into adipogenic, chondrogenic and osteogenic lineages (Pittenger MF et al., 1999). Such type of differentiation assay in some cases were made holding as a negative control fibroblast, which were compared for the differentiative potential with mesenchymal stem cells, and it has been found they could not differentiate under certain stimuli in culture.

Adipogenic differentiation: to induce adipogenic differentiation MSCs are cultured in monolayer with a media containing DMEM (low glucose), 10% FBS and antibiotics and are allowed to become confluent. The cells are cultured for 3-7 days past confluence, then the adipogenic induction medium is added. This medium contains 1μM dexamethasone and 0.5mM methyl - isobutylxanthine, 10μg/ml insulin, 100μM indomethacin and 10% FBS in DMEM low glucose. The cells are maintained in this differentiation medium for 2-3 weeks, changing the medium every three days. Lipid vacuoles are detectable throughout the time in culture, enlarge over the time, and became apparent through the light microscope. The cells can also be stained with the lipophilic dyes Oil Red-O. Pittenger et al. maintained hMSCs for up to three months, and the cells were maintained as mature adipocytes with a single large lipid vacuole that displaces the nucleus to the side.

Chondrogenic differentiation: chondrogenic differentiation of MSC is achieved by placing 2.5×10^5

MSCs into defined chondrogenic medium and subjecting them to gentle centrifugation (800g for 5 min) in a 15ml conical polypropylene tube, where they consolidate into a cells mass or pellet within 24 hours (Pittinger MF et al., 1999). Chondrogenic medium consist of high glucose (4.5g/l) DMEM, supplemented with 6.25μg/ml insulin, 6.2μg/ml transferrin, 6.25μg/ml selenous acid, 5.33μg/ml linoleic acid, 1.25mg/ml bovine serum albumine, 0.1μM dexamethasone, 10ng/ml TGF-β3 50μg/ml ascorbate 2-phosphate, 2mM pyruvate and antibiotics. The chondrogenic cells pellet increase in size 2-3 fold over 3 weeks. When pellets are harvested, the samples are fixed in 4% formaldehyde, embedded and sections cut and analyzed. Extracellular matrix molecules such as type II collagen and aggrecan can be detected by immunoistochemical methods. Sections are also stained with Safranin O to detect the accumulation of proteoglycans.

Ostogenic differentition: in order to obtain ostogenic differentiation of MSCs 3 x 10^4 cells are seeded into 35mm dishes in DMEM with 10% FBS to produce subconfluent, monolayer cultures. After 24 hours, this medium is replaced with the same medium containing ostogenic supplements (50μM ascorbate 2-phosphate, 10mM β-glycerol phosphate, and 100nM dexamethasone). Medium is replaced every three days. Calcium deposition is examined by the von Kossa stain and through a quantitative measurement of calcium deposition.

Cluster of differentiation (CD) expression on Mesenchymal stromal cells:

Several studies aimed at clarify the mechanism underlying the potential of MSCs to differentiate into multiple lineage have encountered difficulties. MSCs are usually harvested as adherent cells from the bone marrow aspirate and are thus heterogeneous cells population (Pittinger MF et al., 1999). Indeed bone marrow contains various types of adherent cells including mesenchymal stromal cells, endothelial cells, osteogenic cells, phagocytotic cells, and others (Wagers AJ and Weissman IL, 2004). For this reason is likely that any of these cells types of adherent cells could contaminate the MSC population, particularly at the initial step of the culture. Moreover MSCs are obtained not only from the bone marrow compartment but also from other adult tissue, and many laboratories have developed methods to isolate and expand MSCs, which invariably have subtle, and occasionally quite significant, differences. These varied tissue sources and methodology of cells preparation beg the question of whether the resulting cells are sufficiently similar to allow for a direct comparison of reported biologic properties and experimental outcomes, especially in the context of cells therapy. Therefore the big picture of MSCs has not yet been clarified (Horwitz EM

et al., 2005) and correct identification of these cells is still a challenge. Pittinger MF et al., (1999) was one of the first to analyze the surface antigens in human MSCs in detail. They described that MSCs are uniformly positive for the following markers: SH2, that is an antibody which recognize an epitope on endogline or CD105 (Mackay AM et al., 1998); SH3 antibody that recognize the epitope CD73; CD29 or integrin beta-1, CD44 (involved in cells-cells interactions, cells adhesion and migration), CD71, CD90 or Thy-1, CD106, CD120a, and CD124. On the other hand MSCs were found negative for hematopoietic lineage markers expression, like CD14 (lipopolysaccaride receptor), CD34, and the common leukocyte antigens CD45 (Kuroda Y et al., 2011). Others researchers groups subsequently attempted to characterize the surface antigens of MSCs. Some common antigens were identified, but other one differed among different paperwork. There are no articles bringing together and summarizing the cell surface markers of adult mesenchymal stem cells, but the review of Mafi et al. (2011) is useful to systematic summarize and provide a good basis for collect the published literature in this regard so far. What clearly emerged from different studies is that CD105, CD90, CD44, CD44, CD73, CD29, CD13, CD34, CD146, CD106, CD54 and CD166 rank the among the most commonly reported positive cell surface markers on mesenchymal cells. Moreover there are a number of cell surface markers that have been reported as being absent in MSCs: among them the most frequently reported are CD34, CD14, CD45, CD11b, CD49d, CD106, CD10 and CD31. In addiction to these, a number of other cell surface markers have been further identified: STRO-1, SH2, SH3, SH4, HLA-A, HLA-B, HLA-c, HLA-DR, HLA-I, DP, EMA, DQ (MHC Class II), CDIO5, Oct4, Oct4A, Nanog Sox-2, TERT, Stat-3, fibroblast surface antigen, smooth muscle alpha-actin, vimentin, integrin subunits alpha4, alpha5, beta1, integrins alphavbeta3 and alphavbeta5 and ICAM-1. Interestingly, several studies have reported conflicting information about some of the cell surface markers including CD10, CD34, CD44, CD45, CD49d, and CD106; moreover, it has emerged that the expression of some surface antigens, such as STRO-1, which is considered one of the most important MSCs markers is dependent on whether the cells are adipose tissue-derived or bone marrow-derived; Stro-1 has recently been found to be expressed also in other cells type, moreover its expression is lost during culture, so that is not possible to be considered a unique marker for the isolation of mesenchymal stem cells. Another factor accounting for the variability in the expression of adult MSC surface markers seems to be the different stages during cell proliferation and culture where the markers have been accessed.

In 2000 the International Society for Cellular Therapy (ISCT) introduced a set of statement whit the aim to define human MSCs for both laboratory-based scientific research, as well as pre-clinical studies. Basically ISCT stated that mesenchymal stem cells must adhere to plastic, have a specific

surface antigen expression and a multiple differentiation potential (Horwitz EM et al., 2005). They stated that human MSCs are defined by their expression of CD105, CD73 and CD90, and lack the expression of CD45, CD34, CD14, CD11b, CD79a, or CD19 and HLA-DR surface molecules (Horwitz EM et al., 2005; Dominici M et al., 2006); they concluded that convincing data for defining "stemness" of un-fractionated plastic-adherent cells was lacking, and proposed that plastic-adherent cells described as mesenchymal stem cells be termed as mesenchymal "stromal" cells. They arrived to this conclusion for several reasons: first, they decided to maintain the acronym MSC to avoid any confusion in the scientific community, since this term has been extensively used in the literature for at least two decades. Second they thought to eliminate the term "stem" from the nomenclature, as this word has specific connotation, i.e. relates with long-term self renewing cells that is capable of differentiation into specific multiple stem cells *in vivo*. Third because the plastic adherent population isolated, is not a uniform population of cells, although this subset clearly may contain mesenchymal stem cells. Fourth the differentiation potential of this cells has still to be clearly elucidated. They are of mesenchymal origin and for this reason, ISCT decided to maintain this designation to imply their origin and not the differentiation potential. Finally mesenchymal stem cells seems to be found *in situ* in the supportive stroma of the resident tissues, thus the un-fractionated population may be termed stromal cells, avoiding any biologic or therapeutic potential. They decided to use the term multipotent mesenchymal stromal cells for the plastic-adherent population because it seems to be the most scientifically accurate descriptor without implying unproven biologic or therapeutic potential (Horwitz EM et al., 2005).

Study	Type of Cell in Study	Cell Surface Character
Dominici *et al*.	Multipotent mesenchymal stromal cells	These must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecole
Gronthos *et al*.	Adult human bone marrow stromal stem cells	Express telomerase activity *in vivo*
Ohgushi *et al*.	Mesenchymal stem cells	Cells were negative for hematopoietic markers (CD14, CD34, CD45) but positive for markers present in mesenchymal cells CD13, CD29, CD90
Wongchuensoontorn *et al.*	Mesenchymal stem cells	Found a population of CD34 and CD45 negative cells which were positive for CD44, CD73 and CD105
Gronthos *et al*.	Human adipose tissue-derived stromal cells	CD9, CD10, CD13, CD29, CD34, CD44, CD 49(d), CD 49(e), CD54, CD55, CD59, CD105, CD106, CD146, and CD166. But no STRO-1 antigen was found as in human adipose tissue derived stromal cells
Tsai *et al*.	Multipotent mesenchymal stem cells	Positive for SH2, SH3, SH4, CD29, CD44 and HLA-ABC (MHC class I), low positive for CD90 and CD105, but negative for CD10, CD11b, CD14, CD34, CD117, HLA-DR, DP, DQ (MHC class II) and EMA
Zvaifler *et al*.	Mesenchymal precursor cells found in the blood	A minority of adult marrow cells express CD34 BMPCs stained strongly with anti-CD44 antibody. Conventional T-cell (CD3), monocyte (CD14, CD68), and B-cell (CD20) antibodies stained neither of the two BMPC populations, nor did they react to anti-LCA (CD45), anti-VCAM (CD106), or MHC-Class II (anti-DR) ADDIN REFMGR.CITE ADDIN EN.CITE.DATA [49]
Igura *et al*.	Placenta-derived mesenchymal progenitor cells (PDMPC)	The PDMPC expressed CD13, CD44, CD73, CD90, CD105 and HLA class I as surface epitopes, but not CD31, CD34, CD45 and HLA-DR.
Zuk *et al*.	Mesenchymal stem cells	Cells expressed CD29, CD44, CD71, CD90, and CD105/SH2 and SH3, expressed STRO-1. No expression of CD31, CD34, and CD45. Positive expression of CD13 and the absence of CD14, CD16, CD56, CD61, CD62(e), CD104 and CD106. CD49(d) was not observed in MSC culture. However MCS expressed CD106 antigen.

Miura *et al.*	Stem cells from human exfoliated deciduous teeth (SHED)	SHED were found to express the cell surface molecules STRO-1 and CD146, which are cell surface markings of adult bone marrow-derived stromal stem cells.
Parte *et al.*	Putative stem cells of menopausal human ovarian surface epithelium	Pluripotent gene transcripts Oct-4, Oct-4A, Nanog, Sox-2, TERT, Stat-3 in human.
Iwata *et al.*	Human bone marrow-derived mesenchymal stem cells	CD29, CD44, CD90 and CD90
Hasebe *et al.*	Dermal tissue stem cells	Express CD44, CD54, CD90, CD105 and CD271 which are stem cell markers.
Yu *et al.*	Adipose-derived stromal/stem cells	CD29(+) CD34(+), CD44(lo) CD45(low) CD73(+) CD90(+) CD105(+)
Kadar *et al.*	Mesenchymal stem cells	STRO-1 positive
Kyurkchiev *et al.*	Multipotent endometrial stromal cells and multipotent decidual stromal cells	Express surface molecules CD73, CD90 and CD105
Royer-Pokora *et al.*	Mesenchymal stem cells	CD105, CD90 and CD73.
Mousavi *et al.*	Mesenchymal stem cells	Expression of CD105, CD166 and CD44, and the absence of CD45, CD34 and CD14 on the surface of mesenchymal stem cells like cells
Orciani *et al.*	Mesenchymal stem cells from human skin biopsies (S-MSCs)	Expression of HLA-A, B, C, CD29, CD44, CD73 and CD90, was strongly positive, while CD105 was low positive, and CD10, CD11b, CD14, CD34, CD49d and HLA-DR were negative
Bühring *et al.*	Mesenchymal stem cells in bone marrow	CD13, CD15, CD73, CD140b, CD144, CD146 and CD164
Latif *et al.*	Mesenchymal stem cells and human cardiac valve ICs (interstitial cells)	Expressed fibroblast surface antigen, smooth muscle alpha-actin, vimentin and CD44. CD105 was weakly expressed by MSCs (> 90%).
Gronthos *et al.*	Bone marrow derived stromal progenitor cells	STRO-1 positive
Gronthos *et al.*	Stromal cells derived from the bone marrow	STRO-1 positive
Stewart *et al.*	Bone marrow-derived mesenchymal stem cells	STRO-1 positive
Simmons and Torok-Storb	BM-derived mesenchymal stem cells STRO-1 positive + CD34 positive	STRO-1 positive, CD34 positive
Simmons *et al.*	Bone marrow derived mesenchymal stem cells	CD106 positive, CD34 positive
Walsh *et al.*	Bone marrow-derived mesenchymal stem cells	STRO-1 positive
Conget and Minguell	Human bone marrow mesenchymal progenitor cells	Express integrin subunits alpha4, alpha5, beta1, integrins alphavbeta3 and alphavbeta5, ICAM-1, and CD44H. These were CD49d (-) and CD34 (-).
Pittenger *et al.*	Adult human mesenchymal stem cells	CD49d (-), CD106 (+), CD34 (-)

Table 1: Summary of the relevant studies for mesenchymal stem cells surface antigen expression. (Mafi et al., 2011).

Clinical application of mesenchymal stromal cells:

Despite the challenges of isolating, expanding and defining stem cells populations, they hold great promise for tissue regeneration at clinical useful level (Fortier LA., 2005). Recent progress especially on the tissue-resident adult stem cells biology has suspired great optimism and given new hopes in offering the possibility to use these undifferentiated cells or their further differentiated progenies for cell replacement in regenerative medicine. With the preceding information, it follows that the regenerative repair of certain mesenchymal tissues will require the augmentation of MSCs

number at the repair sites. Cellular therapies have emerged as leading candidates for regenerative treatment of a variety of diseases; in particular MSCs have shown great promise in numerous clinical trials. Freshly harvested bone marrow has been used for years by surgeons to augment local skeletal tissue healing, and methods to concentrate and enrich the marrow are been introduced commercially as well as scaffold used to concentrate or deliver the cells to the repair site. Such developing technologies lead to the new scientific discipline of tissue engineering. One of the first and most obvious uses of MSCs is in the area of bone regeneration in sites where the body cannot organize this activity, i.e. in non- unions fractures. Critical size defects in non-union models, have clearly demonstrated that culture expanded marrow MSCs in a porous, calcium phosphate, ceramic delivery vehicle are capable of regenerating structurally bone, where whole marrow of the vehicle alone cannot accomplish satisfactory this repair. MSCs have been used also in the cartilage regeneration, since cartilage is an avascular tissue incapable of regeneration or repair of even small defects in adults. Although chondrocytes have been used in attempt to repair large cartilage defects, it is difficult to integrate neotissue with that of the host. Several types of scaffold have been used in combination with MSCs in order to provide an inductive microenviroment for MSCs to enter the chondrogenic lineage and facilitate the integration of the neotissue in the lesion site.

Injected MSCs make it back to the bone marrow to re-fabricate injured marrow stroma. This observation is the basis for a multifocus clinical trial to add back autologous MSCs to chemiotherapy patients receiving bone marrow transplant. MSCs could be injected into a specific muscle of the muscular dystrophy mouse to cure it by providing newly synthesized dystrophin to the affected myotubes. The donor MSCs dedifferentiated into skeletal myoblast, fused with the host myotubes, and caused the synthesis and distribution of the dystrophin. Moreover labelled MSCs injected into injured rat or pig heart appear to differentiate into cardiac myocytes. (Toma C et al., 2002). Several studies have focused on the use of autologous MSCs for tendon repair, as well as the efficacy of these cells in acute graft-versus-host disease (GvHD) (Le Blanc K et al,, 2008), liver disease, diabetic ulcers, cutaneus wounds etc. It is now becoming accepted that MSCs stimulate host recovery and regeneration through the secretion of numerous pro-regenerative factors. *In vitro* studies have documented the secretion of multiple anti-inflammatory, angiogenic, neutrotrophic, immunomodulatory and antifibrotic factor from MSCs. Moreover the secretion of many of these factors can be evoked by cytokines typically present at the site of tissue injury, suggesting that the release of these factor may be contextual, in that the release of factors by MSCs may be dependent on the specific demands of the type and stage of injury (Wagner J et al., 2009).

In addition to the potential clinical use of MSCs, it should be taken into account that diverse poorly differentiated adult stem cells types have been identified in the most mammalian tissues and organs.

The tissue regeneration mediated via adult stem/progenitor cells is usually accompanied by environmental changes in the niche and regulated by several growth factor and cytokine initiated cascades. Hence the tissue–resident adult stem/progenitor cells are unique as compared with other embryonic, umbilical cord, fetal and placenta-derived stem cells sources, in being enriched in an anatomic location that may be easy to access; the supply of new functional adult stem cells or their further differentiated progenies offers great therapeutic potentials for regenerating damaged tissues as well as gene delivery vehicle for treating and even curing diverse diseases.

Hence, it is relevant to summarize the anatomic localization, the known specific biomarkers and functional characteristics of diverse human tissue-resident adult stem/progenitor cell types, highlighting important aspects regarding their niche, their function in tissue regeneration during physiological as well as pathological conditions; moreover refer to the therapeutic potential of adult stem cells to give rise to particular cell lineages in the well-defined culture conditions *ex vivo* as well as in animal models *in vivo*.

Hematopoietic stem cells transplantation: The bone marrow hematopoietic stem cells (HSCs) provide a critical role by continually renewing all of the new mature and differentiated hematopoietic cell lineages in peripheral circulation including leucocytes, erythrocytes and thrombocytes along lifespan of an individual. The immature and quiescent multipotent HSCs which are characterized by the expression of specific biomarkers including $CD34^-$ or $CD34^+/CD38^{-/low}$, $Thy1^+$ $C-Kit^{-/low}$, lin^-, $CD133^+$ are localized with the osteoblast in a specialized niche within a bone marrow region designed as endosteum. Another subpopulation of HSCs, found in a BM microvasculature-sinusoidal endothelium niche, appears to represent the stem cells that may rapidly supply new mature blood cells lineages which have a short live into peripheral circulation (Mimeault M. et al., 2007). Marrow derived hematopoietic stem cells transplantation had been successfully established as a method to restore marrow function in patients whose bone marrows have been obliterated by disease, or by administration of marrow ablative therapies. After the first series of patients reported to receive hematopoietic stem cells transplant in Grain Britain between 1977 and 1983, these source of cells have become the most commonly used autograft product. Self-renewing pluripotent hematopoietic stem cells normally circulate in the human blood stream and can be used to durably reverse bone marrow aplasia (McCarthy DM and Goldmann JM, 1984). Although normally low, the number of circulating HSCs can be increased in response to treatment with drugs, chemokines and cytochines. Mobilization of stem cells from bone marrow to bloodstream has been achieved in both laboratory and clinical setting, and the development of methods to enrich this class of stem cells coupled with the history of successful transplantation in

human, made HSCs a gold standard for all form of stem cells therapies.

Bone marrow derived stem cells /progenitor cells: BM stroma as well as the walls of large and small blood vessels in most tissues or organs contain the multipotent mesenchymal stem cells and endothelial progenitor cells (EPCs). Much of the work conducted on adult stem/progenitor cells has focused on MSCs found within the bone marrow stroma. More particularly, the MSCs expressing CD49a and CD133 are localized in a perivascular niche in BM, and may give rise to the osteoblast that are co-localized with HSCs, and which may support the hematopoiesis by producing the growth factor and cytokines that promote the expansion and/or differentiation of HSCs (Gindraux F et al., 2007). The BM-derived MSCs can generate diverse mesodermal cells lineages involved in osteogenesis, adipogenesis, cartilage and muscle formation under appropriate culturing condition ex vivo and in vivo. MSCs may also be induced to differentiate into fibroblast, neuronal cells, pulmonary cells, pancreatic islet β cells, corneal epithelial cells and cardiomyocytes ex vivo and/or in vivo using specific growth factor and cytokines (Chang YJ et al., 2006; Bobis S et al., 2006). In the case of EPCs, which are derived like HSCs from the embryonic hemangioblast, they may be distinguished by the expression of different biomarkers, including CD34+/-, CD133, vascular endothelial growth factor receptor-2 (VEGFR-2), stem cells factor (SCF) receptor KIT and CXCR4. EPCs may contribuite in a significant manner to give rise to mature endothelial cells that that form new vascular walls of vessels after intense injury and vascular diseases (Balbarini A et al., 2007). The autologous or allogenic transplantation of BM can lead to the homing engraftment of functional MSCs. It has also been reported that MSCs, EPCs and their progenies can contribute to the regenerative process of several tissues including bone, cartilage, tendon, muscle, adipose, brain, lung, heart, pancreas, kidney and eye (Wagner W et al., 2005; Le Blanc K et al., 2007). Thus these cells may constitute a cellular source for the treatment of diverse human disorders including ostogenesis imperfecta, atherosclerotic lesions, ischemic cardiovascular and muscular diseases. Recently it has been found that BM-derived and tissue-resident MSCs are little immunogenic and display immunomodulatory effect in host in vivo. Therefore, these therapeutic properties of MSCs also support their possible clinical applications to prevent the tissue/organ allograft rejection and severe acute graft-versus host diseases, as well as to treat the autoimmune disorders such as inflammatory bowel disease and auto-immune myocarditis. Indeed, MSCs can prolong skin allograft survival and reverse severe acute graft-versus-host disease *in vivo* supporting their use in treating skin disease as well as in the maxillofacial surgery (Shanti, RM et al., 2007). Indeed future studies to optimize the BM-derived cell transplantation strategies and establish the specific mechanism of action and physiological effects of HSCS, MSCs, and EPCs at long term is essential in order to improve their therapeutic and curative benefits.

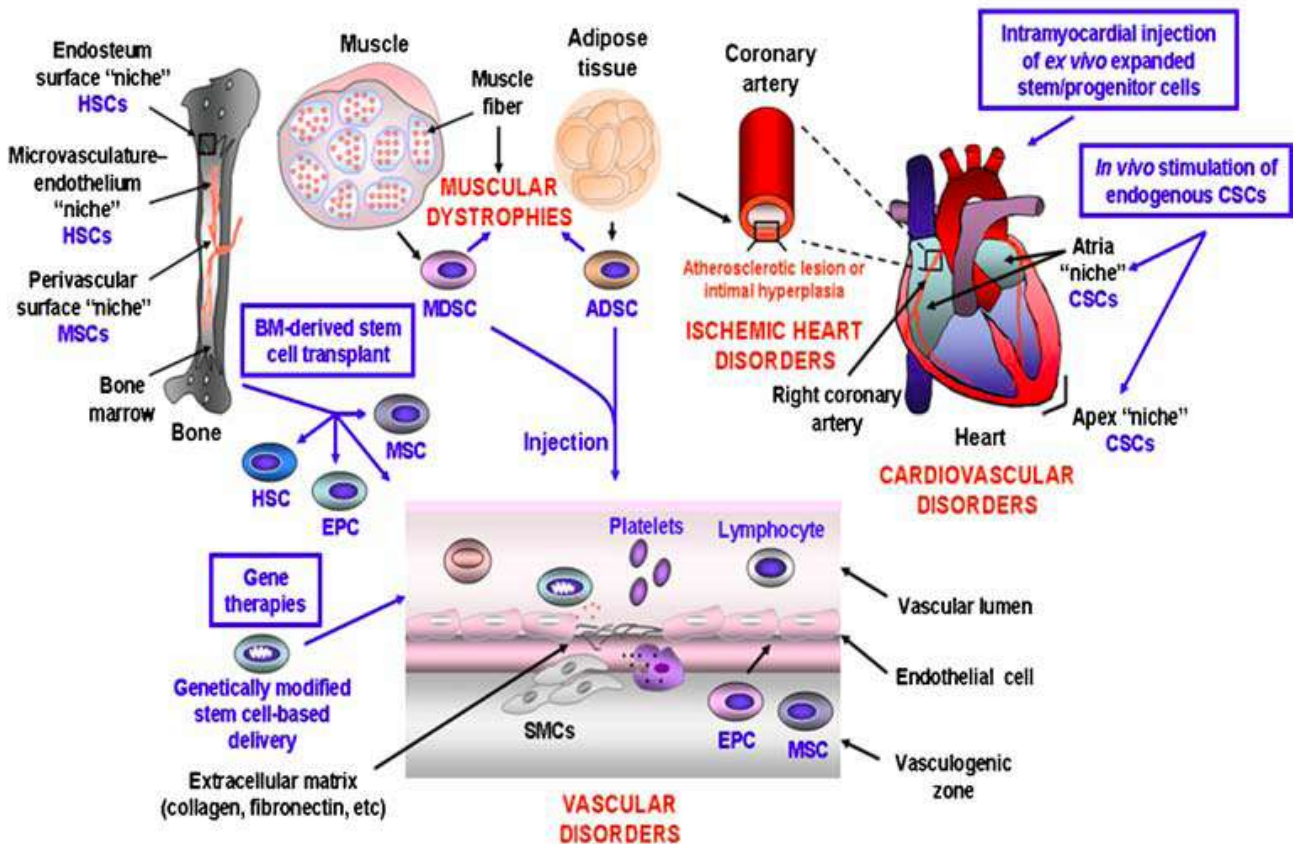**ADULT STEM CELL-BASED THERAPIES FOR VASCULAR AND MUSCULAR DISORDERS**

Fig.3: Diagram showing the possible molecular events and cellular changes associated with the development of vascular disorders and cellular and gene therapies for restoring the damaged walls of blood vessels. (Mimeuolt M et al., 2008).

Adipose tissue-derived stem cells: Adipose tissue is a highly specialized, complex and active metabolic and endocrine structure that contributed to the energy storage under form of fat. In mammals adipose tissue is present in diverse anatomic compartment and designed as subcutaneous adipose tissue, internal organ-surrounding adipose tissue and interstitial adipose tissue (Shen W et al., 2003). Like BM is of mesenchymal origin and contain a stromal vascular fraction. Mature adipocytes, connective tissue matrix, nerve tissue and stromal host cells including immature MSC-like cells, fibroblast, vascular smooth muscle cells, endothelial cells, and immature cells such as the resident hematopoietic progenitor cells and macrophages compose specifically adipose tissue. Recently a putative adult stem/progenitor cells population has been identify within the human adipose compartment and termed as processed lipoaspirate (PLA) or adipose tissue derived stem cells (ADSC) (Zuk PA et al., 2002; Lin Y et al., 2006). The stromal cells isolated from the lipoaspirates express the CD29, CD44, CD71, CD90, CD105/SH2 and SH3 (Zuk PA et al., 2002); they could be distinguished from BM-derived stromal MSCs by its unique expression of antigen CD49d (a4-integrin) and CD106 (VCAM). Indeed, it has been demonstrated that ADSCs may be

differentiated into functional cells expressing the specific markers of mesodermal or ectodermal tissue origin *in vitro* and *in vivo* under well definite culture conditions. The most advantageous property of ADSCs is that they can easily obtained by surgical resection, lipo-aspiration, or ultra-sound assisted lipoaspiration, and this characteristic constitutes another promising source enriched in immature cells for cellular therapy. Among them, there are the clinical management of diverse bone, cartilage and musculoskeletal disorders (Niemela SM et al., 2007; Liu Y et al., 2007).

Muscle-Derived stem cells: Adult skeletal muscle contain two distinct stem/progenitor cells, the muscle-derived stem cells (MDSCs) and satellite cell population that may actively participate to myofiber regenerative process and repair of diseased musculoskeletal tissues (Usas A et al., 2007). Muscle-committed satellite cells expressing the marker such as M-cadherin, myogenic factor 5 (MYF5) and paired box gene 7 (PAX7) transcription factor, and neuronal cell adhesion molecule-1, are quiescent progenitor cells located at the periphery of skeletal myofibers under homeostatic conditions. The satellite cells endowed with self-renew ability may be activated and trigger a migration and differentiation into myogenic cells in vitro and after muscle injuries *in vivo* (Rouger K et al., 2007). The multipotent MDSCs, which may correspond to the more immature progenitor cells, if compared with satellite cells, can give rise to satellite cells and more committed progenies such as musculoskeletal, osteogenic, chondrogenic, vascular, cardiac and peripheral nerve cells lineages *in vitro* under specific conditions, and induce new myofiber formation in animal models *in vivo*. Muscle stem/progenitor cell-based therapy and orthopaedic tissue engineering using *ex vivo* gene therapy, are promising approaches for the treatment of muscle atrophy with aging, muscle wasting (cachexia) and various musculoskeletal and neuromuscular degenerative disorders such as muscular Duchenne and Becker dystrophies and amyotrophic lateral sclerosis (Peault B et al., 2007). At present time, no curative treatment for DMS exist and the current therapies principally consist to delay its progression and provide palliative cares that will result to the death of young patient. Importantly, the results obtained from phase I trial have revealed that the autoulogus transplantation of CD133+ MDSCs was safe, without secondary systemic effects and improved the symptoms of DMS in treated patients. Recently, MDSC or ADSC injection based-therapies have also emerging as a valid alternative therapeutic option for the remedial treatment of deficient urethral functions such as the repair of the damaged urethral sphincter associated with the stress urinary incontinence (Torrente Y et al., 2007). The genetic and/or epigenetic alteration and changes in the microenviroment "niche" of adult MDSCs and/or satellite cells or the embryonic muscle precursor may however lead to defective skeletal muscle differentiation and rabdomyosarcoma development. The metastatic forms of rabdomyosarcomas have a poor clinical management and prognosis. Thus, among the different therapeutical approaches to treat the rabdomyosarcoma, the

targeted therapies consisting in the toxic gene product delivery in satellite tumor cells by the carriers such as MSCs may represent a promising strategy. Recently has emerged that the administration of MDSCs or MDSCs to overexpress vascular endothelial growth factor (VEGF) nto an animal model of acute myocardial infraction induced angiogenesis and improved cardiac function suggesting that the MDSCs could represent an adjuvant therapy for treating the cardiovascular disorders (Payne TR et al., 2007).

Cardiac Stem/progenitor cells and heart disease: the concept that the heart as a terminally differentiated organ unable to replace working myocytes has been at the centre of cardiovascular research and therapeutic development for the last 50 years. Despite the increasing consistency of accumulating data in favour to the formation of new myocytes in a variety of physiologic and pathologic conditions, the notion of myocytes proliferation in the adult heart continues to be challenged. Several reports have provided unequivocal evidence that myocytes die and new one constantly form in the normal human heart at all stages. This process is particularly enhances by pathological states, and the imbalance between cells growth and cell death may be an important determinant of the onset of ventricular dysfunction and its evolution to terminal failure. Recent observations have indicated that the human heart contains a population of primitive cells, and such cardiac stem/progenitor cells (CSCs) found within the specialized niches localized at the apex and the atria of the heart (Leri A et al., 2005). Through growth and differentiation, primitive cells contribute to cells remodelling of the stressed heart by generating myocytes, coronary arterioles, and capillary profiles. These newly formed structures acquire the adult phenotype, are well integrated in the existing myocardium and become indistinguishable from the preexisting tissue components (Anversa P, 2004). CSCs are able to give rise to the three major cell types constituting the myocardium including cardiomyocytes, smooth muscles and vascular endothelial cells in physiological and pathological conditions. In infracted myocardium of patients with heart failure or animals model, the regenerative process appear to occur through the differentiation of resident small interstitial cells expressing nestin, KIT, Sca-1 and efflux transporters, P-glycoprotein and ABCG2 into cardiomyocytes, endothelial cells, smooth muscle cells, neuronal cells and fibroblast (Scobioala S et al., 2008). By consequence the stimulation of these endogenous CSCs or the intravascular, intramyocardial or catheter-based delivery of *ex vivo* expanded CSCs or their further differentiated progenies may constitute the therapeutic strategies for the cardiac cell replacement-based therapies (Dawn B et al., 2005). In particular the CSC-based therapies could be used to replace the aged, dysfunctional or lost CSCs by new functional cardiomyocytes and regenerate coronary vessels after cardiac injury. The transplantation of genetically modified cells also offers great promise by permitting to delivery a specific therapeutic gene product such as angiogenic

factors or survival agents of endogenous cardiomyocytes in the ischemic or non-ischemic heart diseased area. The transplantation of tumour necrosis factor receptor (TNFR) gene-modified MSCs induce an anti-inflammatory effect and inhibited the apoptotic death of resident cardiomyocytes, and thereby improved the left ventricular function in rat with acute myocardial infraction. In a similar manner the transplantation of the adenovirus carrying human vascular endothelial growth factor 165 (Ad-hVEGF165) gene-tranfected MSCs into rats with ischemic heart disease prompted the host derived angiogenesis and produced effective myogenesis as compared to MSCs transplant (Bao C et al., 2008). The transplantation of angiogenin-overexpressing human MSCs obtained after infection with adenovirus containing angiogenin gene (AdAng) also improved the heart perfusion a porcine model of chronic ischemia as compared to MSC (AdNull) (Huang SD et al., 2006). These treatments types alone or in conjunction with the conventional medical therapies should permit to improving the myocardiac regeneration and long-term outcome of patients diagnosed with heart failure, hypertension and myocardial infraction. In addiction some studies have highlighted that other progenitor cells sources, including embryonic stem cells (ESCs), umbilical cord blood cells (UCB)-derived stem cells (CD133+ cells HSCs or MSCs), amniotic epithelial stem cells (AECs), BM-derived stem cells, could restore the functional and contractile cardiomyocytes or vascular endothelial cells under specific differentiation condition (Bonanno G et al., 2007). Several studies have been focused on the benefit use of these stem cells type to repair the damaged myocardium in animal injury models *in vivo*. For instance, it has been observed that the transplantation of ex vivo differentiated cardiomyocites engraftments and improvement of myocardial performance in rat with extensive myocardial infraction. Moreover data from small clinical animal trials have also showed that the transplantation of human BM-derived stem cells, mobilized PB cells or purified CD133+ BM-derived stem cells into patients with advanced ischemic heart disease generally improved the vascularisation process and the myocardial function (Yamada Y et al., 2007). Has been emerged that BM-derived cells, ADSCs and MDSCs may contribute to the repair of the injured cardiovascular system via multiple molecular mechanism. Among them, there are the transdifferentiation of these adult stem cells into new cardiomyocytes, smooth muscle cells and/or endothelial cells as well as their release of diverse paracrine factors such as hepatocyte growth factor (HGF), insulin like growth factor (IGF-1) and VEGF that may in turn stimulate the angiogenesis and endogenous CSCs (Sadat S et al., 2007). BM-derived MSCs recruited to the infracted heart in animal model *in vivo* seem to mediate their cardio-protective effects through the releasing of paracrine growth factors and cytokines including HGF that promote the repair of cardiac lesions by inducing re-vascularisation of diseased vessels and stimulate resident CSCs that contribute to repair of damaged cardiac lesions. In counterbalance, it has been found unfortunately,

that MSCs may also differentiate into smooth muscle cells and contribute to intima hyperplasia development after coronary vascular injury. Moreover MSCs may display cytogenic instability and may differentiate toward progenies endowed with unwanted phenotype *in vivo* such as osteocytes and adipocytes that are undesirable for their therapeutic application in the cardiac repair (Atsma DE et al., 2007). Additional work appears for these reasons necessary for establish more precisely the specific biomarkers, anatomic localization, niche and endogenous CSCs within heart and the intrinsic and extrinsic factors that regulate their self-renewal and differentiation potential.

Neural stem/progenitor cells: anatomical repair and functional recovery after injuries or degeneration in the central nervous system (CSN), require specific integration of novel elements into complex neural circuitries. The mammalian adult central and peripheral nervous system have been considered during long time as non-renewal tissues, but accumulating evidences over the past few years reverse this dogma by showing that the neurogenesis may occur in adult life through self-renewal and multipotent adult neural stem/progenitor cells present in the central and in the peripheral nervous tissues (Watts C et al., 2005). Neural stem cells (NSCs) found in the human brain are localized within two specific neurogenic regions designated as the lateral subventricular zone of lateral ventricle in the forebrain, and dentate gyrus in hippocampus (Lim DA et al., 2007). Multipotent CD133$^{+/nestin}$ NSCs with an astroglia-like cell phenotype are endowed with a self-renewal potential and capable to give rise to the progenitor that can proliferate and migrate at distant damaged areas of brain where they can generate further differentiated and functional progenies (Lim DA et al., 2007). NSCs found in the subventricular zone, can give rise to several neural cell lineages: mature neurons, glial cells, astrocytes and oligodendrocytes, while the ones localized in the subgranular cell layer of the hippocampus generate the granule cell projection neurons. Moreover it has been found that NSCs may give rise to diverse neural and glial cell lineages in appropriate conditions *ex vivo* and *in vivo* (Walton NM et al., 2006). The stringent regulation of the proliferation and cell fate decision of NSCs and astroglial progenitor cells in developing and adult CNS. The local microenvironment of NSCs also may influence their behavior. Indeed, the changes in the niche components, including neighboring endothelial cells co-localized with NSCs in the subraventricular zone, may assume a critical function during regeneration process as well as during the progression of several neuropathologic diseases including the brain cancers which may arise from the alterations occurring in NSCs and their microenvironments (Lindvall O et al., 2004). Recently, the adult stem/progenitor cells derived from neural crest-derived stem cells have also been identified in peripheral nervous system within a germinal center designated carotid body (CB). Multipotent CB-resident adult stem cells, which represent the glia-like sustentacular cells expressing the glial markers can give rise to the dopaminergic glomus cells that produce the

glial cell line-derived neurotrophic factor. Several studies revealed that the *ex vivo* expanded NSCs or their progenies may be transplanted in damaged areas of brain where they can proliferate, survive, migrate, and differentiate into functional neural and glial cell *in vivo*. Moreover it has also been reported that the transplanted dopaminergic neurons derived from mouse ESCs survived for more than 32 weeks and displayed the functional properties into an animal model of Parkinson's disease (Pardal R et al., 2007). Additionally, ESCs, fetal stem/progenitor cells, UC-derived stem cells, AECs, BMSCs including MSCs, ADSCs, and pluripotent epidermal neural crest stem cells (eNCSCs) found in bulge areas within the hair follicle of the skin may also be induced to differentiate or transdifferentiate into functional neurons (tubulin-β and Tuj1), astrocytes (glial fibrillary acidic protein, GFAP) or oligodendrocytes (O4) *in vitro* and/or *in vivo*. These observations support the feasibility to use these immature cells or their further differentiated progenies for treating diverse incurable neurodegenerative diseases (Wu ZY et al. 2006) Nevertheless, before the clinical applications of NSCs, CB-resident stem/progenitor cells and other stem/progenitor cell sources for neurologic therapies, future investigations are required in order to more precisely establish the extrinsic and intrinsic factors that control their behavior within the niche in vivo as well as their therapeutic advantages at long term after treatment initiation.

<u>Corneal and Conjuntival Epithelial stem cells</u>: Several lines of evidence have revealed that the renewal of the mature corneal epithelial cells may be reached by the activation of a small population of corneal epithelial stem cells (CESCs) found within a distant compartment know as the limbus, situated at the junctional zone between the cornea and the conjunctiva (Lavker RM et al., 2004). CESCs are also designed as limbal stem cells and are mainly concentrated within the basal cell layers of the limbal epithelial crypts. The CESCs express several markers, do not form gap junctions and posses the ability to reconstitute an intact and functional corneal epithelium *in vivo* (de Paiva CS et al., 2005). Generally CESCs are maintained under mitotically quiescent state in homeostatic conditions in their specialized "niche", but changes in their local microenviroment, and particularly after corneal epithelium injury, may result in their activation. After an ocular injury, a complex network of growth factor such as EGF, FGF, TGF and PDGF and inflammatory cytokines activates the limbal CESCs and can produce by asymmetric division, the stem cells daughters that remain undifferentiated and replenish the limbal CESC pool and the early intermediate cells (Imanishi J et al., 2000). Hence, a transient renewal of the corneal epithelial cell precursors located in basal cell layer derived from limbal CESCs, may contribute to replace unfunctional or lost corneal epithelial cells after wounding, and thereby repair the damaged areas of corneal surface. The development of novel stem cell-based transplantation strategies has improved considerably the efficacy of the treatments for corneal and retinal disease, and thereby leads to the

new surgical methods to preserve or restore the vision (Mimeault M et al., 2008).

Retinal stem cells: recently has been highlighted that retina may also continue to grow after birth and new cells may be generated during lifespan after injuries due to the presence of retinal stem cells (RSCs) resident in a region adjacent to the retina, the ciliary epithelium (CE) located in the pigmented ciliary bodies of the adult mammalian eye (Moshiri A et al., 2004). These stem cells express several markers including telomerase, neural markers such as nestin, and retinal progenitor markers such as Pax6. The regulation of the signals which promote proliferation versus differentiation of RSCs are strictly regulated by activation of distinct mitogenic or differentiation signalling cascades such as FGF2, EGF, hedgehog, KIT and Notch pathways, which are also known to be important regulators of neurogenesis. Further studies are required to establish the functional properties of RSCs and the niche components as well as their possible implication in the replenishment of the functional retinal cells types in normal and pathological conditions (Ahmad I et al., 2004; Xu H et al., 2007)

Gastrointestinal Stem/progenitor cells: the gastrointestinal epithelium is a complex tissue characterized by an high cell turnover: the cells that reach the villus tip are shed from intestinal and gastric epithelium and constantly replaced by new specialized epithelial cells (Mimeault M. et al., 2008). The regeneration of intestinal epithelial cells in villi is provided by the precursor cells arising from multipotent intestinal stem cells located at the base of each crypt of Lieberkuhn. Epithelial stem cells appear to reside up at the crypt base within a protective niche containing the surrounding mesenchymal cells, expressing marker such as Msi-1, CD24, KIT, and leucine-rich-containing-G-protein-coupled receptor (Lgr5) (Barker N et al., 2007). These stem cells type are able to give rise to all cells type within each crypt and similarly stem/progenitor cells in the colon, which are localized at the crypt base, may also give rise to proliferative progenitors that differentiate into all lineages during epithelium regeneration. Moreover, the numerous glands constituting the stomach also appear to arise from gastric stem/progenitor cels residing in the niche localized in the isthmus region within each gastric gland. Such type of stem/progenitor cells are able to give rise to the progenitors that may differentiate towards all different types of specialized cells lineages that form the functional gastric glands lining the basis of the stomach. Self-renewal or differentiation of gastrointestinal stem/progenitor cells is tightly regulated by different developmental growth factor signalling such as EGF, FGF, IGF, hedgehog, Wnt/β-catenin, Notch, TGF-β cascades. Therefore, the gastrointestinal stem/progenitor cell or BM-derived stem cell-based therapies may constitute a promising approach for improving the repair of the damaged areas of intestinal and gastric epithelium after severe injury and in gastrointestinal disease as well as to develop new gastrointestinal tissue engineering strategies (Schier S et al., 2005; Andoh A et al., 2007).

Hepatic and pancreatic stem/progenitor cells: the liver posses unique and high capacity of tissue repair in order to obtain a rapid regenerative response after hepatic tissue injury and provide a rapid turn over of the mature liver cells mass (Alison MR, et al. 2004). A part from the mature hepatocytes, that can undergo several cell division cycles and are responsible for continuous hepatic cell replacement, a small population of postnatal stem/progenitor cells (designed as hepatic oval cells HOCs) has been found to reside in the smallest units of the intra-hepatic bile ducts within the periportal region of adult liver. The HOCs may express several phenotypic markers of hepatic cells as well as HSCs. The HOCs have been found to contribute to liver repair after the activation of a complex signalling network involving numerous growth factor and cytokine pathways. In addiction the hepatocyte-like cells can also be derived from ESCs, UCB, BM and pancreatic stem/progenitor cells under specific culture condition in vitro and/or in vivo, and thereby these cells could constitute the alternative extra-hepatic sources for liver regeneration in pathological conditions (Cantz T et al., 2008). Finally since hepatic progenitor cells can be easily expanded, genetically manipulated and potentially used in transplantation for a long-term replacement of hepatic cells, they represent a promising strategy that could lead to development of new cell-based therapies for chronic liver disease. Regarding pancreas progenitor cells, recent lines of evidence have revealed that the human and rodent mature insuline-producing islet β-cells could arise from adult pancreatic stem/progenitor cells (PSCs) expressing ductal epithelial cells markers, cytokeratina 19 (CK19), neural (nestin) and endocrine nuclear pancreatic and duodenal homeobox factor-1 (PDX-1) markers and/or more committed nestin^{+}/PDX-1^{+}/CD19^{low} islet precursor localized in the ductal regions and/or within islet compartment (Oertel M et al., 2007). These poorly differentiated adult putative PSCs and their early progenies within the adult pancreas could represent a potential source of β-cells for the cell replacement or gene therapy for treating the type 1 or 2 diabetes mellitus. In addiction the use of functional pancreatic insulin-producing β-cells-like progenitors derived from other stem cells types (embryonic, fetaland UCB stem/progenitor cells, human amniotic epithelial cells (hAECs), placental-derived multipotent stem cells (PDMSCs), and adult stem cells including HSCs, MSCs, HOCs, NSCs, hAECs, ADSCs) also may represent an alternative therapeutic strategy for the treatment of type 1 or 2 diabetes mellitus. Additional in vivo studies appear however to be necessary for establishing the beneficial effects to use these cell types for patient suffering pancreatic disease in the clinics; particularly, it will be essential to establish the effects of the insulin-producing progenitors on the restoration and normalization of the blood sugar levels after long-term treatment (Mimeault M et al., 2008).

Cancer stem/progenitor cells: the recent progress in the stem cells field have also revealed the implication of leukemic and tumorigenic cancer stem/progenitor cells in the initiation and

progression of the most aggressive and recurrent cancers and treatment resistence (Peters BA et al., 2005) Moreover further differentiated tumor cell and activated stromal cells including myofibroblast as well as BM-derived cells including immune cells, macrophages, HSCs and EPCs attracted at the tumoral sites, also may actively collaborate during each step of the tumor formation at the primary and secondary sites. Indeed, the targeting of the cancer stem/progenitor cells and their local microenviroment involved in the cancer development offer great promises for the development of new therapeutic approaches for targeting the aggressive metastatic and recurrent cancers in the clinic. Specifically, the molecular targeting of the oncogenic cascades such as EGFR, hedgehog, and Wnt/β-catenin, oncogenic signalling elements that assume a critical role in regulating the stem cells self-renewal, differentiation, survival and/or drug resistance, is of particular therapeutic interest for eradicating the cancer-initiating cells (Kaplan RN et al., 2005).

Immunosuppressive properties of MSCs: emerging lines of evidence indicate that MSCs posses immunomodulatory properties, and may play specific roles as immunomodulators in maintenance of peripheral tolerance, transplantation tolerance, autoimmunity, tumor evasion as well as fetal-maternal tolerance (Nauta AJ et al., 2007). The first evidence of the immunosuppressive activity on T cells of MSCs derived from studies with human, baboon, and murine MSCs, where they were found to be able to suppress T lymphocyte activation and proliferation *in vitro* (Di Nicola M et al., 2002; Le Blanc K et al., 2003). Most studies agree that soluble factors are involved because separation of MSCs and peripheral blood mononuclear cells (PBMCs) by a semi permeable membrane does not prevent inhibition of proliferation. However, the suppressive factor is not constitutively secreted by MSCs because cell culture supernatants do not suppress T-cell proliferation (Le Blanc K et al., 2006). Dendritic cells (DCs) play a key role in the induction of immunity and tolerance; MSCs have been demonstrated to interfere with DC differentiation, maturation and function. MSCs reduced DC secretion of pro-inflammatory cytokines IFN-γ, IL-12, and TNF-α, whilst production of the suppressive cytokine IL-10 increased (Jiang XX et al., 2005). Moreover MSCs exert an immunosuppressive effect also on B cells, since they have been shown to inhibit lymphocyte proliferation by anti-Ig antibodies, soluble CD40 ligand, and cytokine. In addition, differentiation, antibody production and chemotactic behaviour of B cells were affected by MSCs (Glennie S et al., 2012). The immunomodulatory effects of MSCs have been examined in a variety of animals models related to alloreactive immunity (organ and stem cell transplantation), autoimmunity or tumor immunity. One of the first *in vivo* studies demonstrated that systemic infusion of allogenic MSCs derived from the BM of baboons prolonged the survival of allogenic skin graft to 11 days compared with 7 days in animals not receiving MSCs (Nauta AJ et al., 2007). Indeed, one of the most impressive *in vivo* effects of MSCs has been observed in the treatment of

graft-versus-host disease (GVHD) after allogenic stem cell transplantation. Systemical infusion of ex-vivo expanded MSCs derived from adipose tissue was able to control lethal GVHD in mice transplanted with haploidentical hematopoietic stem cells graft (Yañez R et al., 2006). The current body of data on the immunosuppressive properties of MSCs holds great promise for treating immune-mediated disorders; however, it is important to not overestimate the potential therapeutic effects of MSCs, and indeed many questions need to be addressed before the potential therapeutic promise of these cells can be realized.

Mesenchymal stromal cells in Veterinary Medicine:

Contextually to the interest that over the past few years, MSCs have risen in human medicine, also in the veterinary field there has been an increased interest in understanding the biology and potential clinical application of MSCs. Indeed MSCs research in veterinary medicine has been performed not only in order to find a potential clinical treatment for previous incurable veterinary disease, but also to develop animal models, which could be useful to elucidate the MSCs *in vitro* and *in vivo* behaviour, and use the obtained results as a template for human MSCs research. While widespread clinical use of human adult stem cells is largely restricted to the use of hematopoietic stem cells derived from adult peripheral blood, adult bone marrow, or umbilical cord blood (Koch TG et al., 2009), MSCs in veterinary field are mainly utilized to study their application and their potential regeneration ability in musculoskeletal tissue (Arthur A et al., 2009). MSC within the skeletal tissue contribute to the normal remodelling and repair process by providing the pool of osteoblast necessary to form the mineralized matrix of bone. However, in some pathological conditions, including non-union fractures, osteoporosis, osteoarthritis and infection the normal repair and remodelling process are often impaired. Furthermore, other associated connective tissues such as cartilage, tendon and ligament demonstrate a limited capacity for regeneration in response to damage caused by trauma or disease. For these reasons, MSCs offer promise as novel cell-based therapies to ameliorate the healing process of damaged bones, tendon and ligament (Chang YJ et al., 2006). Preliminary studies carried out in rodents have provided strong evidence for the potential of MSC in skeletal tissue regeneration, and have prepared the way for ostochondral tissue engineering studies in larger animals and more recently human clinical trials (Arthur A et al., 2009). *In vivo* studies employing rabbit as animal model have demonstrated that MSCs transplantation increased bone production and the stiffness of the regenerated structures using mechanical strength testing and animals models where the sight of injury was in weight-bearing location (Tatebe M et

al., 2005). Subsequently canine and sheep models have been used to test the feasibility of stem cell based approach for bone regeneration and repair (Brodke D et al., 2006; Viateau V et al., 2007). In the large animal studies, autologous MSC transplanted alone or in porous ceramic scaffold, significantly increased bone formation. Importantly, the treated bones retained the same strength as the uninjuried control bones by 32 weeks after transplantation in the caprine study, and by 8 weeks in the ovine study (Kon E et al., 2000; Liu G et al., 2008). The study of Brodke et al., (2006) demonstrated enhanced bone formation when MSC were transplanted in the presence of platlet-rich plasma, and the study of Dallari et al., (2006) proved the same. Animal models have been used also to study the vertebral disk regeneration: Muschler et al. (2003) demonstrated that, in a canine model of spinal fusion, an enriched bone matrix containing a bone marrow clot have a greatest union, stiffness and number of osteogenic cells compared to bone marrow alone. Rodent and large animal models including rabbit, dogs, pigs and sheep, have demonstrated that MSC are a reliable alternative for bone repair of cranial bone defects when compared to other cell types, such as spleen stromal cells (Krebsbach PH et al., 1998); the majority of studies investigating the therapeutic potential of culture expanded MSC to regenerate alveolar bone have been carried out in large animal canine and caprine models (Dégano IR et al., 2008). Regarding the cartilage regeneration, research conducted using rabbits demonstrated that MSCs in combination with scaffold or fibrin gels produced hyaline-like cartilage that integrated with the surrounding cartilage and improved the repair of the osteochondral defects created in the knee (Chang F et al., 2008). Similarly, Mrugala (2008) and colleagues demonstrated new cartilage formation 9 weeks following transplantation of MSCs to an ovine model of articular cartilage defect. Another study using a large animal model, reported similar findings, where a chondral defect in sheep treated with autologous MSCs, exhibited an increase in the amount of cartilage-like tissue and greater repair (Dorotka R et al., 2005). In a caprine model of osteoarthiritis of the knee, administration of *ex vivo* expanded autologous MSCs 6 weeks post-injury resulted in the integration of MSCs within the cartilage matrix and reduction of the onset of osteoarthritis up to 20 weeks post MSCs transplantation (Murphy JM et al., 2003). Due to the lack of effective treatments available for tendon and ligament related injuries, researchers have started to investigate the therapeutic potential of MSCs for tendon and ligament repair. The majority of the studies relating to the repair of tendons and ligament by MSCs are limited to *in vitro* assay, rat, rabbit, pig, and horse animal models; from them it appears that MSCs therapy in combination with either a collagen, laminin or fibrin scaffold may be effective in the initial stages of tendon repair (Hirfild-Stein M et al., 2007; Kajikawa Y et al., 2007). Preliminary evidence based on *in vitro* (Liu Y et al., 2007) and small animal studies, (Watanabe N et al., 2002) suggest that both autologous and allogenic derived MSCs display phenotypic characteristics of the endogenous

surrounding tissue following transplantation.

Undoubtedly horse is established as an animal model for focal cartilage injuries and osteoarthritis (Goodrich LR et al., 2007; Frisbie DD et al., 2001). Advantages of horse joint models compared with those of the of the other animals, are their sheer size, which allows for easy manipulation and exploration, and their cartilage thikness and composition, which most closely resemble those of human articular cartilage among the current animal models (Fisbie DD et al., 2001). The horse has also been advocated as an animal model of tendon and ligament injuries, since many of the spontaneous injuries seen in the horses are similar to those seen in human athletes (Smith RK et al., 2005). Other equine tissues and diseases such as wounds and various hypoxic ischemic injuries seem like straightforward candidates for equine stem cells research. It has been argued that indisputable proof of stem cells as curative of animal disorder/disease may provide the "tipping point" for the public debate surrounding human stem cell research (Fiester A et al., 2004). Cell-based therapy using MSCs are increasingly been reported in equine medicine, because races horses, like human athletes often suffer of musculoskeletal disease which currently are not curable. In equine medicine the therapeutic use of MSCs derived from bone marrow has been reported (Guest DJ et al., 2008).

In 2003 Smith et al., were the first to report on implantation of cultured-expanded autologous bone marrow derived MSCs into a spontaneously occurring core lesion of the superficial flexor tendon. This case demonstrated the feasibility of using culture-expanded MSCs therapeutically and more important the absence of adverse reaction at 10 days or 6 weeks post injection. In 2007, Pacini et al. reported that 9 of 11 Italian racehorses with spontaneously occurring incomplete lesions of the superficial digital flexor tendon returned to racing and were still racing two years after treatment with bone marrow-derived MSCs. Crovace et al. in 2007 created core lesions in the superficial digital flexor tendon by injecting collagenase in 3 horses. The lesion were subsequently treated with either culture-expanded BM-MSCs suspended in fibrinogen, freshly isolated mononuclear cells from the bone marrow aspirates suspendend in fibrinogen, or a placebo treatment with as unknown substance. Moreover autologous and allogenic BM-MSC were transfected with green fluorescent protein (GFP) prior to injection into surgically induced lesions of the superficial digital flexor tendon in two horses (Guest DJ et al., 2008). The pilot study was not designed to evaluate treatment efficacy, but it warrants attention because it involved the injection of BM-MSCs of allogenic origin, as well as the use of a cellular marker, and for this reason will greatly strengthen future claims of MSCs treatment efficacy and the mechanism by MSCs exert their effect.

The use of MSCs in equine cartilage repair is increased in the past few years. However most of the work has been restricted to *in vitro* studies, 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 BM-MSCs. In this study it was found that the fibrin/BM-MSC-treated group had improved healing characteristic compared with the group treated with fibrin glue alone; but no difference in treatment outcome between two groups was noted after 8 months after treatment. Indeed more studies are needed to determine if the initial difference was a concrete MSC-mediated effect.

Autologous cancellous bone is often used in equine patients with substantial bone loss in order to enhance bone repair by providing a scaffold of osteoprogenitors cells and various growth factors. However the number of osteoprogenitor cells in equine cancellous bone has been shown to vary between donor sites and due to decrease potency of MSCs. The use of cultured-expanded or otherwise purified and concentrated equine MSCs may therefore be desiderable in selected cases in order to obtain a sufficient number of cells with appropriate osteogenic potential. Even if there are a lots of reports which have evaluated the *in vitro* potential of MSCs from various equine tissue sources to differentiate into the ostognenic cell lineages, the *in vivo* use of cultured-expanded or purified MSCs or fractons of mononuclear cells in equine experimental or clinical cases for enhanced bone repair have not been reported.

Chapter 2: Tendon structure and injuries:

Tendon anatomy:

Structure:

Tendons vary in form, and can be rounded cords, strap-like bands or flattened ribbons (Sharma P et al., 2006). They link muscles to bone at the musculo-tendinous junction and osteo-tendinous junction or enthesis respectively. Tendons have a hierarchical structure and are composed by a cellular unit the tenocytes, a fibroblastic-like cell that produces collagens, lying within a network of extracellular matrix (ECM). Tendon's ECM is predominantly composed by water (55-70%), collagen type I, elastin, proteoglycans, glycosaminoglycans and glycoprotein including fibronectin, thrombospondin, tenascin, decorin, biglycan, cartilage oligomeric matrix protein. The collagen protein forms a triple-helical, rod-shaped molecule that spontaneously associates with other

collagen molecules to form a quarter-straggered fibrillar array that establishes the characteristic tendon matrix. Fibrillar collagen type 1 gives tendons their high tensile strength and is responsible for the hierarchical structure: soluble tropocollagen molecules (a triple helix with two alpha-1 chains and one alpha-2 chain) spontaneously self-assemble after secretion and cross-linking (which result in the formation of insoluble collagen molecules) into collagen miscrofibrils. Microfibrils arrange themselves into larger units, called subfibrils, in ligaments or subfascicles/primary fibre bundles in tendons. The fibrils then gather into collage fibers or fascicles/secondary fibre bundles. A collagen fibre is the smallest tendon unit wich can be mechanically tested and is visible on light microscopy. Although collagen fibers are mainly orientated longitudinally, fibers also run transversely and horizontally, forming spirals and plaits. In addition, tendons are bound together by the endotenon, a loose connective tissue that also includes blood, lymph vessels and nerves and is continuous with the epitenon, which surrounds the whole tendon. Surrounding the epitenon superficially, another thin layer, called paratenon, is present, which allows free movements within the surrounding tissue. Epi- and paratenon together constitute the peritenon. Long tendons such as the digital flexor tendon are additionally enclosed in a synovial sheath that gives lubrification and enhances gliding (Liu CF et al., 2011).

The cellular elements within the tendons are intercalated between the collagen bundles and lie along the long axis. The fibroblast are termed "tenoblast" when they are still immature, they are spindle-shaped, with numerous cytoplasmatic organelles reflecting their high metabolic activity. As they aged tenoblast become elongated and transform into "tenocytes": these have a lower nucleus-to-cytoplasm ratio than tenoblast, with decreased metabolic activity. Together tenoblast and tenocyte account for the 90-95% of the cellular elements of tendons. The remaining 5-10% consist of chondrocytes at the bone attachment and insertion sites, synovial cells of the tendon sheath and vascular cells, including capillary endhotelial cells and smooth muscle cells of arterioles (Sharma P et al., 2006). Tenocytes synthesize collagen and all components of the ECM, and are also active in energy generation. The aerobic Krebs cycle, anaerobic glycolysis and the pentose phosphate shunt are all present in human tenocytes. With increasing age, metabolic pathways shift from aerobic to more anaerobic energy production (Kannus P et al., 2000).
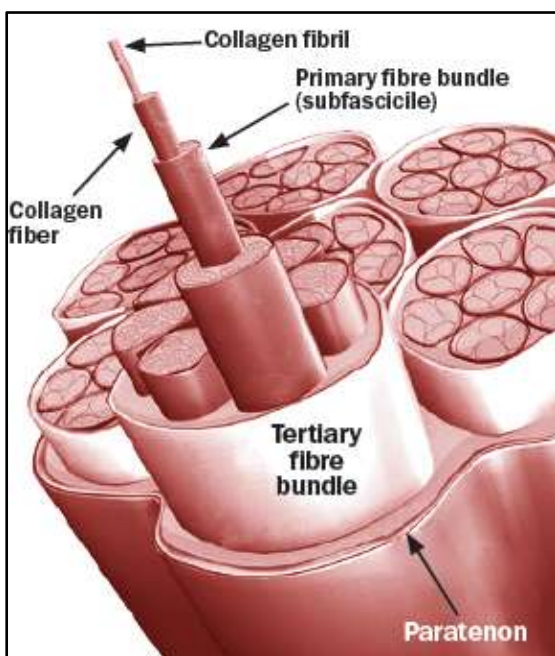
Fig. 4: The hierarchical architecture of tendon.

Tendons are extraordinarily strong in resisting tensile loads. Given to their low metabolic rate and well-developed anaerobic energy generation capacity, tendons are able to carry loads and maintain tension for long periods. This characteristic reduces the incidence of ischemia and necrosis, but on the other hand the low metabolic rate entails slow healing after injury. Tendons are visco-elastic tissues in exhibiting time-dependent strain and relaxation rates. A stress-strain curve helps to demonstrate the behaviour of the tendon (Fig. 5). At rest collagen fibers and fibrils display a crimped configuration. The initial concave portion of the curve (toe region), where the tendon in strained up to 2%, represents flattering of the crimp pattern. Beyond this point, the tendon deforms in a linear fashion due to intramolecular sliding of collagen triple helices, and the fibers become more parallel. Below a strain of about 4% a tendon behaves in an elastic way, which means that it returns to its original length and performance upon strain release. A strain excess of 4% causes microscopic or macroscopic failure (Hoffmann A et al., 2007).
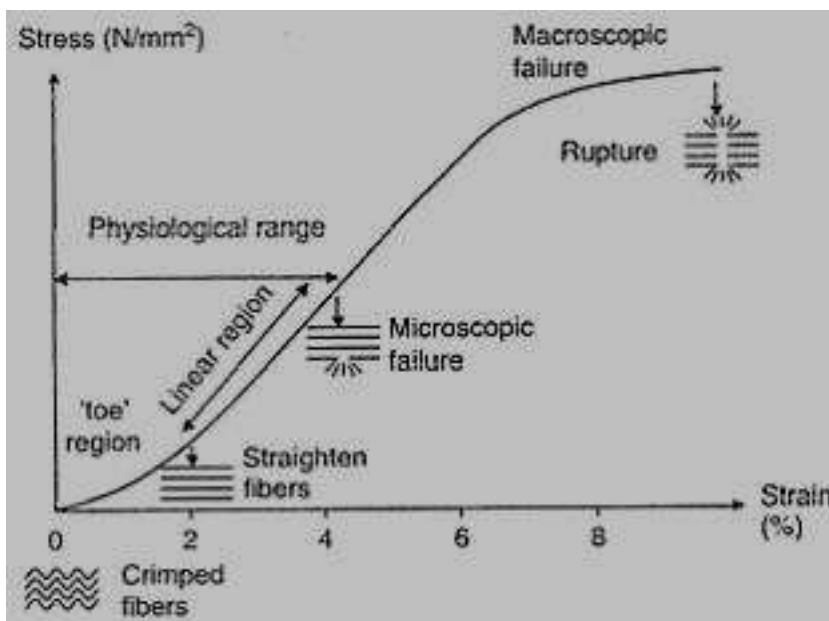

Fig. 5: Stress-strain curve demonstrating the basic physical properties of the normal tendons.

A second and less mentioned function of tendinous tissue is the propioception: mechanoreceptors serve to protect from extremes of motion, thereby maintaining the stability of joints or provide feedback control that changes muscle activity when resistance to movement is encountered.

At the myotendinous junction (MTJ) tendinous coolagen fibrils are inserted into deep recesses formed by myocyte process, allowing the tension generated by intracellular contractile proteins of

muscle fibers to be transmitted to the collagen fibrils. This complex architecture reduces the tensile stress exerted on the tendon during muscle contraction. However the MTJ still remains the weakest point of the muscle-tendon unit (Kvist M et al., 1991). The osteotendineus junction (OTJ) is composed of three zones: a dense tendon zone, fibrocartilage, and bone; its specialized structure prevents collagen fibre bending, fraying, shearing, and failure.

Blood supply:

Tendons receive their blood supply from three main sources: the intrinsic system at the MTJ and OTJ, and from the extrinsic system via the paratenon or the synovial sheath (Carr AJ et al., 1989). Tendons enveloped by sheaths to reduce friction, branches from major vessels pass through the vincula (mesotenon) to reach the visceral sheet of the synovial sheath, where they form a plexus. This plexus supplies the superficial part of the tendon, while some vessels from the vinculae penetrate the epitenon. These penetrating vessels course in the endotenon septae, and form a connection between the peri- and intra-tendinous vascular networks. In the absence of synovial sheaths, the paratenon the extrinsic component of the vasculature. Vessels entering the paratenon course transversely and branch repeatedly to form a complex vascular network (Jozsa L et al., 1997).

Tendon innervation:

Tendon innervation derived from cutaneous, muscular, and peritendineous nerve trunks. Nerve fibers from rich plexus in the paratenon, and branches penetrate the epitenon. Most nerve fibers do not actually enter the main body of the tendon, but terminate as nerve ending on its surface. Nerve ending of myelinated fibers function as specialised mechanoreceptors to detect changes in pressure or tension (the Golgi tendon organs). Unmyelinated nerve endings act as nociceptors, and sense and transmit pain. Both sympathetic and para-sympathetetic fibers are present in tendon (Lephart SM et al., 1997; Ackermann PW et al., 2001).

Tendon formation during embryonic development:

Until recently there was no comparable understanding of the origins of the axial tendons that connect axial muscle and skeleton. However, the discovery of the basic-helix-loop-helix transcription factor (SCX), both identified a molecular marker for tendon progenitor cells and allowed more mechanistic studies of axial tendon formation. SCX mRNA is expressed both in fully formed tenocytes and in the progenitor cells of tendons in the embryo (Schweitzer R et al., 2001). Brent et al. (2003) demonstrated that SCX-expressing progenitor cells of trunk tendons first appeared between the myotome and sclerotome during somite development. During embryogenesis,

a unique compartment of the somite, called the syndetome, provides tenocyte progenitors for the development of the axial tendons. Inductive interactions between the well-described myotome and sclerotome layers generate the syndetome, demarcated at the earliest stages of development by the expression of the SCX (Brent AE et al., 2003). However, although SCX expression has been implicated as necessary, it seems not sufficient to trigger tendon formation; other transcription factors involved in tendon formation are still to be elucidated but may be Six1, Six2, as well as Eya proteins, wich are expressed during limb formation in developing tendons. However no master genes and not many real markers genes for tendon development have yet been identified.

Tendon injury:

Tendon injuries, degenerative tendinophaty and overuse tendinitis are very common both in human and veterinary field. It has been estimated that 30 billion dollars are spent on musculoskeletal injuries in the United States each year, and tendon/ligament injuries represent about 45% of these injuries (Praemer A et al., 1999); in addiction surgical repair is often unsuccessful. Approximately 50% of the population by the age of 60 will have suffered a degenerative rotator cuff tear. Although small rotator cuff tears have better outcomes, surgical repairs of large tears show failure rates as high as 90% due to muscle contraction, decreased range of joint motion, neurovascular damage, or altered shoulder mechanics (Jost B et al., 2000; Klepps S. et al., 2004).

In the veterinary field, overstrain injuries to weight-bearing tendons are common in racing animals that can run fast for long distances. The horse is particularly predisposed to overstrain injury of the palmar soft tissue structures of the distal limb due to hyperextension of the metacarpophalangeal joint during weight-bearing. Of these structures, the superficial digital flexor tendon suffers the highest frequency of injury (Genovese R et al. 1996; Goodship AE et al. 1994; Kasashima Y et al. 2004). Strains of the superficial digital flexor tendon (SDFT) account for up to 46% of limb injuries in racing Thoroughbreds (Williams RB et al., 2001). The strain in this tendon is proportional to the force on the limb and hence increases with speed. The most recent epidemiological data suggest that approximately one-quarter of National Hunt racehorses in training are affected by the disease (Dyson S et al., 2004; Avella CS et al., 2009; Ely ER et al., 2009), with individual yards reaching frequencies of 40% or more (Pickersgill G et al., 2000; Avella CS et al., 2009). In younger flat racehorses the frequency of injury is less at 11%, but increases with age from 6% in 2-year-olds to 16% in >5-year-olds (Kasashima Y et al. 2004). This age-related incidence, together with experimental studies on the influence of exercise on equine tendon suggests that, while injury appears to be spontaneous, occurring most commonly during high speed exercise, it is preceded by degenerative changes occurring within the extracellular matrix (Goodship AE et al., 1994; Smith RKW et al., 2003; Birch HL et al., 2008).

Tendon injuries can be acute or chronic, and are caused by intrinsic or extrinsic factor, either alone or in combination. In acute trauma, extrinsic factors predominate. Whilst in chronic cases intrinsic factors also play a role.

Tendinopathy: The aetiology of tendinopathy remains unclear, and many causes have been theorised. Hypoxia, ischaemic damage, oxidative stress, hyperthermia, impaired apoptosis, inflammatory mediators, fluoroquinolones, and matrix metalloproteinase imbalance have all been implicated as mechanisms of tendon de generation (Goodship AE et al., 1994). Histologically, tendinopathy shows a picture of disordered haphazard healing with absence of inflammatory cells, poor healing response, non-inflammatory intratendinous collagen degeneration, fibre disorientation and thinning, hypercellularity, scattered vascular ingrowth, and increased interfibrillar glycosaminoglycans. Inflammatory lesions and granulation tissue are infrequent, and are mostly associated with tendon ruptures. Macroscopically, the affected portions of the tendon lose their normal glistening white appearance and become greybrown and amorphous. Tendon thickening, which can be diffuse, fusiform or nodular, occurs. Tendinosis is often clinically silent, and its only manifestation may be a rupture, but it may also co-exist with symptomatic paratendinopathy.

Tendon rupture: Tendon rupture is an acute injury in which extrinsic factors predominate, although intrinsic factors are also important. Malfunction of the normal protective inhibitory

pathway of the musculo-tendinous unit may result in injury. The aetiology of tendon rupture remains unclear. Degenerative tendinopathy is the most common histological finding in spontaneous tendon ruptures. Tendon degeneration may lead to reduced tensile strength and a predisposition to rupture.

Tendon healing:

Tendon healing studies have predominantly been performed on ruptured animal or human tendons, and their relevance to human tendinopathy its associated with failure and healing response remains unclear. Tendon healing occurs in three overlapping phases. In the initial inflammatory phase, erythrocytes and inflammatory cells, particularly neutrophils, enter the site of injury. In the first 24 hours, monocytes and macrophages predominate, and phagocytosis of necrotic materials occurs. Vasoactive and chemotactic factors are released with increased vascular permeability, initiation of angiogenesis, stimulation of tenocyte proliferation, and recruitment of more inflammatory cells. Tenocytes gradually migrate to the wound, and type III collagen synthesis is initiated. After a few days, the remodeling stage begins. Synthesis of type III collagen peaks during this stage, which lasts for a few weeks. Water content and glycosaminoglycan concentrations remain high during this stage. After approximately 6 weeks, the modeling stage commences. During this stage, the healing

tissue is resized and reshaped. A corresponding decrease in cellularity, collagen and glycosaminoglycan synthesis occurs. The modelling phase can be divided into a consolidation and maturation stage. The consolidation stage commences at about 6 weeks and continues up to 10 weeks. In this period, the repair tissue changes from cellular to fibrous. Tenocyte metabolism remains high during this period, and tenocytes and collagen fibres become aligned in the direction of stress (Murphy PG et al., 1994; Oakes BW et al., 2003; Tillman LJ 1996). A higher proportion of type I collagen is synthesized during this stage. After 10 weeks, the maturation stage occurs, with gradual change of fibrous tissue to scar-like tendon tissue over the course of one year. During the latter half of this stage, tenocyte metabolism and tendon vascularity decline. Tendon healing can occur intrinsically, via proliferation of epitenon and endotenon tenocytes, or extrinsically, by invasion of cells from the surrounding sheath and synovium (Gelberman RH et al., 1984). Epitenon tenoblasts initiate the repair process through proliferation and migration. Healing in severed tendons can be performed by cells from the epitenon alone, without relying on adhesions for vascularity or cellular support. Internal tenocytes contribute to the intrinsic repair process and secrete larger and more mature collagen than epitenon cells. Despite this, fibroblasts in the epitenon and tenocytes synthesize collagen during repair, and different cells probably produce different collagen types at different time points. Initially, collagen is produced by epitenon cells, with endotenon cells later synthesizing collagen. The relative contribution of each cell type may be influenced by the type of trauma sustained, anatomical position, presence of a synovial sheath, and the amount of stress induced by motion after repair has taken place. Tenocyte function may vary depending on the region of origin. Cells from the tendon sheath produce less collagen and GAG compared to epitenon and endotenon cells. However, fibroblasts from the flexor tendon sheath proliferate more rapidly (Ingraham JM et al., 2003). The variation in phenotypic expression of tenocytes has not been extensively investigated, and this information may prove useful for optimizing repair strategies. Intrinsic healing results in improved biomechanics and fewer complications. In particular, a normal gliding mechanism within the tendon sheath is preserved. In extrinsic healing, scar tissue results in adhesion formation, which disrupts tendon gliding. Adhesion formation after intra-synovial tendon injury possess a major clinical problem. Synovial sheath disruption at the time of injury or surgery allows granulation tissue and

tenocytes from surrounding tissue to invade the repair site. Exogenous cells predominate over endogenous tenocytes, allowing the surrounding tissues to attach to the repair site

resulting in adhesion formation (Riederer-Henderson MA et al., 1983). Despite remodeling, the biochemical and mechanical

properties of healed tendon tissue never match those of intact tendon. Therefore, the ultimate aim

of all cellular and gene therapies would be to restore functional tissue. In order to pursue this aim, different studies have been undertaken. These include: local delivery of growth factors, stem cell and tendon-derived cell therapy, and gene-therapeutic approaches based on vehicles encoding selected factors, or combination of these (Sharma P et al., 2006).

IN VIVO BIOLOGICAL TREATMENTS OF TENDON INJURIES:

Treatment	Type of tendon	Deliver methods	Results
bFGF	ACL in dog	Implantation of bFGF pellet	Enhanced the healing process of the injured ACL
bFGF	MCL in rabbit	Carried by fibrin gel with recombinant human bFGF	Promoted early formation of repair tissue
bFGF	PT in rat	Injected with increasing doses	Increased the expression of collagen type 3 but there was no significant difference on ultimate stress and the pyridinoline content between healing tendon and control groups.
bFGF	Flexor tendon in dog	Fibrin-heparin-based delivery	Failed to produce improvements in either the mechanical or functional properties of the repair. Increased cellular activity resulted in peritendinous scar formation and diminished range of motion.
GDF5	Zone II flexor tendon repairs in a rabbit flexor tendon	Sutures coated with GDF5	All tendons were failed at the repair site but the GDF5 treatment group showed better outcomes on maximum load at early treatment
GDF6	Achilles tendon in rat	Injected GDF6 locally in the defect site	Tendons were 39% stronger than the controls
IGF-1	Achilles tendon in rat	Injected LR3-IGF01	Increased the healing rate by reducing inflammation
MSCs from bone marrow	MCL in rat	Injected 10^6 nucleated cells of bone marrow	MSC from bone marrow may serve as a vehicle for therapeutic molecules and to be a source in enhancing healing of ligaments.
MSCs from bone marrow	Achilles tendon in rabbit	Implanted with autologous, culture-expanded MSC constructs	Delivered MSC-contracted, organized collagen implants to large tendon defects. Significantly improeved the biomechanics, structure, and probably the function of the tendon after injury.
MSCs from bone marrow	PT in rabbit	Seeded in collagen-based construct and mechanically stimulated in culture	Matched normal tendon tangent stiffness up to 50% beyond peak in vivo forces measured during activities of daily living.

IGF-1 and TGF-β1	PT in rabbit	Mixed with fibrin sealant as a delivery vehicle	Significant increase in force at failure, ultimate stress, stiffness, and energy uptake at 2 weeks comparing to the control group.
PDGF, PDGF+IGF, PDGF+bFGF	MLC in rat	Directly injected	Increased the healed ligament strength, stiffness and breaking energy in three treatment groups compared to controls.
PDGF-BB	Achilles tendon in rat	Delivered using nanoparticles	Increased the healing process.
SDF-1	Achilles tendon in rat	Surgically implanted knitted silk-collagen sponge scaffold	The expression of tendon repair gene markers and endogenous SDF-1 were increased.
TGF-β1	ACL in rabbit	Adenoviral vactor containing TGF-β1	Induced relatively rapid and continous proliferation of ACL fibroblast and high gene expression of collagen type I, collagen type III, and fibronectin mRNA among matrix markers.
TGF-β2	MCL in rabbit	Adenoviral vector conraining TGF-β2	Increased type I collagen expression and profoundly increased early scar mass.
VEGF	Flexor tendon in rabbit	Adenoviral vector containing VEGF165	Induced relatively rapid and continous proliferation of ACL fibroblast and high gene expression of collagen type I, collagen type III, and fibronectin mRNA among matrix markers.

Table 2: bFGF, basic fibroblas growth factor; ACL, anterior cruciate ligament; MCL, medial collateral ligament; PT, patellar tendon; GDF, growth and differentiation factor; IGF, insuline-like growth factor; MSC, mesenchymal stem cells; TGFβ, transforming growth factor β; PDGF, platelet derived growth factor; VEGF, vacular endothelial growth factor; SDF-1, stromal cell-derived factor-1.

GENERAL AIM

The present work has been focused on two main subject correlated between each other: the study of adult mesenchymal stromal cells (MSCs) isolated from horses and from dog tissues, and the application of MSCs in musculoskeletal disease, specifically in tendon healing process.

Adult MSCs gained general attention, both in the human and veterinary research field, due to the great promise they offer as a resolute alternative for the treatment of disease that currently lack a satisfactory clinical resolution. Moreover adult MSCs posses considerable advantages respect to embryonic stem cells (ES), since the latter receives attention because of the ethical controversies associated with the destruction of human embryos, and the possibility to give rise tumors, when clinically applicated. Despite stem cells may direct a new era in medical and surgical therapies, understand the basic biology of stem cells remains of fundamental importance in both human and veterinary medicine. The major aim of the first part of this work has been to better characterize adult stem cells isolated from peripheral blood of horses (ePB-MSCs) and from adipose tissue of dog (cA-MSCs), by analyzing some features like the cluster of differentiation expression, the population doubling time, the telomerase activity. Moreover, further investigations have been performed to study the plasticity of these cells, which means their ability to differentiate, under proper culture stimulations, into different lineages respect to the original one. The notion of unidirectional tissue-lineage commitment of stem cells is being challenged by evidence of adult conversion in adult stem cells (Wagers AJ et al., 2004). With the increasing perspective to use MSCs for clinical purposes, several techniques to preserve and deliver MSCs has been developed in human medicine, as well as the creation of cells bank. Cryopreservation for long-term storage of MSCs became a necessity to facilitate the application of cell-based therapy. Thanks to the technical advancements, cryopreservation protocols have been improved: in fact HSCs as well as UCB-MSCs have been extensively cryopreserved and successfully used for transplantation. In veterinary field there is a lack of studies focusing on cryopreservation and delivery of MSCs that should be utilized in clinical trials. Indeed the effect of one year of cryopreservation on ePB-MSCs and on cA-MSCs have been evaluated in this work, as a consequence of the positive results obtained from their isolation and characterization, and with the aim to verify the possibility to successful cryopreserve these cells in order to use them for clinical purposes. The "stemness" properties of one year cryopreserved ePB-MSCs and of cA-MSCs have been verified and compared with not cryopreserved cells, using the same parameters utilized for characterization and differentiation

potential above mentioned. To further investigate alternative delivery methods for MSCs in the short-term period, a third study was carried on, with the aim to study the maintenance of MSCs in suspension, under the influence of parameter that likely influence cells survival in non-frozen conditions. The final goal of this study was to provide useful guidelines for MSCs delivery in the short term period, optimizing their survival and ensuring their number for their potential use in clinical trials.

The second major topic of this work was to study the regenerative capacity of stromal cells derived from mesenchymal tissue in tendon healing process. Specifically, MSCs isolated from adipose tissue (human lipoaspirate) or from peripheral blood of sheep (sPB-MSCs), where respectively applied in tendon scaffold or in experimental induced tendon injuries.

Tendon injuries are common in adults, necessitating over 300,000 surgical tendon repairs each year in the United States. At the same time tendons heal capacity is poor, because of the limited endogenous regenerative potential. By consequence the new tissue regeneration strategies, aim to improve the outcome of tendon repair. The goal of the first work focused on tendon healing, was to obtain a human biocompatible tendon to be recellularized with MSCs derived from human adipose tissue with the future perspective to implant the recellularized scaffold in human full thickness lesions of flexor tendons of the hand. Overstrain injuries of flexor tendons are very common also in veterinary field, particularly in races horses, due to the hyperextension of the metacarpophalangeal joint during weightbearing. For this reason a second project on tendon healing has been carried on, and Bergamasca sheep has been utilized as an experimental model for horses, since among the other experimental animals, sheep present better anatomical and physical characteristic for a comparison with horses. Specifically, the effect of three different treatment: sPB-MSC, sPB-MSC + PRP (platlet rich plasma) and PRP, were compared among each other and after 1 month and 4 months from the creation of an experimental lesion on left hind DDFT. Overall, the major aim of this second part of the present work, focused on tendon healing, was to better understand the capacity of MSCs to integrate in the tendon ECM, both in a tendon biocompatible scaffold, and in an experimental injured tendon *in vivo*, and measure the contribution of MSCs on tendon regeneration process.

Isolation, characterization, cryopreservation and shipping of adult mesenchymal stem cells derived from peripheral blood of horses (ePB-MSC) and from adipose tissue of dog (cA-MSC):

Adult mesenchymal stromal cells are being reported with increasing frequency for their potential clinical application in veterinary clinic. Among the several animals of veterinary interest, competitive horses are the first candidates for cell-based therapies using mesenchymal stromal cells, due to the frequency of musculoskeletal wastage injures, associated with failure to return to a previous level of performance (De Schauwer C et al., 2011). On the other hand recently research on adult stromal cells is increasing also in pets: dog represent an important patient in veterinary medicine, and often a good experimental model for human disease (Sampaolesi M et al., 2006). Although mesenchymal stromal cells have been isolated from different sources, the most utilized derivation of adult MSCs remains bone marrow. The present study focused on adult mesenchymal stromal cells derived from peripheral blood of horses (ePB-MSC) as an alternative cells source respect to bone marrow, and because of the easier accessibility and lower invasively compared with bone marrow harvesting technique. Furthermore adipose tissue of dog was choose as a source of adult stromal cells for this species, due to the availability of visceral adipose tissue during routinely ovaristerectomies surgery, and for the large number of stromal cells which can be obtained from fat in relatively short time.

The aim of the following studies was to investigate adult MSCs derived from peripheral blood of horses and from adipose tissue of dog, provide a full characterization and study their plasticity, since no available data providing full information on MSCs derived from ePB-MSC and cA-MSC, were present in literature. In order to issue such goal, several assays have been performed in stromal cells derived from both species: to investigate the characterization of both ePB-MSC and cA-MSC, population doubling time has been performed, as a feature that distinguish stromal cells from other type of cells; adult stromal cells, as well as ES cells, appear to possess a distinct cell cycle control to maintain their stemness, compared with the well known cell cycle regulators operating in mammalian cells cycle (cheng and Scadden from book). Moreover cluster of differentiation profiles (CD) has been analyzed, by using FACS sorting, in order to verify the expression of known typical MSCs CD markes on ePB-MSC and cA-MSC. A karyotyping analysis and telomerase activity was

examined, in order to verify the self-renew activity on ePB-MSC and cA-MSC. Finally to define their ability to undergo differentiation in different lineages, ePB-MSC and cA-MSC were *in vitro* induced to differentiate, under proper culture stimulation conditions, into osteogenic lineage, adipogenic lineage and muscular lineage, since no reports previously studied the ability of adult stem cells to undergo muscular differentiation.

Because of the presence of promising supporting preclinical data, clinical trials for stromal cell regenerative treatment of various diseases have been initiated. However to facilitate these applications, cryopreservation and long-term storage of MSCs becomes an absolute necessity (Haack-sørensen & Kastrup, 2011). An effective cryopreservation technique is crucial for any stem cell laboratory with future perspective for clinical application. The use of cryopreserved cells has become common, above all in human medicine, but the quality and utility of cryopreserved cells continue to have significant challenges. Then the critical aim of these studies was to compare fresh and one year cryopreserved ePB-MSC and cA-MSC cells, in order to verify the viability of the cells after thawing.

Moreover, because of the increased use of cell-based therapy in veterinary medicine, the present work has been focused also on the delivery of MSCs for their potential use in clinical trials in non-freezing conditions. Among several methods of delivering cells, cryopreservation is the most studied and, undoubtedly, represent the best choice for long-term storage of MSCs, even if some cell death occurs due to the freezing and thawing processes (Haack-Sørensen M et al., 2011). However, because long-term storage is not necessary for local shipment of cells, it is essential to determine the optimal conditions under which MSCs may be maintained in a non-frozen state, to facilitate their shipment and use in veterinary medicine. This purpose acquires importance also because it is essential to improve, by proper and standardized tests, the stemness of MSCs and establish precise GMP (Good Manufacturing Practice) procedures for their use. Indeed, regulations that focus on the safe production of MSCs, by providing roles for their recovery, storage, receipt, pre-distribution and shipment for use in human medicine, are still scarce in the veterinary field (De Schauwer C et al., 2011). Then the maintenance of ePB-MSC and cA-MSC in different experimental shipping condition has been evaluated, and guidelines on delivery of not cryopreserved ePB-MSC and cA-MSC has been suggested as a result of this investigation.

It follows the published paperworks focused on cryopreservation and shipping of ePB-MSC and cA-MSC:

Cryopreservation does not affect the stem characteristics of multipotent cells isolated from equine peripheral blood.
Martinello T, **Bronzini I**, Maccatrozzo L, Iacopetti I, Sampaolesi M, Mascarello F, Patruno M.
Tissue Eng Part C Methods. 2010 Aug;16(4):771-81.

Canine adipose-derived-mesenchymal stem cells do not lose stem features after a long-term cryopreservation.
Martinello T, **Bronzini I**, Maccatrozzo L, Mollo A, Sampaolesi M, Mascarello F, Decaminada M, Patruno M. Res Vet Sci. 2011 Aug;91(1):18-24. .

Influence of temperature, time and different media on mesenchymal stromal cells shipped for clinical application.
Ilaria Bronzini, DVM; Marco Patruno, PhD; Ilaria Iacopetti, DVM; Tiziana Martinello, PhD. Accepted The Veterinary Journal. 2012 Jan.

Cryopreservation Does Not Affect the Stem Characteristics of Multipotent Cells Isolated from Equine Peripheral Blood

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Mammalian adult stem cells show, *in vitro*, extensive differentiative ability and may represent a versatile tool for tissue regenerative purposes, even after long-term storage. Multipotent stem cells isolated from horse blood have been shown to possess the capacity to differentiate into diverse mesenchymal lineages although their full characterization is still at an early stage. The aim of this study was to examine the effects of cryopreservation on stemness characteristics of adult equine mesenchymal stem cells isolated from peripheral blood (ePB-MSC). Each sample of ePB-MSC was analyzed immediately and then after being frozen in liquid nitrogen for 10–12 months. After cryopreservation, cells conserved their morphology, alkaline phosphatase positivity, telomerase activity, karyotype profile, proliferation rate, and CD expression pattern. We characterized ePB-MSC as cells expressing CD44, CD90, CD117, and CD13, but not CD34 and CD45. Finally, freezing and storing ePB-MSC did not change their adipogenic, osteogenic, and myogenic differentiative potential, as analyzed by histochemistry, immunofluorescence, and polymerase chain reaction expression analyses. Overall, our results demonstrate that cryopreservation of ePB-MSC provides a convenient tool for *in vitro* applications, because cryopreserved cells possess the same stem characteristics as freshly isolated cells. Moreover, the feasibility of maintaining stem cell features of ePB-MSC after long-term storage has important implications for autologous cellular-based therapy in veterinary medicine.

Introduction

ADULT MESENCHYMAL STEM CELLS (MSC) are characterized by a fibroblast-like morphology, long-term self-renewing capacity, and an ability to generate many mature and specialized cell types.[1] MSC have been isolated from bone marrow,[2] peripheral blood,[3] umbilical cord,[4,5] adipose tissue,[6] and dermis.[7] MSC derived from bone marrow, umbilical cord blood, and adipose tissue have been defined as cells expressing stem cell markers CD44, CD29, CD90, and CD73, but not expressing hematopoietic markers (CD14, CD34, and CD45) or the stem cell marker CD133.[8,9] Further, these cells possess the peculiar ability to differentiate along several cell lineages, including chondrocytes,[10] adipocytes, and myoblasts.[11,12] Bone-marrow-derived stem cells may also be attracted to distant peripheral sites after intense injuries and participate in the tissue repair of damaged areas.[1,13] The clinical potential of MSC has been demonstrated in the treatment of bone defects[14] and infarcted heart,[15] and in tracheal regeneration.[16] Moreover, other studies showed that stem cells can regenerate the retina,[17] the cornea,[18] and muscle.[19] Among different sources of adult stem cells of mesenchymal origin, peripheral blood hematopoietic stem cells (HSCs) are the major source of stem cells used for transplantation studies.[20]

In equine medicine, the tissue of choice as source of stem cells is still bone marrow, but peripheral blood may provide a promising tool for achieving clinical advantages because of its lower invasivity, easier accessibility, and plasticity. Indeed, the ability to differentiate toward nonhematopoietic tissue was recently proven for equine multipotent stem cells isolated from peripheral blood.[21] Nevertheless, expression of CD markers by equine MSC that are isolated from peripheral blood (ePB-MSC) is still unknown, and only stem cells isolated from horse umbilical cord blood have been thus characterized by flow cytometry.[22]

The horse is an important animal model because of its inherent value in sports, breeding, and leisure activities, and because it is particularly inclined to develop skeletal muscle diseases. Therefore, ePB-MSC could be used in cell-based therapies for the treatment of tendon, ligament, bone pathologies, and cartilage defects, because it is often not possible to achieve a *restitutio ad integrum*.

Another crucial point for applicability of stem cells in clinical veterinary medicine is the possibility of long-term storage, as functional cryopreservation could provide an

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available pool of cells for basic research and feasibility testing. Although the cryopreservation of HSCs and umbilical cord blood cells is a common method that provides advantages for cellular therapies[23] in human medicine, in equine veterinary medicine, only adipose-tissue-derived progenitors cells have been demonstrated to maintain their properties after cryopreservation.[24] A fundamental objective of this work was to verify the proliferative capacity of ePB-MSC and their ability to differentiate into different cell types and to assess their ability to maintain stem features after long-term cryopreservation.

In this study, we have examined the effect of cryopreservation on ePB-MSC stemness by means of a complete cellular characterization, utilizing phenotype morphology, vitality assay, telomerase activity, and flow cytometry. Further, the capacity of these multipotent cells to differentiate into adipocytes, osteoblasts, and myoblasts has been compared in both fresh and cryopreserved cells. The possibility of cryopreserving ePB-MSC for a long time may make use autologous stem cells for equine therapies feasible, and the establishment of a veterinary cell bank a priority.

Materials and Methods

Isolation and culture of ePB-MSC

Peripheral blood samples obtained from horses ($n = 25$) aged 4–20 years were collected in tubes containing anticoagulants Li-heparin. To isolate mononuclear cells, peripheral blood was diluted 1:1 with phosphate-buffered saline (PBS) and carefully placed on Ficoll-paque solution (Amersham Biosciences, Piscataway, NJ). After density centrifugation (R.C.F. Meter4233R; A.L.C International S.r.l., Cologno Monzese, Italy) at 1600 g for 20 min without brake, cells were removed from the interphase and washed twice with PBS. Cells obtained from each 100 mL of blood were plated in 25 cm² culture flasks (Falcon; BD Biosciences) in noninductive medium consisting of Dulbecco's modified Eagle's medium (DMEM D5671; Sigma-Aldrich S.r.l., Milan, Italy) with 20% fetal bovine serum (FBS; ECS0180-L; Euroclone, Milan, Italy), penicillin and streptomycin (Sigma-Aldrich S.r.l.), and glutamine (Sigma-Aldrich S.r.l.), and incubated (Jouan IG150; Laborgerateborse, Burladingen, Germany) in an atmosphere of humidified air and 5% CO_2 at 37°C. After overnight incubation, nonadherent cells were removed and fresh medium was added to the flasks. After 2–3 weeks, the developed colony-forming units of adherent cells were recovered and replated for amplification, and the medium was changed every 3 days. Isolated cells close to confluence were quantified and subcultured in 10% FBS. To evaluate MSC properties, human dermal fibroblasts were used as negative controls in differentiation studies.

Cryopreservation and thawing of ePB-MSC

At passages 2–3, ePB-MSC were frozen at a concentration of 2×10^6 cells/cryovial, which we refer to as cryopreserved P1 cells. ePB-MSC were resuspended in cryopreservation medium (90% FBS and 10% dimethyl sulfoxide) (Sigma-Aldrich S.r.l., Milan, Italy) using Mr. Frosty (Nalgene, Roskilde, Denmark) decreasing −1°C/min until −80°C; at this temperature, cells were held for 1 week and then transferred to a liquid nitrogen tank for long-term storage.

The thawing procedure was performed after 10 or 12 months: cryovials with ePB-MSC were placed in a 37°C water bath for 1–2 min and then centrifuged at 1600 g for 10 min and washed with noninductive medium to eliminate dimethyl sulfoxide. The cells were then seeded into 25 cm² flasks in noninducted medium.

Proliferation assay

To determinate the cellular proliferation rate a cell proliferation kit II (XTT)-based (Roche, Milan, Italy) colorimetric assay was used. Cells (5×10^3) were grown for 9 days in 96-well tissue culture plates. Each day, cells in selected wells were incubated with the yellow XTT solution for 2 h. After incubation with XTT, the metabolically active cells develop an orange formazan product, which was quantified using an enzyme-linked immunosorbent assay plate reader (Spectra Count; Perkin Elmer, Milan, Italy). The amount of orange formazan formed was directly correlated to the number of living cells.

The population doubling time

The population doubling level (pdl) was determined in continual subculture from a known number of cells. At each subcultivation the pdl was calculated with the equation log (Nf/Ni)/log2, where Ni and Nf are initial and final cell numbers, respectively. The population doubling time was calculated using the equation $x = t(h)/pdl$.

Alkaline phosphatase detection

The ePB-MSC were washed at 50% confluence with PBS and fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature. The alkaline phosphatase (AP) enzyme activity was assayed using a commercial AP detection kit (Kit SIGMA FAST BCIP/NBT; 5-bromo-4chloro-3-indolyl phosphate/nitro blue tetrazolium; Sigma-Aldrich S.r.l.).

Karyotyping

Karyotype studies were performed on metaphase cells derived from ePB-MSC (among passage 1 or P1 and passage 8 or P8). Dividing cells were arrested in metaphase with 0.1 mg/mL colcemid (Sigma-Aldrich S.r.l.) overnight; cells were subsequently incubated in hypotonic solution for 15 min (0.075 M KCl, pH 7), and fixed with methanol/acetic acid (3/1, vol/vol). Cells were then stained with Giemsa solution and analyzed for chromosome number.

Telomerase assay

Telomerase activity was detected by TeloExpress Quantitative Telomerase Detection Kit (Express Biotech International, Thurmont, MD). Appropriate amount of cells were resuspended in TeloExpress lysing buffer according to the manufacturer's instruction. Samples ($n = 3$) were analyzed in triplicate using an AB 7500 real-time polymerase chain reaction (PCR) instrument (Applied Biosystem, Foster City, CA). The telomerase positive control was provided in the kit, and negative controls were ePB-MSC heat-inactivated samples.

Flow cytometry assay

Cultured ePB-MSC ($n = 5$) were analyzed between the first and eighth passage using a fluorescence-activated cell sort-

ing (FACS) technique. Cells were harvested, washed with PBS, and incubated for 10 min at 4°C in PBS containing 2% FBS and 2 mM ethylenediaminetetraacetic acid. The cell suspension was then incubated with primary antibodies directed against the following cell surface markers: CD90 (BD555596; BD Biosciences), CD44 (BD550989; BD Biosciences), CD13 (BD555394; BD Biosciences), CD140a (BD556002; BD Biosciences), and CD117 (BD553355; BD Biosciences), each of which was conjugated with phycoerythrin, and against CD34 (ABD MCA1825F) and CD45 (BD553079; BD Biosciences) conjugated with fluorescein isothiocyanate (FITC). Cells were suspended in 300 µL PBS in FACS tubes (Falcon; BD Bioscience). Mouse isotype IgG1 and IgG2a and rat isotype IgG2a were employed as controls (BD Biosciences). For each sample, 200,000 cells were passed through a FACScan Canto Flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star; Oregon Corporation, Ashland, OR).

Differentiative potential of ePB-MSC

Osteogenic differentiation. Osteogenesis was induced using culture medium supplemented with 1 nM dexamethasone (Sigma D1756; Sigma-Aldrich S.r.l.), 10 mM β-glycerolphosphate (1-800-854; MP Biomedicals Europe, Illkirch, France), and 50 µM ascorbate-2-phosphate (Sigma A4544; Sigma-Aldrich S.r.l.). Cells were induced in this medium for approximately 21 days and osteogenic medium was replaced every 2–3 days. After fixation in a 4% solution of PFA for 30 min, the mineralization of cells was assessed by von Kòssa staining.

Adipogenic differentiation. When cells reached about 80–90% confluence, the ePB-MSC may be induced to differentiate using culture medium (DMEM-LG D5921; Sigma-Aldrich S.r.l.) supplemented with 0.5 mM isobutyl-methylxanthine (Sigma I5879; Sigma-Aldrich S.r.l.), 200 µM indomethacin (Sigma I7378; Sigma-Aldrich S.r.l.), 0.5 µM dexamethasone (Sigma D1756; Sigma-Aldrich S.r.l.), and 10 µM insulin (Sigma I6634; Sigma-Aldrich S.r.l.). The medium was changed every 3 days until adipocyte-like cells were obtained after 21 days of differentiation. Oil Red O staining was performed to evaluate the degree of ePB-MSC differentiation. Cells were fixed in a 4% solution of PFA for 30 min, washed with 60% isopropanol, and stained with Oil Red O solution for 10 min followed by repeated washing with water.

Myogenic differentiation. The ePB-MSC were cultured in differentiation medium consisting of high glucose DMEM (DMEM D5671; Sigma-Aldrich S.r.l.) supplemented with 2% horse serum (CCVCHL00-01; Eurobio, Courtaboeuf, France) and 50 µM hydrocortisone (H888; Sigma-Aldrich S.r.l.) for 15 days. Cells were fixed with 4% PFA, and after an incubation with 0.1% Triton X-100, a primary antibody against Myf5 (Santa Cruz sc-320; D.B.A., Milan, Italy) or MyoD (Santa Cruz m-318; D.B.A.) was added at 1:100 dilution and incubated for 1 h at room temperature. After washing three times with PBS, primary antibodies were detected using species-specific fluorochrome-conjugated antibodies (FITC) diluted 1:400 in PBS (Alexa A11001; Invitrogen Life Technologies). Immunostaining controls were performed using the same conditions but omitting primary antibody.

RNA isolation, standard PCR, and DNA sequencing

ePB-MSC were maintained in adipogenic and osteogenic differentiation medium for 3 weeks or in myogenic differentiation medium for 2 weeks. From each cell population total RNA was extracted using Trizol® reagent (Gibco-BRL, Gaithersburg, MD). Total RNA was reverse-transcribed using Superscript® protocols (Invitrogen Life Technologies) and a mixture of random hexamers. The cDNAs were used as template for ordinary PCR. Different PCRs were conducted using specific primers designed with Primer Express software and ordered online (www.eurofinsdna.com). Peroxisome proliferator-activated receptor-gamma (PPARγ2) 153 bp product (forward primer: 5′-ATTACGGAGTTCAT GCCTGTG-3′; reverse primer: 5′-GTTTTCACAAGTGCCTT TCGG-3′) was analyzed for adipogenic samples; SPP1 (secreted phosphoprotein 1, osteopontin) 285 bp product (forward primer: 5′-AAGCCTGATCCGTCTCAGAA-3′; reverse primer: 5′-ACGTCGGTGGAAAAATCAG-3′) was analyzed for osteogenic samples; desmin 186 bp product (forward primer: 5′-GCTTCAGGAACAGCAAGTCC-3′; reverse primer: 5′-CGCAGTGCATCATTGTTCTT-3′) was analyzed for myogenic differentiation. A β-actin fragment of 185 bp product (forward primer: 5′-CCATCTACGAGGGGTACG CCC-3′; reverse primer: 5′-TGCTCGAAGTCCAGGGCGA CGTA-3′) was amplified to exclude a genomic contamination and to validate the purity of cDNA. PCR products were sequenced by the BMR Genomics (Bio Molecular Research) (sequencing service) to confirm the identity of PPARγ2, SPP1, and desmin. As negative controls human fibroblasts were maintained in adipogenic, osteogenic, and myogenic differentiation medium for 3 weeks, and expression of PPARγ2, SPP1, and desmin was analyzed, respectively, using the following specific primers—forward: 5′-TTCTTCAT GGAAGTGTTGGCTAT-3′, reverse: 5′-TTTCTCGATCTTTG GGCATG-3′; forward: 5′-CATATGATGGCCGAGGTGAT AG-3′, reverse: 5′-GAATGGTGCATACAAGGCC-3′; forward: 5-TTGACCTGGAGCGCAGAATT-3′, reverse: 5′-TAA GCCAGACCTCACTGCCG-3′.

Statistical analysis

Data are expressed as the mean ± standard deviation. Normality of the data was confirmed using the Kolmogorov–Smirnov test ($\alpha = 5\%$). Statistical analysis was performed using the paired Student t-test (SPSS software, version 11.0; SPSS, Chicago, IL). The level of statistical significance was set at $p < 0.05$ for all analyses.

Results

Isolation, expansion, and morphology of ePB-MSC

ePB-MSC were successfully isolated from peripheral blood samples of 11 horses (from total sampling of 25 horses). Fifteen to 20 days after isolation and with only 3 medium changes, some clones were growing. However, the efficiency of colony formation was quite low, with about 1.7 ± 0.6 (colony-forming unit) per 10^6 nucleated cells. Cells were re-cultured in growth medium for further expansion, with regular passages when cultures reached a 70–80% confluence. The ePB-MSC achieved confluence in 3–4 days for cells of passages 1 to 4, and in 5–6 days for later passages. Each sample that was successfully isolated was cryopreserved.
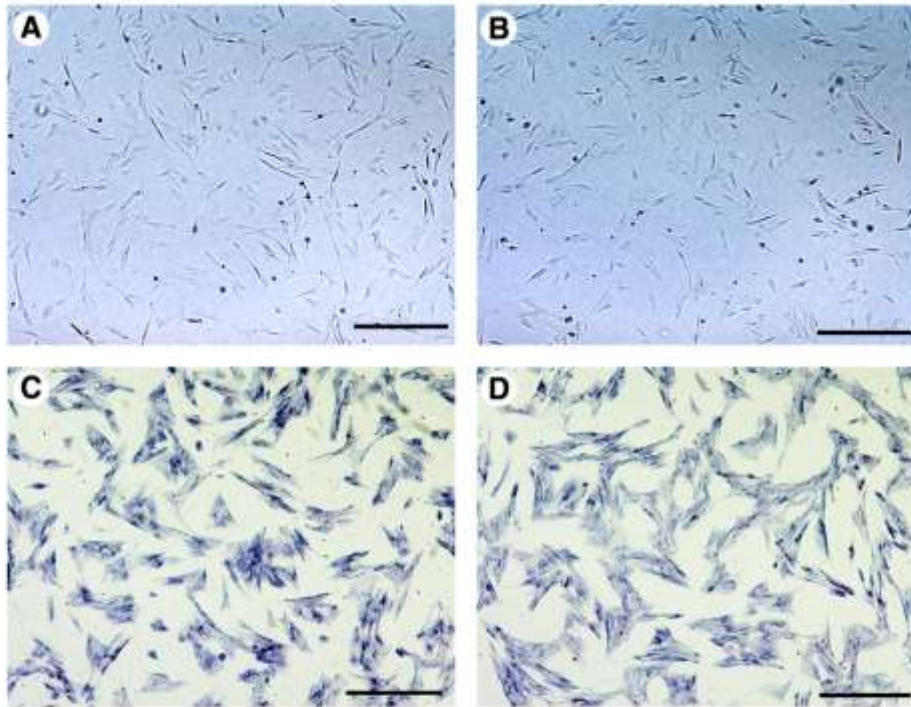
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FIG. 1. Morphology of adherent PB-MSC. **(A)** Fresh stem cells isolated from peripheral blood showed a fibroblast-like phenotype. **(B)** After 12 months of cryopreservation, cells presented the same morphology as shown in **(A)**. **(C)** Fresh ePB-MSC showed a strong positivity for alkaline phosphatase. **(D)** Cryopreserved cells presented an equal staining intensity. Scale bars: **(A, B)** 300 μm; **(C, D)** 500 μm. ePB-MSC, equine peripheral blood mesenchymal stem cells. Color images available online at www.liebertonline.com/ten.

After thawing, cell passages were compared with fresh cells isolated from the same horse. These cells maintained a fibroblast-like phenotype when expanded in culture (Fig. 1A). The cryopreserved ePB-MSC, after being thawed, seeded, and cultured for 2 days, presented the same fibroblast-like morphology as fresh cells (Fig. 1B). Both fresh and cryopreserved cells showed strong AP staining, an embryonic marker for stemness (Fig. 1C, D). The cell viability of ePB-MSC was determined by XTT proliferation assay. Fresh and cryopreserved cells (before P8) were analyzed for their proliferation potential. The growth curve for fresh cell populations ($n = 7$) was characterized by an initial lag phase (48 h) followed by a log phase, whereas cryopreserved cell populations ($n = 7$) immediately grew logarithmically (Fig. 2A). To determine the proliferation potential, the population doubling time of fresh and cryopreserved cells was calculated at passages P1, P4, and P8 (Fig. 2B). Our results showed that the doubling time of cells increased with passage number and that cryopreserved cells doubled significantly more rapidly than fresh cells at P1 (fresh: P1, 30.57 ± 1.3; P4, 40.37 ± 2.5; P8, 47.35 ± 4.3; cryopreserved: P1, 18.39 ± 2.4; P4, 30.21 ± 3.6; P8, 46.77 ± 4.4).

To investigate and compare the self-renewal ability of fresh and cryopreserved ePB-MSC, we evaluated telomerase activity and its stability at passages 1 and 8. Three samples of fresh and cryopreserved cells presented a telomerase activity similar to the positive control supplied by TeloExpress Quantitative Telomerase Detection Kit and this activity did not change between passage 1 and 8. Although telomerase activity was numerically higher in fresh cells than in cryopreserved, this difference was not significant (Fig. 3). We also

observed a normal karyotype in both fresh ePB-MSC and cryopreserved cells at passage 8, for which time 64 chromosomes were counted (Supplemental Fig. 1 available online at www.liebertonline.com/ten).

Immunophenotype

Expression of cell surface antigens was characterized in fresh and cryopreserved ePB-MSC between P3 and P8 ($n = 5$) by flow cytometry analysis. Cell populations, defined on the basis of forward and side scatter, showed similar cell complexity in fresh and cryopreserved ePB-MSC (Supplemental Fig. 2; supplemental figures available online at www.liebertonline.com/ten). Shown in Figure 4 are representative histograms of fresh and cryopreserved ePB-MSC immunophenotypes, together with a percentage analysis of cell antigens, confirming the conserved immunophenotype of frozen cells. Fresh and cryopreserved cells were highly positive for extracellular matrix protein CD90 ($100\% \pm 0$ for fresh, $100\% \pm 0$ for cryopreserved), and the hyaluronate receptor CD44 ($97.9\% \pm 2.9$ for fresh, $98.3\% \pm 2.9$ for cryopreserved). The ePB-MSC expressed membrane glycoprotein CD13 antigen, although its expression decreased slightly after cryopreservation ($78\% \pm 11$ for fresh, $61\% \pm 9.9$ for cryopreserved). Cells expressed the CD117 antigen (c-Kit receptor) ($63.8\% \pm 35.7$ and $73.5\% \pm 36$ for fresh and cryopreserved cells, respectively). This cytokine receptor is expressed on the surface of HSCs, although with high variability. Expression of all analyzed antigen markers did not change significantly between fresh and cryopreserved cells. In addition, the analysis
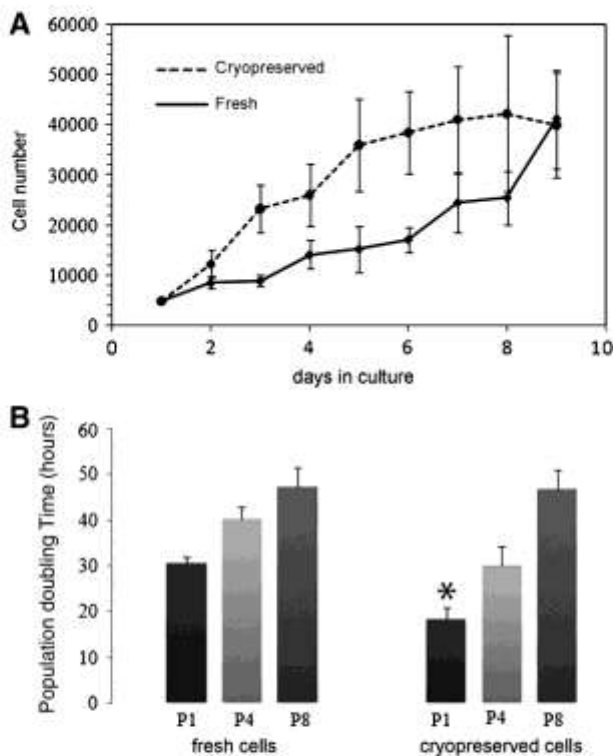
FIG. 2. Growth kinetics and population doubling time of ePB-MSC. (**A**) Fresh cells isolated from horse blood samples and cryopreserved cells ($n = 7$) thawed after 12 months were seeded in triplicates into 96-well dishes and analyzed with a colorimetric assay cell proliferation kit II (XTT). The graph shows the cell number average respect to culture time. (**B**) Values shown are the average ± deviation standard of the population doubling time of fresh and cryopreserved cells ($n = 7$) at passages P1, P4, and P8. The paired Student's t-test t was used for each passage to compare fresh versus cryopreserved cells (*$p < 0.05$).

showed no staining for hematopoietic lineage markers CD34 and CD45.

Differentiative potential

The multilineage differentiation capability of ePB-MSC was evaluated before and after cryopreservation using lineage-specific induction factors to examine differentiation toward the adipogenic, osteogenic, and myogenic lineages. Differentiative pathways were qualitatively assessed using gene expression and imunohistochemistry analyses.

We examined expression of mRNA for specific marker genes in fresh and cryopreserved ePB-MSC after induction of differentiation with adipogenic-, osteogenic-, and myogenic-specific media (Fig. 5). Human fibroblasts were used as a negative control, and β-actin mRNA was used as an internal PCR control (Supplemental Figs. 3 and 4; supplemental figures available online at www.liebertonline.com/ten). The cells induced into an adipogenic fate expressed PPARγ2, a nuclear receptor isoform present exclusively in adipose tissue. Expression of osteopontin (SPP1), an extracellular structural protein and an organic component of bone, was analyzed to evaluate osteogenic induction. Expression of

desmin, a protein of intermediate filaments present in skeletal muscle tissue, was investigated to evaluate the myogenic differentiation of ePB-MSC. Among investigated genes, PCR results did not show differences between fresh and cryopreserved ePB-MSC (Fig. 5). Further, the same tissue-specific gene markers were not expressed in noninduced cells or in fibroblasts that were treated with induction (differentiation) medium (Supplemental Figs. 3 and 4). To confirm the identity of the gene markers employed, all PCR bands were subcloned, sequenced, and BLASTed against GenBank database; all sequences showed a total identity with the targeted equine genes (data not shown).

The ePB-MSC cultivated in adipogenic differentiation medium showed the presence of lipid vacuoles as evidenced by Oil Red O staining (Fig. 6A, B). Osteogenic differentiation resulted in an increase of calcium deposits, as assessed by von Kossa staining (Fig. 6C, D). Myogenic commitment was indicated by nuclear Myf5 positivity together with myoblast alignment (Fig. 6E, F). Another early marker of myogenic differentiation (MyoD, data not shown) was tested and provided supportive evidence for myogenic differentiation by staining nuclei of myoblast-induced cells.

Discussion

Adult stem cells have been isolated from several tissues[25] and their huge potential is still being tested in both human and veterinary medicine.[26] HSCs are used in human medicine for autologous and allogenic transplantation to restore bone marrow cell populations because they provide a faster and more efficient outcome than that obtained by transplantation of marrow-derived stem cells.[27] Circulating stem cells may also traffic to sites of tissue injuries, which may lead to infiltration of the damaged tissue and regeneration of nonhematopoietic cell types.[28] In the horse, Giovannini *et al.*[21] and Koerner *et al.*[29] proved, *in vitro*, the capacity of putative stem cells isolated from peripheral blood to differentiate into nonhematopoietic lineages; the latter authors referred to these cells as fibroblast-like[21] or progenitor[29] cells, and nomenclature is still inconsistent. In the current study, we have used the acronym ePB-MSC (equine peripheral blood mesenchymal stem cells) because the term "mesenchymal" has gained wider consensus in the scientific community.[30] The plasticity of these cells raises the possibility of using them as an alternative source of stem cells to treat many pathologies of competitive horses that are difficult to resolve, such as osteochondrosis, subchondral cystis, and tenodesmic injuries.

In this work we presented comparative data for fresh and cryopreserved ePB-MSC, a well known class of adult stem cells of mesenchymal origin. Our study provided a full characterization of these cells, including a CD expression assay, not previously performed in horse peripheral blood. We confirmed that ePB-MSC are able to differentiate into adipocytes, osteoblasts, and myoblasts. To our knowledge, this is the first demonstration of myoblast differentiation from ePB-MSC. We have also demonstrated that cryopreserved cells show similar stem characteristics as fresh cells.

We have analyzed morphological characteristics and proliferative ability of different clones from fresh and cryopreserved ePB-MSC. Generally, these cells presented a fibroblastoid appearance and possessed proliferative behavior
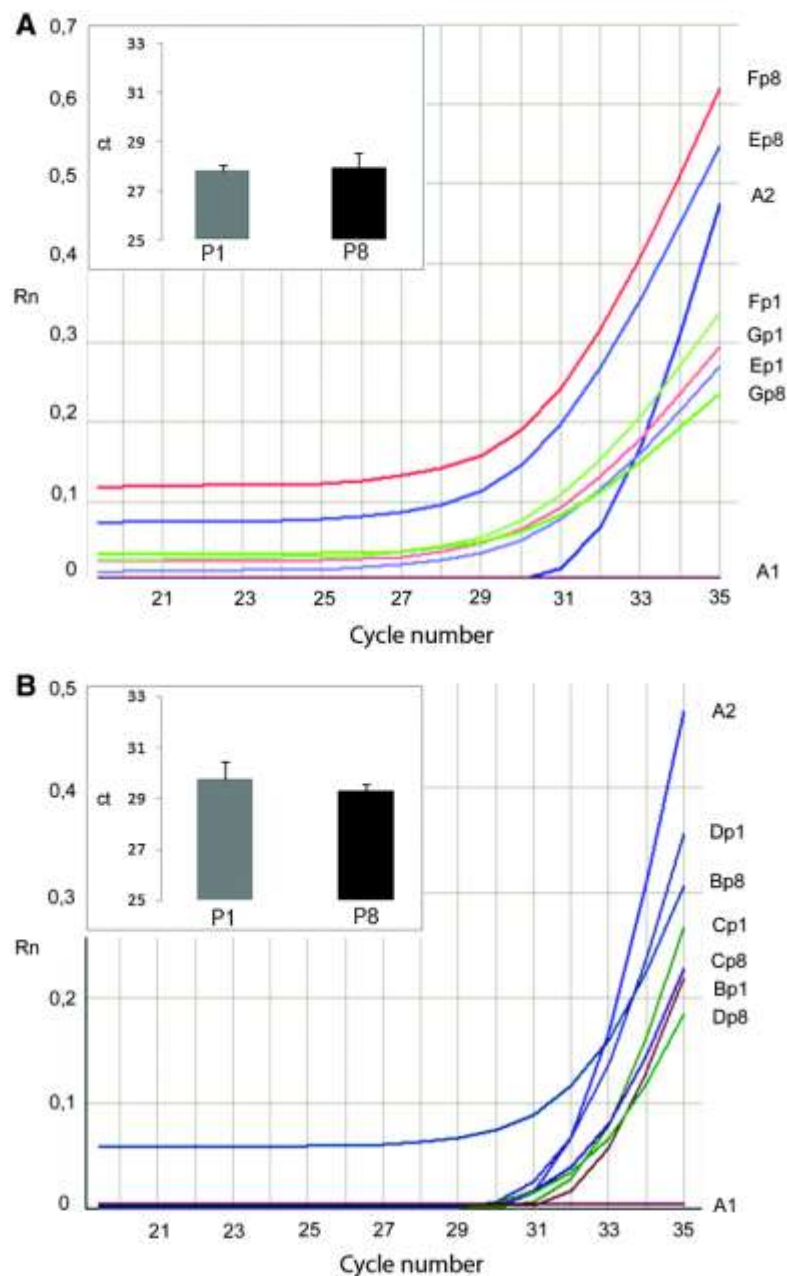
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FIG. 3. Telomerase activity of ePB-MSC. (**A**) Three fresh cell populations (E, F, and G) were analyzed at passages P1 and P8. (**B**) Three cryopreserved cell populations (B, C, and D) were analyzed at passages P1 and P8. A1, negative control; A2, positive control. In graphs (**A**) and (**B**), x-axis corresponds to number of cycles and y-axis corresponds to normalized reporter (Rn = the ratio of fluorescence emission intensity of the reporter dye to the fluorescence intensity of the passive reference dye). The histograms show the Ct average ± standard deviation of the three populations at passages 1 and 8. The paired Student's *t*-test *t* was used for each passage to compare fresh versus cryopreserved cells. Color images available online at www .liebertonline.com/ten.

that is similar to other mammalian MSC.[31] Thawed cells did not show a lag phase of proliferation that is typical of freshly cultured MSC, apparently because these cryopreserved cells were already growing logarithmically when frozen. Indeed, to obtain optimal cryopreservation it is necessary to utilize a very high number of cells obtained from passages 2–3 (which correspond to log phase). Nevertheless, based on growth kinetics and population doubling time, our data suggest that ePB-MSC maybe easily expanded while maintaining their viability even after cryopreservation. We found that ePB-MSC presented a similar AP activity in both fresh and cryopreserved cells. Because AP is a characteristic enzyme
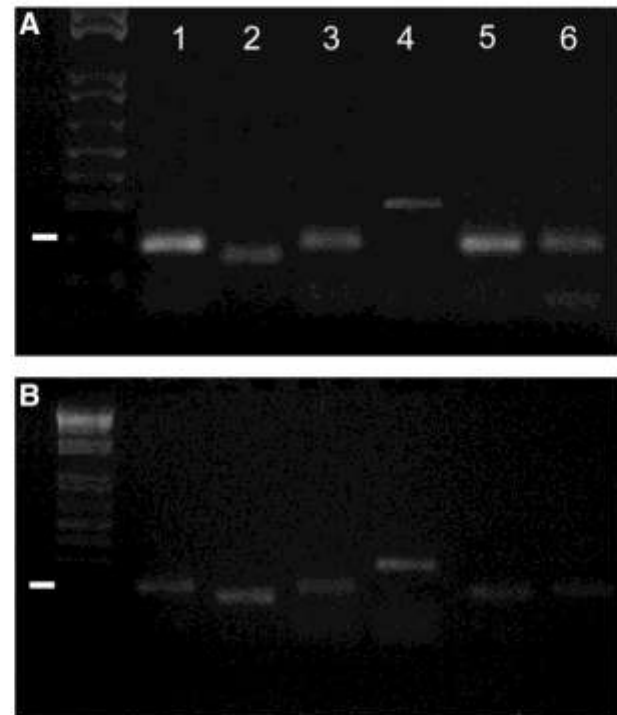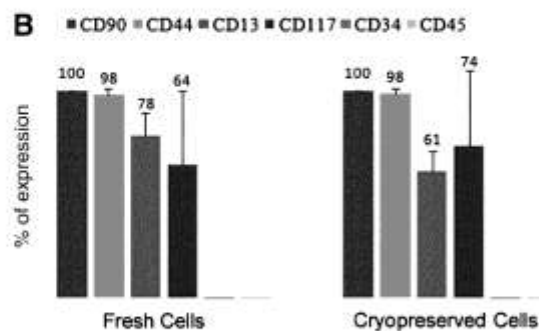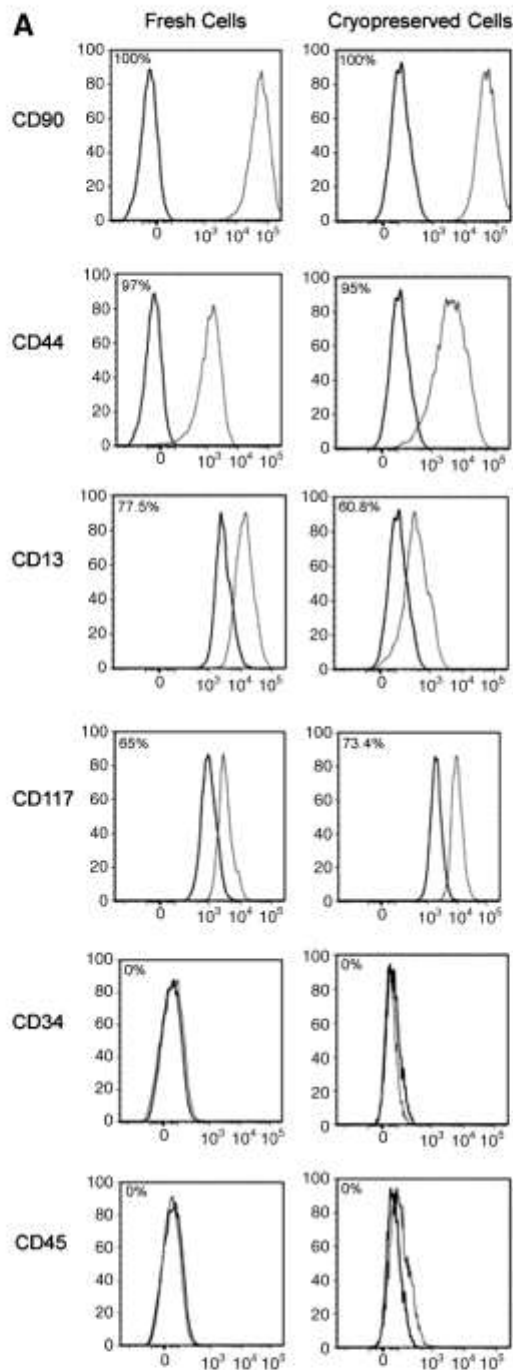
FIG. 5. Expression profile of differentiative gene markers in fresh and cryopreserved cells induced toward the adipogenic (lanes 1 and 2), osteogenic (lanes 3 and 4), and myogenic (lanes 5 and 6) fate. (**A**) Fresh cells were tested with specific primers for peroxisome proliferator-activated receptor-gamma2 (lane 2), secreted phosphoprotein 1 (lane 4), and desmin (lane 6). (**B**) Cryopreserved cells were tested with the same specific primers for peroxisome proliferator-activated receptor-gamma2 (lane 2), secreted phosphoprotein 1 (lane 4), and desmin (lane 6). β-Actin was used as internal control (lanes 1, 3, and 5). The molecular size standard used was the 1Kb Plus DNA Ladder (Invitrogen, Gaithersburg, MD). Scale bars indicate 200 bp.

for bone tissue, the observed enzymatic activity may indicate an inherent natural tendency toward osteogenic differentiation.[32] Moreover, AP is considered an embryonic marker because increased activity in embryonic stem cells corresponds to higher capacity for colony formation.[33] Normal karyotypic profiles indicated that freshly isolated and cryopreserved ePB-MSC did not possess chromosome abnormalities and measures of telomerase activity were indicative of the stem cell character of the cell populations evaluated. Stem cells require *de novo* synthesis of telomere repeats

FIG. 4. Detection of CD markers on cell membrane. Both fresh and cryopreserved cells were tested for several antibodies. Cells were positive for CD90, CD44, CD13, and CD117, whereas no expression was detected for CD34 and CD45. (**A**) Representative diagrams showing fluorescence-activated cell sorting evaluation in fresh and cryopreserved cells. The isotype control is shown as a black-line histogram. (**B**) The histograms show the average, expressed in percentage, of CD presence in fresh and cryopreserved ePB-MSC. The paired Student's *t*-test was used for each antigen to compare fresh versus cryopreserved cells.

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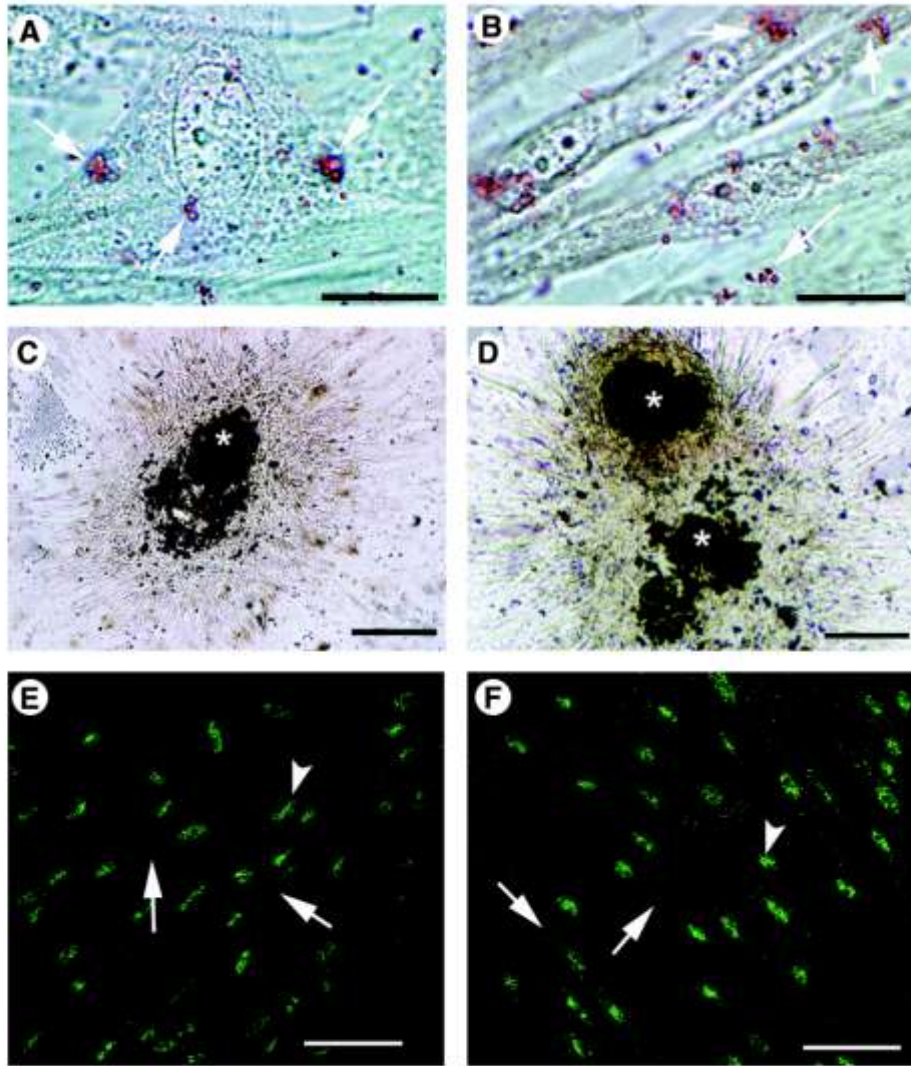
FIG. 6. Differentiative potential of horse ePB-MSC. Adipogenic induction in fresh (**A**) and cryopreserved (**B**) cells is evident in ePB-MSC with Oil Red O staining; in both images small lipid droplets are visible inside the cells (arrows). Osteogenic induction in ePB-MSC was verified with von Kossa staining: both fresh (**C**) and cryopreserved (**D**) cells showed a strong positive reaction since the calcium deposits (asterisks) increased in the culture. Myogenic induction in fresh (**E**) and cryopreserved (**F**) ePB-MSC. The myogenic commitment was evident in ePB-MSC since the nuclear positivity for Myf5 (arrowheads) and myoblasts alignment (arrows). Scale bars: (**A, B**) 40 μm; (**C, D**) 100 μm; (**E, F**) 75 μm. Color images available online at www.liebertonline.com/ten.

to maintain their ability to divide; this is achieved by the transcriptase activity of telomerase, which is low in normal somatic cells but increased in a variety of malignancies and in immortalized/stem cells.[34] The capacity of telomerase to elongate telomeres (telomerase enzymatic activity) was found in cell lysates of fresh and cryopreserved cells, and it was unchanged between passage 1 and 8. Although fresh cells presented numerically greater telomerase activity than cryopreserved cells, the means were not significantly different, and both fresh and cryopreserved cells maintained an elevated growth potential. The normal, healthy status of frozen cells was also confirmed by their normal karyotype.

Another key finding of the present research pertains to the characterization of ePB-MSC using flow cytometry (FACS).

The surface markers for human and mouse HSCs have been identified, whereas in the horse, the CD assay has been performed only in mesenchymal stem cells derived from umbilical cord blood.[22] We employed commonly used mesenchymal markers to define the horse stem cell population found in peripheral blood.[12] Our study showed, for the first time in horse peripheral blood, that ePB-MSC express CD44, CD90, and CD13, whereas the progenitor hematopoietic markers CD34 and CD45 were not detected. CD117 was expressed in horse ePB-MSC although its expression in mesenchymal stem cells (MSC) from other mammals has been controversial. Some authors showed that MSC do not express CD117,[35] whereas others determined that embryonic stem cells, HSCs, and MSC are positive for this marker.[36] Importantly, we did not find significant differences between

fresh and cryopreserved ePB-MSC concerning cell size (Supplemental Fig. 2; supplemental figures available online at www.liebertonline.com/ten) or CD expression pattern.

As suggested by several studies,[10,12,37] MSC possess the potential to differentiate into multiple mesodermal lineages after exposure to specific culture conditions. We verified that horse multipotent stem cells are able to differentiate into three different lineages. Adipogenic induction of the ePB-MSC in culture for 21 days resulted in formation of small lipid vacuoles, suggesting that differentiation was in its initial stages. Expression of PPARγ2, a fat-specific transcriptional factor that functions in preadipocyte commitment, confirmed the adipogenic differentiation. Incubation of ePB-MSC with osteogenic induction medium caused the formation of cell aggregates and matrix mineralization that was indicated by the calcium specific staining (using von Kossa stain) and by expression of SPP1 mRNA (osteopontin). Finally, hydrocortisone addition provoked the differentiation of ePB-MSC toward the myogenic pathway. After 15 days of induction, ePB-MSC formed multinucleated fibers that morphologically resembled myotubes and that expressed the structural protein desmin. The myogenic commitment was further manifested by expression of two nuclear myogenic transcription factors: Myf5, which promotes the myogenic specification of undifferentiated cells, and MyoD, which initiates the differentiative myogenic program.[38] Additionally, this is the first report to investigate the stemness features of cryopreserved ePB-MSC. We observed the following similar characteristics, at least up to passage 8, between fresh and ePB-MSC cryopreserved cells: (i) growth and morphology was not affected; (ii) the immunophenotype expression pattern was the same; (iii) the plasticity of ePB-MSC was not reduced.

Recent progress in the isolation and characterization of adult stem cells led to the development and testing of therapeutic strategies in a variety of human clinical applications. Stem cell therapy involves the transplantation of autologous or allogenic stem cells. Some examples of the therapeutic use of different tissue-derived MSC included cartilage repair,[39] orthopedic[40] and coronary artery diseases,[41] cornea injure repair,[18] and muscular dystrophy.[42] In veterinary medicine, proof of the efficacy of stem cell therapy is still lacking, although efforts have been conducted in horses affected by orthopedic and tendon injuries.[43] A common cryopreservation method was used in this study that resulted in a low cell mortality and maintenance of stem cell properties after thawing. Nevertheless, implementation of different strategies to improve the cryotolerance of equine MSC is worthy of investigation. For instance, it is known that cells grown in monolayer are generally more susceptible to cryoinjuries than cells grown in suspension.[44] The possibility of using ePB-MSC instead of bone marrow cells for cell-based therapies prompted us to better define the characteristics of undifferentiated cells present in peripheral blood even after long-term storage in liquid nitrogen. Considering that the percentage of circulating stem cells in mammalian peripheral blood is very low and their successful isolation in equine is between 33% (Koerner et al.[29]) and 44% (this study), cryopreservation might be regarded as a key tool for enabling access to MSC from domestic animals. The long-term stability of cryopreserved stem cells is not very well known, although in vitro functions of HSCs derived from human umbilical cord blood was not lost after 5 years of cryopreservation. Moreover, human bone marrow MSC frozen for 2 to 8 years did not show any alterations of their in vivo engraftment capability.[45]

In summary, our data suggest that ePB-MSC provide an accessible and effective source of adult stem cells. These cells hold the promise of value for regenerative veterinary medicine as well as for laboratory in vitro study, because they maintain their differentiative potential after cryopreservation.

Acknowledgments

Disclosure Statement

No competing financial interests exist.

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Canine adipose-derived-mesenchymal stem cells do not lose stem features after a long-term cryopreservation

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ABSTRACT

Adult stem cells are nowadays used for treating several pathologies. A putative stem cell population was found in the adipose tissue of mammals and canine adipose tissue-derived-mesenchymal stem cells (cA-MSC) have been shown to possess the capacity to differentiate into several lineages. The main goal of our research was to fully characterize cA-MSC and examine the effects of cryopreservation on their *stemness* features. Each sample of cA-MSC was analyzed immediately and then again after being frozen in liquid nitrogen for one year. After the cryopreservation period cells conserved their fibroblast-like morphology, alkaline phosphatase positivity and CD expression but showed a lower proliferation ratio and a lower telomerase activity in comparison with fresh cells. Finally, the cryopreservation protocol did not change the cA-MSC adipogenic, osteogenic and myogenic differentiative potential. Our data demonstrate that stored cA-MSC might represent a promising type of progenitor cell for autologous cellular-based therapies in veterinary medicine.

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1. Introduction

Mesenchymal stem cells (MSC) are found in several adult tissues and represent an attractive stem cell category for regenerative medicine and cell therapy purposes (Mimeault and Batra, 2008; Strauer et al., 2009). MSC possess a robust self-renewal potential, show a constant growth and a known proliferation kinetic; furthermore, MSC are able, *in vitro*, to differentiate into numerous specialized cell types (Zuk et al., 2002; Yamamoto et al., 2007). An adult mesenchymal progenitor cell population was identified in human adipose tissue and lipoaspirate (Zuk et al., 2002; Lee et al., 2004; Kern et al., 2006; Rebelatto et al., 2008). Numerous studies (Lee et al., 2004; Kern et al., 2006; Rebelatto et al., 2008) have demonstrated that human mesenchymal stem cells derived from adipose tissue (A-MSC) express classic MSC markers as CD90, CD44, CD73, CD105, CD117, CD106 and possess the same capacity of bone marrow-derived stem cells (BMSC) to differentiate into osteogenic, adipogenic and chondrogenic lineages. Moreover, less than 1% of genes are differentially expressed between A-MSC and BMSC; this data should support the notion that A-MSC and BMSC are originated from a common precursor (Bianco et al., 2008). The adipose tissue presents some advantage with respect to other investigated stem cells sources because the resulting cell numbers obtained from the isolation is very high and, indeed, it is sufficient a small chunk of fat for their isolation (Kern et al., 2006). In mammals the adipose tissue is found in different anatomical compartments and in dogs the visceral fat is particularly easy to collect because this species is subjected to a large number of ovariohysterectomies.

The plasticity of mesenchymal stem cells has made it possible to develop cell-based therapies and for this purpose it is very useful to cryopreserve these cells in order to gain a ready source of abundant autologous stem cells. Mesenchymal stem cells may be frozen to preserve their vitality and when thawed MSC have to maintain their pluripotent phenotype (Attarian et al., 1996; Gonda et al., 2008); obviously, the possible clinical application should be based on the abundance, frequency and expansion potential of the cells.

In the veterinary field the experimental stem cell therapy has been applied mainly in horses while in pets the research is still in progress (Richardson et al., 2007; Martinello et al., 2009). The dog is an important patient in veterinary medicine and it is often used as an experimental model for human diseases. Indeed, the golden retriever dog has been used as a muscular dystrophy model and was successfully treated with mesoangioblast stem cells (Sampaolesi et al., 2006).

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(CCVCHL00-01, Eurobio, Courtaboeuf, France) and 50 μM hydro-chortisone, (H888, Sigma–Aldrich) for 15 days. Cells were then fixed with 4% PFA, and after an incubation with Triton X-100 a primary antibody against Myf5 (Santa Cruz sc-320, D.B.A. Italy S.r.l. Milan, Italy) was added at dilution of 1:100 and incubated for 1 h at room temperature. After washing three times with PBS 1X, primary antibodies were detected by specie-specific fluorochrome-conjugated antibodies (FITC) diluted 1:400 in PBS 1X (Alexa A11001, Invitrogen Life Technologies, Cambridge, UK). Immuno-staining controls were performed in the same conditions but omitting primary antibody.

2.8. RNA isolation standard PCR and DNA sequencing

cA-MSCs were maintained in osteogenic and adipogenic differentiation medium for 3 weeks and in myogenic medium for 2 weeks. From each cell population total RNA was extracted using Trizol® reagent (Gibco-BRL Gaithesburg, MD, USA). Total RNA was reverse-transcribed using Superscript® protocols (Invitrogen Life Technologies) and a mixture of random hexamers. cDNA was used as a template for ordinary PCR.

Different PCR reactions were conducted using specific primers designed by means of Primer Express software and purchased on-line (http://www.eurofinsdna.com). For adipogenic sample: PPARγ2 (peroxisome proliferator-activated receptor-gamma) 197 bp product (forward primers: 5'-CATTGACCCAGAAAGCGACT-3'; reverse primer: 5'-TGGAAATGCTGGAGAAATCC-3'); for osteogenic sample: SPP1 (secreted phosphoprotein 1, osteopontin) 300 bp product (forward primers: 5'-ACGATGTGGATAGCCAGGAC-3'; reverse primer: 5'-GGACGGCATTGAAGTCATCT-3'); for myogenic sample: desmin 166 bp product (forward primers: 5'-ATTCCC-TGATGAGGCAGATG-3'; reverse primer: 5'-AGGGCCATCTT GACA-TTGAG-3'). A β-actin fragment of 185 bp (forward primers: 5'-CCATCTACGAGGGGTACGCCC'3'; reverse primers: 5'-TGCTCGAA GTCCAGGGCGACGTA'3') was amplified to exclude a genomic contamination and to validate the purity of cDNA. PCR products were sequenced by the CRIBI (http://www.bmr.cribi.unipd.it) sequencing service to confirm the identity of all primers tested. Human dermal fibroblasts were used as negative control since they were maintained in culture for the same period of adipose-derived stem cells and analyzed for the expression of respectively PPARγ2, SPP1 and desmin mRNA with the following specific primers: forward, 5'-TTCTTCATGGAAGTGTTGGCTAT-3', reverse, 5'-TTTCTCGATCTTTG GGCATG'3'; forward, 5'-CATATGATGGCCGAGGTGATAG'3', reverse, 5'-GAATGGTGCATACAAGGCC'3'; forward, 5-TTGACCTGGAGCGCA-GAATT'3', reverse, 5'-TAAGCCAGACCTCACTGCCG-3'.

2.9. Statistical analysis

Data are expressed as the mean ± standard deviation. Normality of the data was confirmed using the Kolmogorov–Smirnov test ($\alpha = 5\%$). Statistical analysis was performed using the paired Student t-test (SPSS software, version 11.0, SPSS Inc., Chicago, Illinois, USA). The level of statistical significance was set at $p \leqslant 0.05$ for all analyses.

3. Results

3.1. In vitro cA-MSC isolation and characterization

The cA-MSC were isolated as previously described in Section 2; as soon as the cells reached semiconfluence they were further expanded and cryopreserved. After thawing cA-MSC were compared with fresh cells isolated from the same animal. All cA-MSC obtained from 20 different dogs presented fibroblast-like phenotype (Fig. 1A) and showed strong alkaline phosphatase (AP) staining (Fig. 1B) detected by an enzyme activity assay. The cryopreservation of cells in liquid nitrogen and their thawing after 10–12 months did not affect the cell morphology and the AP activity (Fig. 1C and D).

3.2. Measurement of population-doubling time of cA-MSC

To examine the population-doubling time of cA-MSC samples ($n = 10$) the proliferation potential was measured according to the passage number (the experiment lasted about 40 days, which corresponds to eight passages). The analysis of proliferation capacities of fresh and cryopreserved cells showed that cryopreservation caused a significant delay of cell divisions at passage P1 and P4 (Fig. 2).
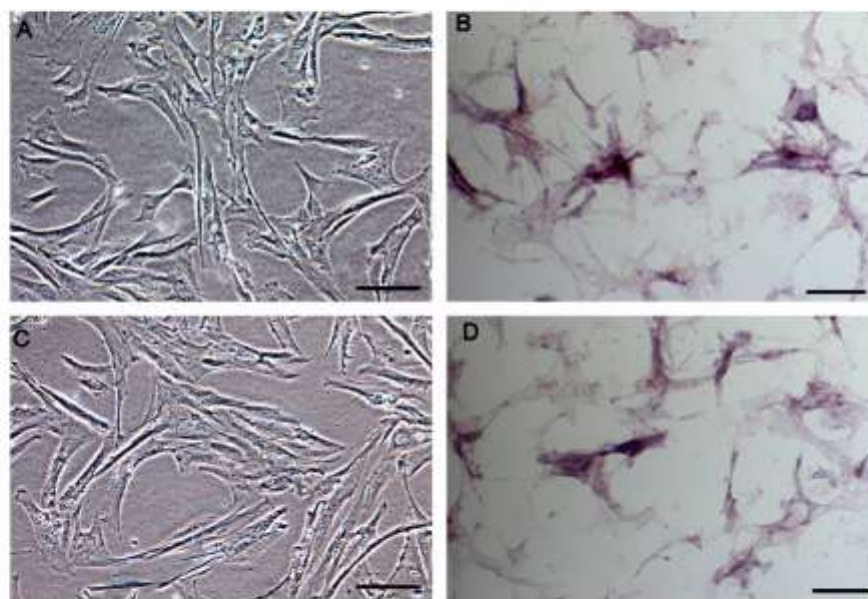

Fig. 1. (A) Fibroblast-like morphology of fresh cA-MSC after isolation and expansion. (B) Strong reactivity to alkaline phosphatase enzymatic staining of fresh cA-MSC. Cryopreserved cells (C and D) showed the same phenotype of fresh isolated cells and the same positivity to alkaline phosphatase (D). Scale Bars (A–C) 200 μm. Scale Bars (B-D) 300 μm.

3.3. Telomerase assay

In order to evaluate the replicative ability of fresh and cryopreserved cA-MSC ($n = 6$) we measured the concentration of telomerase protein and verified its amount at passage 2 and 8. The telomerase concentration decreased after cryopreservation and with the senescence of cells at passage 8 (Fig. 3). The telomerase concentration found was lower in cA-MSC respect to the protein level observed in HeLa immortalized cells (Fig. 3).

3.4. cA-MSC cell-surface antigen profile

To identify markers that might indicate the differentiative potential of cA-MSC the cell-surface antigen expression analysis was evaluated by flow cytometry in six fresh and six cryopreserved samples between P3 and P6 (Fig. 4). The presence of surface mole-

	fresh cells	cryopreserved cells
P2	$1,53 \pm 0,49$	$4,00 \pm 0,05$
P4	$4,93 \pm 1,74$	$11,60 \pm 0,31$
P8	$22,20 \pm 7,40$	$22,37 \pm 1,26$
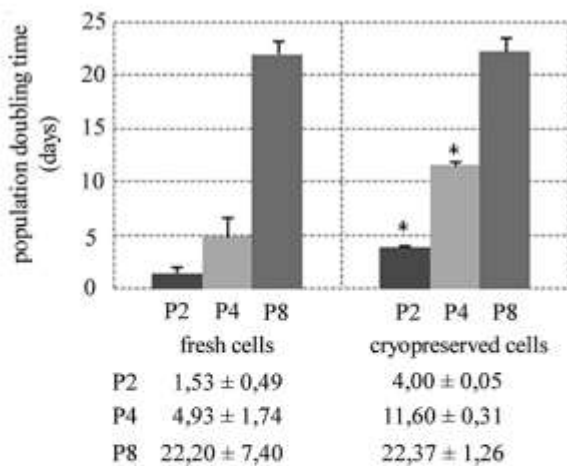
Fig. 2. Analysis of population-doubling time (pdt) of fresh and cryopreserved cells ($n = 10$) at passages 2, 4 and 8. cA-MSC showed a increase of pdt between P4 and P8. Cryopreserved cA-MSC displayed a significant decrease of pdt respect to fresh cells. The paired Student t-test was used for each passage in order to compare fresh vs. cryopreserved cells ($* = p < 0.05$).
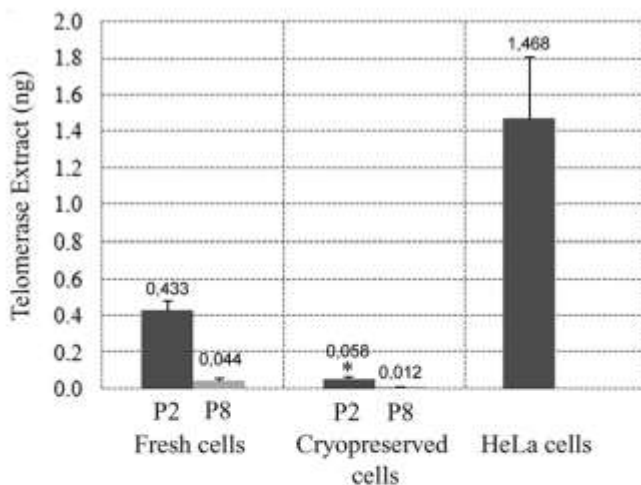
Fig. 3. Telomerase assay was performed between P2 and P8. Fresh cA-MSC showed telomerase activity at early passages (P2) that progressively decreased at P8. Cryopreserved cA-MSC presented a lower telomerase activity compared to fresh cells both at P2 and P8 passages. Immortalized HeLa cells were utilized as positive control. The paired Student t-test was used for each passage to compare fresh vs. cryopreserved cells ($* = p < 0.05$).
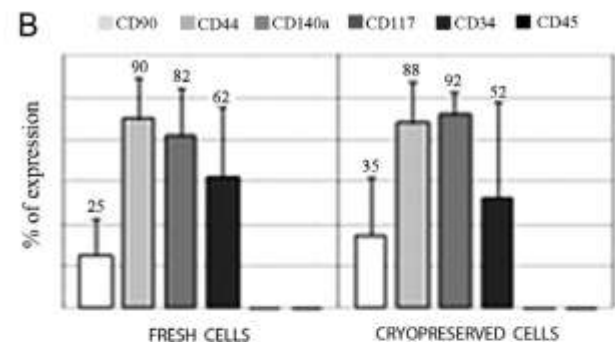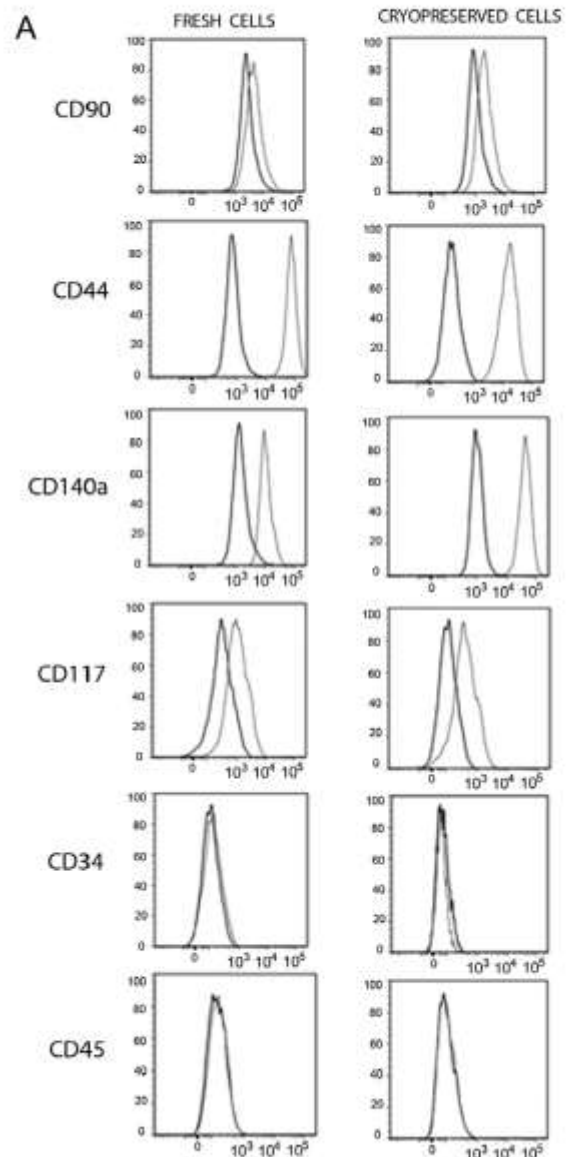
Fig. 4. Detection of CD markers on cA-MSC by FACS. Both fresh and cryopreserved cells showed a corresponding positivity for monoclonal antibodies against CD90, CD44, CD 140a, CD117, tested between P3 and P6. No significant differences were found in the five samples analyzed; both fresh and cryopreserved population were negative to CD34 and CD45. (A) Representative diagrams showing FACS evaluation in fresh and cryopreserved cells: the isotype control is highlighted by a black line. (B) The histograms show the average expression of CD positivity in fresh and cryopreserved cA-MSC. The paired Student t-test was used for each antigen to compare fresh vs. cryopreserved cells.

In this study we have analyzed specific features and plasticity of mesenchymal stem cells isolated from adipose tissue of *Canis familiaris* (cA-MSC). Furthermore, we have examined the effect of cryopreservation on cA-MSC "stemness" by means of a complete cellular characterization investigating phenotype morphology, vitality assay, telomerase activity and flow cytometry. The capacity of these multipotent cells to differentiate into adipocytes, osteoblasts and myoblasts has been compared in both fresh and cryopreserved cells. Our results demonstrate that cryopreservation alters some characteristics of *stemness* of cA-MSC although it does not cause any changes in their differentiation potential. Therefore, the opportunity to cryopreserve cA-MSC may offer the prospective for clinicians to use autologous stem cells in canine cell-based therapies.

2. Materials and methods

2.1. Cell culture

Samples were collected from visceral adipose tissue of female dogs during ovariohysterectomy surgery. The average age of the dogs spanned from 1 to 5 years; a total of 20 different samples were collected and processed. The weight of sampled adipose tissue was on average 7 g. Each sample was cleaned of large blood vessels, chopped adding 5–10 ml of phosphate-buffer saline (PBS) and incubated with collagenase I (Sigma–Aldrich S.r.l. Milan, Italy) at 40 µg/ml for 2 h at 37 °C. Subsequently, each sample was filtered with 100 µm filter and centrifuged at 1400g for 20 min. The supernatant was removed and the pellet washed with PBS. After a second centrifugation at 1600g for 10 min the supernatant was removed and the pellet was set in T25 flask (Falcon, BD Bioscience, Basel, Switzerland) in non-inductive medium consisting of Dulbecco modified Eagle's medium (DMEM D5671, Sigma–Aldrich) with 10% fetal serum (FBS) (ECS0180-L, Euroclone, Milan, Italy), containing penicillin, streptomycin (Sigma–Aldrich) and glutamine (Sigma–Aldrich). The isolated cells were put in an incubator, (Jouan IG150, labogerateborse, Burladingen, Deutschland) supplied with humidified air and 5% CO_2. After the overnight incubation, non-adherent cells and lipid droplets were removed and fresh medium was added to the flask. After three days of incubation canine adipose-derived-mesenchymal stem cells (cA-MSC) were developed, recovered, counted and set for amplification. In order to evaluate stemness characteristics of MSC both human dermal fibroblasts and HeLa cells were used as controls, respectively for differentiation studies and for telomerase assay.

2.2. Cryopreservation and thawing

cA-MSC were collected and resuspended in cryopreservation medium (90% FBS and 10% of dimethyl sulfoxide, DMSO, Sigma–Aldrich) at density of 3×10^6 cells/cryovial. Cells were frozen by Mr. Frosty (Nalgene, Roskilde, Denmark) decreasing 1 °C/min until 80 °C for 1 week and then they were transferred to the liquid nitrogen tank for long-term storage. Thawing of cells was performed after 10/12 months; cA-MSC were placed into a 37 °C water-bath for 1–2 min and washed in 10% FBS and 90% DMEM to eliminate DMSO. Finally, the cells were plated into T25 flasks with non-inductive medium.

2.3. Alkaline phosphatase

The AP (alkaline phosphatase) enzyme activity was assayed using a commercial AP detection kit (Kit SIGMA FAST BCIP/NBT; 5-bromo-4chloro-3-indolyl phosphate/nitro blue tetrazolium, Sigma–Aldrich, B5655-5TAB). In order to detect the AP enzyme activity, adipose-derived stem cells at 50% of confluence were washed with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature.

2.4. Measurement of population-doubling time (pdt)

We defined the population-doubling time as the time required for a colony area to expand twofold. Population-doubling was determined in continual subculture and growth of cA-MSC harvested at subconfluence, and were calculated using the formula $x = [\log_{10}(N_H) \quad \log_{10}(N_t)]/\log_{10}(2)$ (Cristofalo et al., 1998), where N_t is the inoculums cell number and N_H the cell harvest number. To yield the pdt, the population doubling for each passage was divided by the time (days). The pdt is showed at passages 2, 4 and 8.

2.5. Telomerase assay

TeloExpress Quantitative Telomerase Detection Kit (Express Biotech International, Thurmount, USA) was used for the detection of telomerase expression. Samples ($n = 6$) were assayed with real-time PCR (ABI 7500 Real-Time PCR System) to measure the enzyme presence.

2.6. Flow Cytometry Assay (FACS)

Adipose-derived stem cells ($n = 6$) were analyzed between passage P3 and P6 using FACS technique. Cells were collected, washed with PBS 1X, and incubated for 10 min at 4 °C in PBS 1X, FCS 2%, EDTA 2 mM. Subsequently the cell suspension was incubated with the primary antibodies directed against the following cell surface markers, each of which was conjugated with phycoerythrin (PE) and conjugated with fluorescein isothiocyanate (FITC). Cells were suspended in 300 µl of PBS in FACS tubes (Falcon, BD Bioscience, Basel, Switzerland). As control mouse isotype IgG1, IgG2a and rat isotype IgG2a (BD Bioscience, Erembodegem, Belgium) were used. For each sample 200,000 cells were passed through a FACScan Canto Flow cytometric, (BD Bioscience, Erembodegem, Belgium) and results were analyzed by FlowJo software (Tree Star, Inc., Oregon Corporation, USA).

2.7. Differentiation assay

cA-MSC at 80–90% of confluence were induced to differentiate towards different lineages by appropriate culture medium.

Osteogenic differentiation: to induce adipose-derived stem cells to differentiate into osteogenic tissue was added to the culture medium 1 nM dexamethasone, (Sigma D1756, Sigma–Aldrich S.r.l. Milan, Italy, http://www.sigmaaldrich.com) 10 mM β-glycerophosphate, (1-800-854, MP Biomedicels Europe, Illkirch, France) and 50 µM ascorbate-2-phosphate (Sigma A4544, Sigma–Aldrich). Cells were maintained in differentiation medium for 21 days and in this period the medium was changed every 2–3 days. After fixation in a 4% solution of formaldehyde (PFA) for 30 min the presence of mineralizated matrix was assessed by von Kossa staining.

Adipogenic differentiation: adipogenesis was induced using culture medium (DMEM-LG D5921 Sigma–Aldrich) supplemented with 0.5 mM isobutylmethylxanthine (Sigma I5879, Sigma–Aldrich), 200 µM indomethacin (Sigma I7378, Sigma–Aldrich), 0.5 µM dexamethasone (Sigma D1756, Sigma–Aldrich), and 10 µM insulin (Sigma I6634, Sigma–Aldrich). The medium was changed every 3 days and cells were maintained in culture differentiation medium for 21 days. The formation of lipid droplets was verified with Oil Red O staining, after fixation of cells in 4% PFA solution for 10 min. *Myogenic differentiation:* adipose-derived stem cells were cultured in differentiation medium consisting of high glucose DMEM, (Sigma–Aldrich) supplemented with 2% HS

cules was analyzed using specific monoclonal antibodies against CD90, CD44, CD140a, CD117, CD34 and CD45. None of the cells population examined was found to express hematopoietic lineage markers CD34 and CD45. In contrast, cells were uniformly positive for the adhesion marker CD90 ($25\% \pm 17.7$ for fresh; $35\% \pm 26.9$ for cryopreserved), for the hyaluronate receptor CD44 ($90\% \pm 17.3$ for fresh; $88\% \pm 20.2$ for cryopreserved), for platelet derived growth factor receptor CD140a ($82\% \pm 22.6$ for fresh; $92\% \pm 11.1$ for cryopreserved) and for c-Kit receptor CD117 ($62\% \pm 34.2$ for fresh; $52\% \pm 46.7$ for cryopreserved) (Fig. 4). Fresh cells did not present differences in the expression levels of these surface antigens in comparison to cryopreserved cells. All cell populations examined, defined on the basis of forward and side scatter, showed a similar cell complexity (in fresh and cryopreserved cA-MSC, Supplemental material Fig. 1).

3.5. Differentiative potential of cA-MSC examined by mRNA analysis

Fresh and cryopreserved cA-MSC were compared to check possible differences regarding their multilineage differentiation, by means of an in vitro assay. The mRNA expression of specific marker genes was examined after differentiation induction with adipogenic, osteogenic and myogenic specific media (Fig. 5). As a marker of adipogenic differentiation mRNA levels of PPARγ2 (peroxisome proliferation-activated receptor) were analyzed; in fact PPARγ2 is a nuclear receptor isoform present exclusively in adipose tissue. To verify osteogenic induction osteopontin expression (SPP1, secreted phosphoprotein 1) was evaluated; SPP1 is an extracellular structural protein and an organic component of bone. The expression of desmin, a protein of intermediate filaments present in skeletal muscle tissue was investigated in order to evaluate the myogenic differentiation. Fresh cA-MSC were able to differentiate towards adipogenic, osteogenic and myogenic lineages (Fig. 5A) and cryopreservation did not affect this potentiality (Fig. 5B). cA-MSC did not express these specific genes when not induced with differentiative media (Fig. 5C). The latter culture media did not induce the differentiation of human fibroblasts (Fig. 5D). In order to confirm the identity of the gene markers employed in this study all PCR bands were subcloned, sequenced and blasted in GeneBank database; all sequences showed a total identity with *C. familiaris* genes (data not shown).

3.6. Differentiative potential of cA-MSC examined by histochemical staining

Differentiation to adipogenic, osteogenic and myogenic lineages was qualitatively assessed on the basis of cell morphology, hystochemistry and immunohystochemistry. Induction of cA-MSC with adipogenic medium resulted in the presence of lipid vacuoles as shown by Oil Red O staining; no differences were found in the adipogenic potential between fresh and frozen cells (Fig. 6A and B). After the induction toward the osteogenic differentiation a mineralization of extracellular matrix, marked by von Kossa staining, was evident in cA-MSC; the same calcium deposits were present in fresh and cryopreserved cells with the same time of induction (Fig. 6C and D). The myogenic induction of fresh cA-MSC resulted in myoblast alignments with an initial fusion and formation of multinucleated cells; the myogenic commitment was indicated by nuclear Myf5 positivity and similar results were obtained after cryopreservation (Fig. 6E and F).

4. Discussion and conclusion

Mesenchymal stem cells are present in several connective compartments and have been isolated from various tissues (Kuznetsov et al., 2001; Jiang et al., 2002; Bieback et al., 2004; Crigler et al., 2007; Yamamoto et al., 2007). In order to develop cell-based therapies and autologous tissue transplants an important requirement is to obtain a sufficient number of MSC; for this reason the possibility to cryopreserve MSC cells in order to stock a high amount of quickly available cells will play a crucial role for the success of these treatments (Attarian et al., 1996; Park et al., 2008; Malpique et al., 2009). Therefore, it is fundamental to verify that the cryopreservation do not alter the *stemness* characteristics and the differentiative potential of isolated MSC (Martinello et al., 2009).
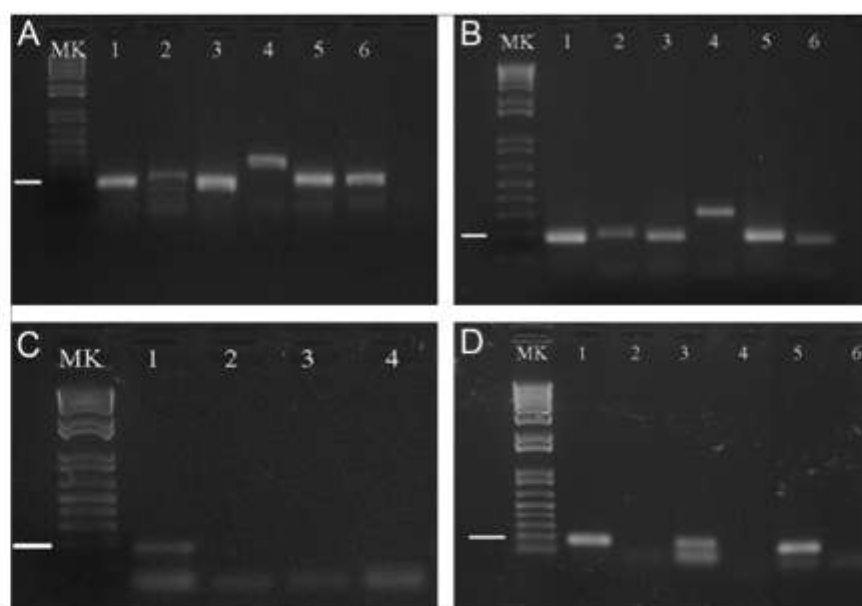

Fig. 5. mRNA level detection after the cA-MSC differentiation induction: PCR analysis on fresh cells (A) and cryopreserved cells (B): cells showed expression of PPARγ2 (lane 2), of SPP1 (lane 4) and desmin (lane 6); β-actin was used as internal control (lanes 1, 3 and 5). (C) cA-MSC maintained in culture with normal growth medium did not express any marker of differentiation (lanes 2, 3 and 4). (D) Fibroblasts were used as negative controls; they were maintained in the same differentiative culture condition of cA-MSC and did not show expression of PPARγ2 (lane 2), of SPP1 (lane 4) and desmin (lane 6).
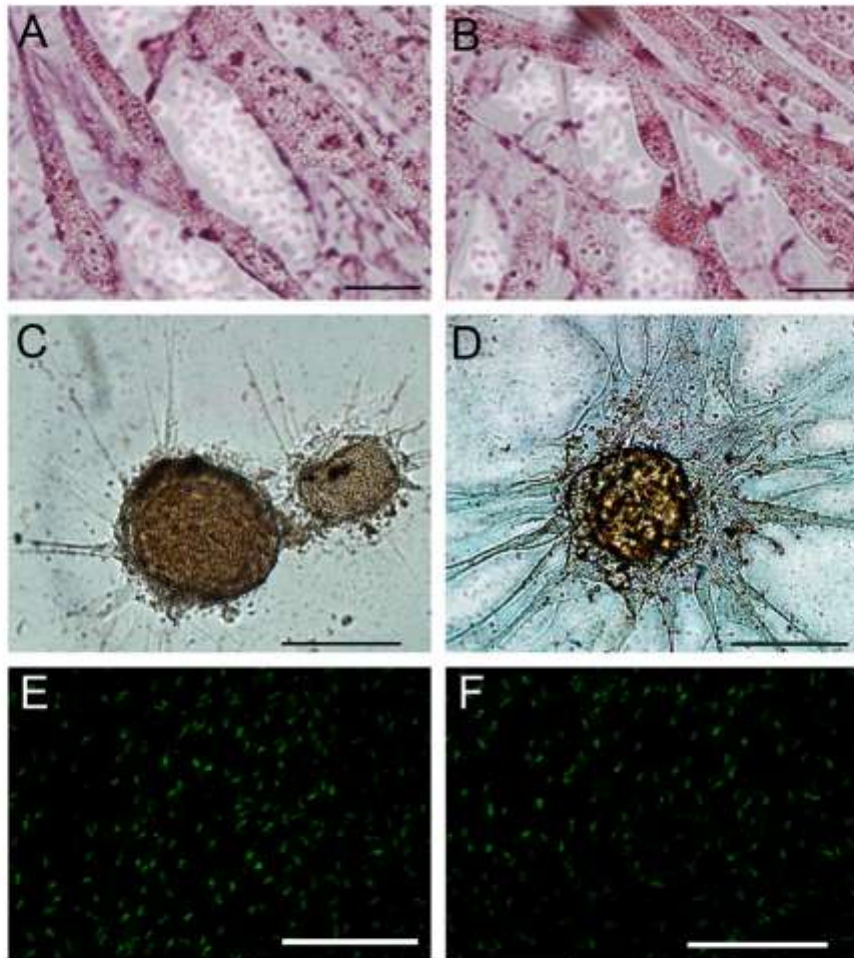
Fig. 6. Differentiative potential of cA-MSC. Adipogenic induction in fresh (A) and cryopreserved cells (B) was evident with Oil Red O staining; in both images small lipid droplets are visible inside the cells. Osteogenic induction in cA-MSC was verified with von Kossa staining: both fresh (C) and cryopreserved cells (D) showed a strong positive reaction since the calcium deposits increased in the culture. Myogenic induction in fresh (E) and cryopreserved ePB-MSC (F). The myogenic commitment was evident in cA-MSC since the nuclear positivity for Myf5. Scale Bars (A and B) 90 μm; (C and D) 100 μm; (E and F) 75 μm.

Adipose tissue is an attractive source of MSC because of its abundance and easy accessibility and this is especially true for dogs due to size limitation. We isolated MSC from visceral adipose tissue of 20 dogs without any complication and we expanded these cells for about 40 days. cA-MSC maintained a fibroblast-like morphology up to day 30 and the cryopreservation did not alter this phenotype. We demonstrated, for the first time, the presence of the alkaline phosphatase (AP) activity in cA-MSC; this enzyme is considered an embryonic marker because to an increase of its activity in embryonic stem cells it corresponds an higher capacity to colony formation (O'Connor et al., 2008). Our results showed that fresh and cryopreserved cA-MSC possessed a similar AP positivity and this fact prompted us to study other parameters to test the effect of cryopreservation on stocked mesenchymal stem cells.

MSC *in vitro* culture usually possess a limited life span and undergoes senescence as indicated by the loss of proliferation ratio and the altered morphology. The fresh cA-MSC investigated in this study reduced their population-doubling time during *in vitro* culture as well as after cryopreservation; passage 8 represents the upper limit step at which senescence occurs as demonstrated also by other researchers (Kern et al., 2006) in human MSC adipose tissue-derived cells. cA-MSC differ from equine A- and BM-derived-mesenchymal cells because presented a very high proliferative capacity and cell viability even after cryopreservation; the latter process seems to affect cell duplication only after 30 days of

culture (Mambelli et al., 2009). The hypothesis that cryopreservation may affect MSC life span was supported by telomerase expression analysis. Cells with the highest telomerase level have the greatest proliferative potential (Lansdorp, 2004) and our data indicated that cryopreservation caused a decrease of telomerase expression at passage 2. Although the reduced telomerase activity found in our study confirmed the influence of freezing protocols on the biology of stem cells, we considered, of more scientific value, to set up a differentiative protocol in order to test the potential of frozen cells when placed in an adequate microenvironment. Our results demonstrated that fresh cA-MSC showed great stem potentials because these cells were able to differentiate into three mesodermal lineages as well as cryopreserved cells. Although the potential of cA-MSC to differentiate into adipogenic and osteogenic fates was proved (Neupane et al., 2008) our study demonstrated, in addition, that their multilineage potential was not altered after cryopreservation. Furthermore, we demonstrated that cA-MSC could differentiate into myogenic lineage as observed in MSC isolated from human lipoaspirate (Zuk et al., 2002). Adipogenic differentiation of cA-MSC was confirmed by the formation of small lipid vacuoles, indicating that differentiation could be at initial stage, and by the expression of the adipocyte specific transcription factor PPARγ2 that functions in preadipocyte commitment (Tontonoz et al., 1994). Induction of cA-MSC towards the osteogenic fate led to morphological changes since the elongated fibroblast-like cells

turned into short, and cuboidal cells. The latter treatment caused the formation of cell aggregations and matrix mineralization as confirmed by calcium specific staining von Kossa together with the expression of SPP1(osteopontin) mRNA. Furthermore, the positivity of alkaline phosphatase demonstrated that undifferentiated cA-MSC may undergo osteogenic differentiation (Couchourel et al., 2009). Finally, the myogenic lineage potential of cA-MSC was confirmed by nuclei alignment, by the expression of nuclear myogenic transcription factor Myf5 and of the desmin mRNA. These results, although very promising, should be performed *in vivo* in future experiments in order to obtain a confident and definitive outcome of injected A-MSC cells.

Another important aspect of this study was to identify markers for canine mesenchymal progenitor cells and the development of protocol modifications to isolate dog stem cells in order to gain more data useful when dogs are used as models for human diseases. In this work we characterized cA-MSC by FACS analysis. However, it is important to consider that, in literature, data regarding the expression of surface antigens in canine adipose-derived MSC were not present, and also mesenchymal stem cell specific cellular markers are lacking. Human and murine progenitor cells may be isolated based on positive selection of cells which express surface markers as well as a negative selection whereby cells expressing lineage markers are removed. In this study, we achieved characterized cA-MSC thanks to a combination of positive and negative signals using different antigen markers.cA-MSC did not present significant differences concerning the expression of analyzed markers after cryopreservation; indeed both sets of cells expressed: CD44, a glycoprotein involved in cell adhesion and migration of mesenchymal stem cells; CD90, a surface protein used as a marker for a variety of stem cells; CD140a, the cPDGF receptor expressed by embryonic tissues and mesenchymal-derived cells of adult tissues and CD117, a cytokine receptor, expressed on the surface of hematopoietic stem cell. Our results supported data present in literature about mesenchymal stem cells isolated from human and mouse adipose tissue (Yamamoto et al., 2007; Lee et al., 2004; Kern et al., 2006; Rebelatto et al., 2008). However, cA-MSC lack hematopoietic marker CD45 and CD34 as demonstrated in human A-MSC and in dog bone marrow-derived MSC (Csaki et al., 2007).

Overall, this study has demonstrated for the first time that MSC isolated from adipose tissue of dogs could be cryopreserved maintaining their stemness characteristics. Telomerase expression decreased significantly at passage 2 after cryopreservation; nevertheless, this fact does not affect the differentiative potential of cA-MSC. The slower pdt at passage 2 and 4 of cryopreserved cells does not really affect their phenotype or differentiative potential and suggests to cryopreserve cA-MSC at early passages. The observation that at passage 8 we did not evidence significant changes regarding doubling time parameters between fresh vs. cryopreserved cells is probably due to the fact that after much time spent in culture, cells are all slow and biologically "exhausted". In conclusion, our data indicates that for cell-based tissue engineering therapies and tissue transplants it would be advisable to use cA-MSC fresh or just thawed at no more than 20–25 days of culture.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.rvsc.2010.07.024.

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Title: Influence of temperature, time and different media on mesenchymal stromal cells shipped for clinical application.

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Abstract: Cell-based therapies, as the use of mesenchymal stromal cells (MSCs), are becoming popular in veterinary medicine. When MSCs are not cryopreserved, they are shipped in suspension although no previous studies analyzed MSCs viability during delivery. Here, the impact of several experimental shipping conditions on the number of equine blood-derived (ePB-MSC) and canine adipose-derived mesenchymal stromal cells (cA-MSC) were evaluated. Among different parameters tested, only time and temperature influenced MSCs number during experimental shipping condition. Furthermore, cells were monitored during different time intervals for gene expression of typical MSCs markers and to evaluate acquired resistance to apoptosis and beta-galactosidase activity. Overall, results obtained from this study indicate that ePB-MSC and cA-MSC should be delivered in PBS at room temperature within 9-12 hrs.

1 **Influence of temperature, time and different media on mesenchymal stromal cells**
2 **shipped for clinical application**

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10

11 **Abstract**

12   Cell-based therapies, such as the use of mesenchymal stromal cells (MSCs), are becoming

13   popular in veterinary medicine. When MSCs are not cryopreserved, they are shipped in

14   suspension although no previous studies have analyzed MSCs viability during delivery. Here,

15   the impact of several experimental shipping conditions on the number of equine blood-

16   derived (ePB-MSC) and canine adipose-derived mesenchymal stromal cells (cA-MSC) were

17   evaluated. Among different parameters tested, only time and temperature influenced MSCs

18   number during experimental shipping condition. Furthermore, cells were monitored during

19   different time intervals for gene expression of typical MSCs markers and to evaluate acquired

20   resistance to apoptosis and beta-galactosidase activity. Overall, these results indicate that

21   ePB-MSC and cA-MSC should be delivered in PBS at room temperature within 9-12 h.

22

23   *Keywords:* Horse; Canine; Mesenchymal stromal cells; Validation of cell shipping;

24   Senescence

25

26   Mesenchymal stromal cells (MSCs) are receiving considerable attention in veterinary

27   medicine because of their potential for cell-based regenerative therapies (Fortier, 2005). They

28   have been isolated and characterized from several tissues and have been widely studied to

29   define their characteristics and plasticity in vitro (Koch et al., 2009). In order to use MSCs for

30   clinical purposes, cells are often delivered cryopreserved; our previous studies, focused on

31   cryopreservation of equine blood-derived (ePB-MSC) and canine adipose-derived (cA-MSC)

32   MSCs, showed that stemness characteristics were identical for fresh and cryopreserved cells

33   (Martinello at al., 2009; Martinello et al., 2010).

34   Undoubtedly, cryopreservation is the best choice for long-term storage of MSCs, even

35   if some cell death occurs due to the freezing and thawing processes (Haack-Sørensen and

36   Kastrup et al., 2011). However, because long-term storage is not necessary for local shipment

37   of cells, it is essential to determine the optimal conditions under which MSCs may be

38   maintained in a non-frozen state to facilitate their shipment and use in veterinary medicine.

39   Such data are currently lacking. The aim of this study was to provide an analysis of the

40   influence of parameters likely to be relevant during short-term shipment of ePB-MSC and cA-

41   MSC. Specifically, cell number, CD (cluster of differentiation) expression, acquired apoptotic

42   resistance and senescence were monitored as relevant parameter for short-term shipment of

43   cells. ePB-MSCs were obtained from 100 mL of peripheral blood of 10 horses and plated at

44   1.6×10^5 cells/cm^2; cA-MSC were isolated by enzymatic digestion from approximately 7 g of

45   adipose tissue of 10 female dogs, and both cell type plated in 25 cm^2 culture flasks (Falcon;

46   BD Bioscience) in non-inductive medium for further expansion. Five cell samples of each

47   species were utilized to test all experimental shipping conditions: ePB-MSC and cA-MSC

48   were collected at passage 3, placed in sterile tubes at a concentration of 5×10^5 cells/mL, and

49   maintained in different types of medium, containing 1% penicillin and streptomycin, at

50   different time intervals and temperature conditions (Table 1). After exposure to different

51   experimental shipping conditions cells were counted with Scepter 2.0 Automated Cells

52 Counter (PHCC20040 Millipore) and analyzed using Scepter Software pro 2.0 (Millipore).

53 Subsequently ePB-MSC and cA-MSC were plated and left to adhere to 25 cm^2 culture flasks

54 for an additional 10 h in non-inductive medium. Adherent cells were trypsinized and counted,

55 gating the Scepter 2.0 for cells comprised between 15-22 μ. Statistical analysis of ePB-MSC

56 and cA-MSC exposed to previous experimental shipping condition were performed, and cells

57 that maintained the highest number respect to the selected parameters, were subjected to

58 further analysis: five cells sample of ePB-MSC and cA-MSC were used for CD expression,

59 apoptosis resistance, and beta-galactosidase assays, after maintaining these cells in PBS at RT

60 and for the time request by the three different studies. In order to perform CD expression

61 analysis, real time PCR (Applied Biosystems) was used on 3, 6, 9 and 12 h maintained ePB-

62 MSC and cA-MSC. Primers were designed using the Primer Express 3.0 software (Applied

63 Biosystems) as listed in Table 2. HT TiterTACS Colorimetric Apoptosis Detection Kits

64 (Trevigen) was used on ePB-MSC and cA-MSC to determine acquired resistance to apoptosis

65 after 12 and 24 h of maintaining in shipping condition. Cells resuspended in PBS at RT for 12

66 and 24 h were plated, fixed and stained for senescence-associated β-galactosidase (SA β-gal)

67 activity by using Mirus β-Gal Staining Kit (Madison, U.S.A).

68   The statistical analyses performed after keeping ePB-MSC and cA-MSC in different

69 types of medium showed no significant differences in cell survival rate among the ten

70 different media utilized, consequently, serum did not affect cell survival over several ranges

71 of times and temperatures tested (Fig.1A). A dramatic decline in cell number after 12 h for

72 both ePB-MSC and cA-MSC were found (Fig.1B). Our study clearly indicates that

73 maintenance of ePB-MSCs and cA-MSCs number is greater at RT (22°-25°C) compared with

74 low (4°) or physiological temperatures (37 °C) (Fig.1C). However, data obtained from the

75 exposure of ePB-MSC and cA-MSC to the above experimental shipping conditions, showed

76 that neither the different media formulations, nor temperature were able to prevent the decline

77 in viable cells during short incubation periods, since about 30/40% mortality occurred.

78   As shown in Fig.2 level of expression of CD44 and CD105 in cA-MSC during all time

79   intervals did not differ from freshly isolated cells; ePB-MSC showed a decrease in CD90

80   expression at 9 and 12 hrs. Kozhhevnikova et al. (2008) and Radcliffe et al. (2010) showed

81   that the expression of CD90 was constant in in vitro culture during time. In our study, a strong

82   decrease of the stemness MSC marker CD90 was observed in cells at 9 and 12 h; this fact

83   might suggest an inability of ePB-MSC to retain the properties of multipotent stromal cells

84   after this period. Both ePB-MSC and cA-MSC showed an enlarged and irregular shape after

85   12 h of suspension, similar characteristics are observed in senescent cells which remain

86   metabolically active, resistant to apoptosis, (Dimri et al. 1995) and possess an "egg-shape"

87   morphology (Wagner et al. 2010). Campisi et al. (2007) describes that some cells type

88   acquires resistance to certain apoptotic signals when they become senescent; this resistance

89   may partly explained why senescent cells are so stable in culture.

90   In conclusion, our results showed that at 24 h of treatment, both ePB-MSC and cA-

91   MSC cells were more resistant to apoptotic stimuli than control cells (fresh cells) (Fig.3).

92   ePB-MSC and cA-MSC maintained in suspension at RT in PBS were positive for beta-

93   galactosidase at 24 h (Fig.4), confirming the hypothesis that 24 h shipped cells become

94   senescent. Collectively, these data provided by this study indicate that both ePB-MSC and

95   cA-MSC should be shipped and used within 9-12 h maintaining them in PBS at RT.

96

97   **Conflict of interest statement**

98   None of the authors has any financial or personal relationships that could

99   inappropriately influence or bias the content of the paper.

100

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149 **Table 1.** Experimental conditions of cells cultures. 5×10^5 cells were maintained in ten

150 different media for 3 to 72 hrs at three different temperatures.

Type of medium	Time Intervals	Temperature
PBS	3, 6, 9, 12, 24, 48, 72 hrs	4°C, 37°C, 20-22°C (RT)
DMEM	3, 6, 9, 12, 24, 48, 72 hrs	4°C, 37°C, 20-22°C (RT)
PBS + HS 20%	3, 6, 9, 12, 24, 48, 72 hrs	4°C, 37°C, 20-22°C (RT)
PBS + HS 80%	3, 6, 9, 12, 24, 48, 72 hrs	4°C, 37°C, 20-22°C (RT)
PBS + FCS 20%	3, 6, 9, 12, 24, 48, 72 hrs	4°C, 37°C, 20-22°C (RT)
PBS + FCS 80%	3, 6, 9, 12, 24, 48, 72 hrs	4°C, 37°C, 20-22°C (RT)
DMEM + HS 20%	3, 6, 9, 12, 24, 48, 72 hrs	4°C, 37°C, 20-22°C (RT)
DMEM + HS 80%	3, 6, 9, 12, 24, 48, 72 hrs	4°C, 37°C, 20-22°C (RT)
DMEM + FCS 20%	3, 6, 9, 12, 24, 48, 72 hrs	4°C, 37°C, 20-22°C (RT)
DMEM + FCS 80%	3, 6, 9, 12, 24, 48, 72 hrs	4°C, 37°C, 20-22°C (RT)

151

152 **Table 2. Primers used in the real time-PCR reaction**

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Dog		
B2M	TCTACATTGGGCACTGTGTCAC	TGAAGAGTTCAGGTCTGACCAAG
CD44	CCCAGAATGGCTCATCATCT	GTTGGAAGGTGGGTGTGGAA
CD105	CCAATGCTACCGTGGAAGTT	GTGCCATTTTGCTTGGACTT
Horse		
18S	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT
CD44	TCCACCCCAACTCCATCTGT	TGAAGCAATAGGTGTCGTACTGAGA
CD90	CCGTGAGACAAAGAAGCATGTG	CCTGATGTTGTACTTGCTGGTGAAG

153

154

155 **Figure legends**

156

157 Fig. 1. The influence of shipping conditions on ePB-MSC and cA-MSC were statistically

158 evaluated for the medium (A) time (B) and temperature (C); medium did not significantly

159 (P>0.05) effect cell survival. Both types of MSC significantly decreased (P<0.05) their

160 number after 12 hrs. RT provided the greatest survivability (P<0.05) of both ePB-MSC and

161 cA-MSC. Data presented are mean cell counts ± st.er. for each type of medium (A), time

162 point (B) and each temperature (C).

163

164 Fig. 2. Real time PCR analysis of CD markers expression on ePB-MSC and cA-MSC at

165 different time intervals. No significant differences were detected in cA-MSC for CD105 and

166 CD44 expression; in ePB-MSC CD44 expression did not change while a significant decrease

167 in CD90 was found at 9 and 12 hrs (*). Data were derived from one-way analysis of variance

168 (ANOVA), statistical significance was set at P≤0.05.

169

170 Fig. 3. Apoptosis detection assay performed on fresh and treated cA-MSC and ePB-MSC

171 following 0.01 µM staurosporine stimulation. After 24 hrs of maintenance in suspension

172 medium, both horse and dog MSC were less susceptible to apoptotic stimuli than were freshly

173 isolated cells (control). After 12 hrs of maintenance in suspension medium, cA-MSC treated

174 cells showed greater sensitivity to apoptotic stimuli than control cells. *Differs from control,

175 P≤0.05.

176

177 Fig. 4. Beta-Galactosidase assay on cA-MSC (A, C), and ePB-MSC (B, D) cells. After 24 hrs

178 of treatement, MSC from both species (C, D) were positive for X-Gal staining. No Beta-

179 Galactosidase activity was detected in treated cells at 12 hrs (A, B). Scale bars: 300 µm.

180

Figure3
Click here to download high resolution image
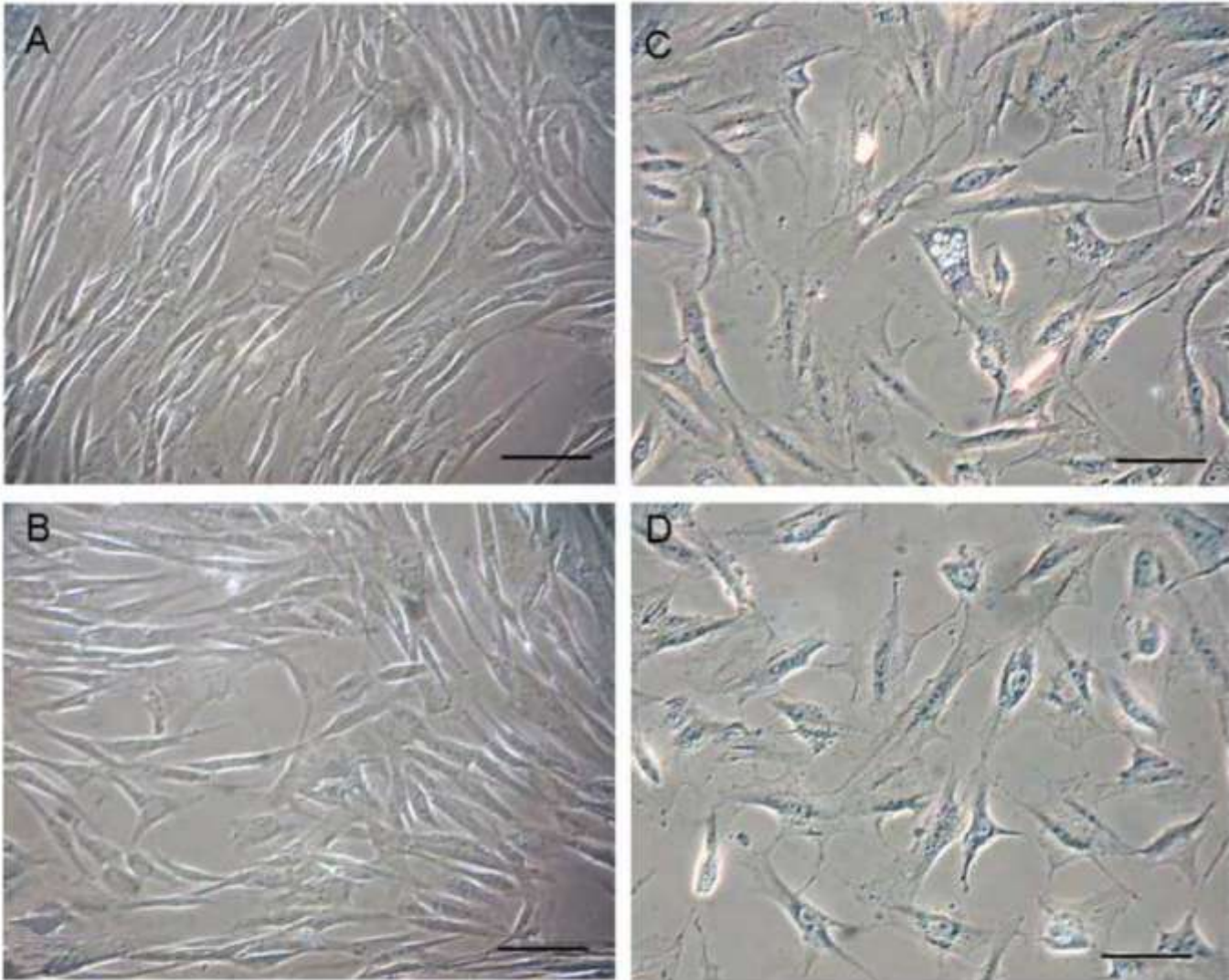
Applications of autologous adult mesenchymal stromal cells (MSCs) in tendon healing:

- **Human adipose tissue derived MSCs applied for recellularization of human tendon scaffold.**

- **Tendon repair in a sheep model: comparison of three different treatment protocols, using MSCs, PRP or MSCs+PRP after short and long time period of recovery from induced lesion**

Tendon injuries are common clinical problems both in human and in veterinary field; they can be acute or chronic, and caused by intrinsic or extrinsic factors. In acute trauma, extrinsic factor are predominant, even if intrinsic factor are important, for example disfunction of the normal protective inhibitory pathway of the musculo-tendinous unit may result in injury. The aetiology of tendon rupture remains unclear and degenerative tendinopathy remain the main common histological findings in spontaneous tendon rupture. In chronic tendon disorders, interaction between intrinsic and extrinsic factors is common. <u>Excessive loading of tendons during physical training is the main pathological stimulus for tendon degeneration</u>. In fact the majority of the tendon lesions in veterinary field are observed in races horses; superficial digital flexor tendonitis (SDF) constitute a career-limiting injury, with a high incidence of reinjury, due to the intrinsic healing poor capacity of tendons (Genovese R et al., 1996; Palmer S et al., 1994; Yovich J et al., 1995). Most of the reported SDF tendonitis relate to Thoroughbred flat (Gibson K et al., 1997) and National Hunt (Marr C et al., 1993; Ordidge R, 1996) racehorses, Standardbred racehorses (Hawkins J and Ross M, 1995), and Grand Prix showjumpers (Gibson K et al., 2002; Bathe A, 2003; Dyson S, 2003). On the other hand tendon injuries are common also in human medicine, it has been estimated that in the USA alone, more than 32 million traumatic and repetitive motion injuries to tendon and ligament occur each year (Shoen DC et al., 2005); the majority of such lesions are related to rotator cuff disease (Pegreffi F et al., 2011), anterior crucial ligament (ACL) of the knee (Steinert AF et al., 2011), flexor and extensor tendon of the hands (Schöffl V et al., 2010).

Tendon healing can occur intrinsically, by proliferation of epitenon and endotenon tenocytes, or extrinsically by migration of cells from the surrounding sheath and synovium. However despite the healing process, the biochemical and mechanical features of tendon tissue never match those of not injured tendon, and often adhesion formation is present and represents a clinical problem. Therefore

the aim of all new treatment strategies for tendon healing would be to restore functional tissue. Experimental studies using a collagenase-induce tendonitis model have investigated the effect of intralesional injection of hyaluronan or beta aminopropionitrile fumarate (BPAN) or systemically administred polysulphate glycosaminoglycan (PSGAG) (Redding WR et al., 1992). More recently, enhanced biological approaches, including platelet rich plasma, bone marrow aspirate, growth factor supplements and cell- and gene-modified therapy has been investigated, in order to effort the challenge to improve tendon healing.

Delivery of local growth factors by injection of recombinant growth factors or on carriers as supporting materials was performed in injured tendon. Factors like platelet-derived growth factor (PDGF), epidermal growth factor (EGF), or insuline like growth factor (IGF-1), alone or in combination, not only directly influence tissue regeneration, but also exert an anti-inflammatory function and are chemo-attractive for cells. Despite added complexity, cell-derived therapies form an important part of the enhanced repair, and tendon-derived cells or mesenchymal stem cells have been proposed as able to regenerate injured tendon tissue, and the mechanical loading of stem cell or tendon derived cells appear to be a very promising alternative to the classical treatment of tendon disorders, even if needs further clinical evaluation. Moreover the combination of carrier vehicles, growth factor, and implanted cells provide the best opportunity for robust tendon repair (Nixon AJ et al., 2012). The gene-modified cell therapy open further possibility to study tendon regeneration: it has been demonstrated that transfected MSCs with growth and differentiation factor (GDF7) contributed to the healing of tendon defect. Moreover it has been shown that MSCs adopted a tenogenic fate *in vitro* and *in vivo* when transfected with bone morphogenic protein-2 (BMP-12) and a variant of the Smad8 signalling mediator protein.

In the works that follow, MSCs derived from human lipoaspirate have been used in attempt to recellularize a human tendon scaffold to be used in hand flexor tendons failure. Several type of synthetic materials have been used to construct extracellular matrices for *in vitro* cell culture and *in vivo* tendon tissue regeneration, however, there is currently no scaffolding material that simultaneously offers superior biocompatibility, bio-functionality, mechanical property and tractability. Indeed in the study a natural acellularized scaffold from cadaveric tissue has been used in order to preserve physiological and mechanical properties and ECM proteins for attachment, migration and proliferation of cells. The integration of MSCs into the scaffold has been evaluated by histological, immunohistochemical assay.

Furthermore, in a second study, the tendon healing has been evaluated using sheep as a model for horses. Specifically, the effect of mesenchymal stromal cells derived from peripheral blood of sheep (sPB-MSCs), the platlet rich plasma, and the combination of both, were utilized to treat

experimental lesions induced by Collagenase 1A in deep digital flexor tendon (DDFT) of ovine model. Several studies reported the use of bone marrow derived MSCs or PRP both *in vitro* and *in vivo* in tendon healing, however this is the first report that focus on an alternative source of MSCs to treat tendon injuries *in vivo*, and that analyzed the long term regeneration (4 months) comparing three different typology of treatment.

It follows the submitted paperwork focused on recellularization of human tendon scaffold, and the paperwork aimed to compare short and long term tendon healing on DDFT of sheep using sPB-MSC, PRP and combination of both:

Successful recellularization of human tendon's scaffold using adipose derived mesenchymal stem cells and a collgen gel.
Martinello T., Bronzini I., Volpin A., Vindigni V., Caporale G., Mascarello F., Bassetto R., patruno M. Submitted Journal of tissue engineering and regenerative medicine. 2011

Effect of peripheral blood derived mesenchymal stromal cells (PB-MSCs), platlet rich plasma (PRP) or combination of both on deep digital flexor tendon repair of ovine experimental model.
Bronzini I., Martinello T., Perazzi A., Iacopetti I., Testoni S., DeBenedictis G., Caporale G., Mascarello F., Patruno M. Ready to submition.

SUCCESSFUL RECELLULARIZATION OF HUMAN TENDON'S SCAFFOLDS USING ADIPOSE-DERIVED MESENCHIMAL STEM CELLS AND A COLLAGEN GEL.

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ADIPOSE-DERIVED MESENCHIMAL STEM CELLS AND A COLLAGEN GEL.

ABSTRACT

The major aim of regenerative medicine is to excogitate experimental techniques that take maximal advantage of reparative processes that occur naturally in the animal body. Injection of mesenchymal stem cells into the core of a damaged tendon represents a form of seeding which aims to cope with the above statement. Nowadays, the acellularization of native tendons seems a possible target while the seeding protocols are still under investigation. The results presented in this paper concerning the use of adipose derived mesenchymal stem cells for reseeding an acellularized tendon should reduce this gap. Indeed, the aim of our study was to obtain a recellularized biocompatible scaffold from cadaveric tissue to be used in total or partial tendon injuries in order to promote a better cellular regeneration. Our results show, for the first time, that it is possible to introduce proliferating cells in the core of a decellularized tendon treating the scaffold with a collagen gel.

INTRODUCTION

The major aim of regenerative medicine is to develop regenerative techniques that take maximal advantage of reparative processes that occur naturally in the animal body. Regeneration in mammalian tendons is scarce and not epimorphic or tissue reparative mechanisms take place, rather edema and swelling is followed by the inflammation response. These processes induce a complex and slow healing process that does not lead to a total functional *restitutio ad integrum* (Liu *et al.* 2011; Liu *et al.* 2008). Tendon pathologies are frequent and debilitating and, in the US alone, more than 32 million traumatic and repetitive motion injuries to tendons and ligaments occur each year (Shoen, 2005). For example, hand flexor tendons may undergo two different kinds of failure, rupture due to excessive loads and accidental laceration. Tendons suffer various degrees of injury,

ranging from relatively mild inflammation to full thickness transaction (Liu *et al.* 2008). Surgical techniques employ the use of autogenous tendons (biological grafts) to repair these deficits in an effective way although the tissue defects may lead to patient morbidity and diminished functional performances. However, the mechanical strength of such grafts never returns to complete functional recovery following their insertion and tendon repairs are often weak and susceptible to relapses (Liu *et al.* 2011). Furthermore, the use of biological grafts is limited by availability, the possible risk of an adverse immunological reaction or to disease transmission.

Tendon tissue engineering therefore represents a more promising approach since its aim is to promote full tendon regeneration rather than simply replacing damaged tendons with partially functional external substitutes. Various natural and synthetic materials have been used to construct extracellular matrices for *in vitro* cell culture and *in vivo* tissue regeneration (Cooper *et al.* 2005; Liu *et al.* 2006; Cao *et al.* 2002; Ouyang *et al.* 2003; Awad *et al.* 2003; Juncosa-Melvin *et al.* 2006; Gentleman *et al.* 2006; Bagnaninchi *et al.* 2007; Funakoski *et al.* 2005; Funakoski *et al.* 2005; Majima *et al.* 2005); however, there is currently no scaffolding material that simultaneously offers superior biocompatibility, bio-functionality, mechanical property and tractability.

The use of a natural acellularized scaffold from cadaveric tissue might preserve physiological and mechanical properties and ECM proteins for attachment, migration and proliferation of the cells (Gilbert *et al.* 2006). Moreover, it has been observed that the de-cellularization of natural scaffolds reduces immunogenicity and preserves their histological structures (Hudson *et al.* 2004). Several approaches to de-cellularization have been investigated including peracetic acid, *t*-octyl-phenoxypolyethoxyethanol (TritonX-100), sodium dodecyl sulfate, tri(n-butyl) phosphate (TnBP) (Gilbert *et al.* 2006; Woods *et al.* 2005; Cartmell *et al.* 2000; Deeken *et al.* 2011; Cartmell and Dunn, 2004; Harrison and Gratzer, 2005; Ingram *et al.* 2007) . Some authors have demonstrated that 0.1% (w/v) sodium dodecyl sulfate (SDS) was a very effective tendon cell removal agent and tendon fascicles treated with SDS were similar to control values with no alterations of ECM (Ingram *et al.* 2007; Pridgen *et al.* 2011). Previous studies have suggested that different cell types

might be used to engineer tendons in order to repopulate scaffolds such as tenocytes or adipo-derived mesenchymal stem cells, although some technical problems have still to be solved (Kryger *et al.* 2007; Chen *et al.* 2009). Indeed, the isolation of tenocytes from patient for autologous clinical use requires long culture times and invasive sampling while mesenchymal stem cells showed an easier collection and proliferated faster in cell culture (Kryger *et al.* 2007).

In order to reseed scaffolds, the tissue engineering approach has provided several treatments including static culture, injection, pulsatile perfusion, centrifugal force and ultrasonication but re-cellularization was never homogeneous in all cases (Ingram *et al.* 2007; Kryger *et al.* 2007; Thevenot *et al.* 2008). Generally, the seeded cells formed only a monolayer over the scaffold surface (Pridgen *et al.* 2011; Kryger *et al.* 2007) or, if they succeed in penetrating the matrix, the number and density was significantly lower than those seen in the native tendon (Ingram *et al.* 2007). The aim of our study was to develop a re-cellularized biocompatible scaffold from cadaveric tissue for use in total or partial tendon injuries in order to promote better cellular regeneration. Here, we describe for the first time, that it is possible to introduce proliferating cells in the core of a de-cellularized tendon treating the scaffold with a collagen gel.

METHODS

Sampling of tendons

Human flexor tendons were harvested from body parts removed during surgery for severe hand and digits traumas following ethic guidelines of the General Hospital of Padova.

Acellularization of tendons

Flexor tendons used for acellularization were harvested as described above and washed with phosphate buffered saline. The whole tendons were placed for 2 h at 37°C with agitation in 50 mL of hypotonic Tris buffer (10 mM, pH 8) containing 0,1% (w/v) ethylenediaminetetraacetic acid (EDTA) and two proteases inhibitor, phenylmethylsufonyl fluoride (100 mM) and leupeptin

(1mg/ml). Subsequently, tendons were placed in 0.1% SDS (w/v) in hypotonic buffer for 5 h at 37°C along with agitation. Finally, the tendons were rinsed 3x30 min in PBS at room temperature. After washing tendons were incubated in 2000 KU DNase (D4263, Sigma-Aldrich, Milan, Italy) in 1M NaCl to remove residual cell detritus and shaken for 1h at room temperature to solubilize nuclear contents and degrade DNA. After two washing steps with PBS tendon samples were stored in PBS containing 1% antibiotic solution at 4°C. After the decellularization protocol the scaffolds were sterilized under UV light overnight.

Cell culture

Adipose-derived stem cells (ADSCs) were extracted from human adipose tissues of healthy female patients undergoing cosmetic surgery procedures (liposuction), following guidelines from the Clinic of Plastic Surgery, University of Padova. Cells were cultured as described in a previous study (Martinello et al. 2010) . Briefly, samples were washed extensively with sterile phosphate-buffered saline (PBS) to remove contaminating debris and red blood cells. Washed aspirates were treated with 40 µg/ml collagenase (type I; Sigma-Aldrich, Milan, Italy) in PBS at 37°C with gentle agitation until complete disaggregation. The collagenase was inactivated with an equal volume of DMEM, 10% fetal bovine serum (FBS) and filtered through a 100 µm mesh filter and centrifuged at 1400g for 20 min The supernatant was removed and the pellet washed with PBS. After a second centrifugation at 1600g for 10 min the supernatant was removed and plated onto conventional tissue culture plates in control medium. After three days of incubation adipose-derived stem cells were developed, recovered and set for amplification.

Recellularization

Two seeding methods were performed and $1x10^6$ cells/scaffold were used for each tendons. First method: acellularized tendon scaffolds were reseeded by injecting a concentrated cell solution (in

200 μl of medium) into the center of the scaffold using a 27-guage needle. The scaffold was

allowed to incubate for 2hrs waiting for cell attachment and then growth medium was added.

Second method: it consisted in injecting in the core of acellularized tendon 200 μl of collagen

solution (C34243, Sigma-Aldrich, Milan, Italy) which, at 37°C for 1hr, forms a gel; after this step

the cell solution was injected into the scaffold. After 1hr of incubation growth medium was added.

All cell-scaffold constructs were incubated at 37°C in a humidified tissue culture chamber with 5%

CO_2 in medium for 7 days before analyses; the medium was changed every third day.

Histological analysis of the scaffolds

Human tendon and decellularized/recellularized scaffolds were fixed for 24 h in 4%

Paraformaldeyde solution in PBS (pH 7.4) at 4 °C. Samples were washed in distilled water,

dehydrated through a graded alcohol series, embedded in paraffin and sectioned at a thickness of 5

μm. Several serial sections were stained with different procedures such as haematoxylin and eosin,

Masson's trichrome (HT15, Sigma-Aldrich, Milan, Italy) and periodic acid Schiff-Alcian blue,

PAS-AB. Images were taken with an Olympus BX50 photomicroscope.

Immunohistochemistry

The immunohistochemical reactions were performed using the Vectastain elite ABC Kit Peroxidase

(PK-6100, Vector laboratories, Milan, Italy). The primary antisera anti-collagen type 1 antibody

(monoclonal antibody anti-COL-1, C2456, SIGMA Aldrich) and anti-anticartilage

oligomeric matrix protein (Rabbit polyclonal antibody anti-COMP, ab74524, Abcam) were applied

overnight at 4° C in a humid chamber, both at 1:100 dilution in PBS/Tween20 (0,01%) and the

secondary antibody Biotinylated antimouse IgG (BA-9200, Vector laboratories, Milan, Italy) and

Biotinylated antirabbit IgG (BA-1000, Vector laboratories, Milan, Italy) were used. After rinsing in

PBS buffer, sections were incubated in ABC Kit. After washes in PBS, the immunoreactive sites

were visualized using DAB substrate Kit 3,3'-diaminobenzidine tetrahydrochloride (SK-4100, Vector laboratories, Milan, Italy). In order to ascertain structural details, sections were dehydrated, mounted in Eukitt and examined under an Olympus BX50 photomicroscope.

The specificity of the immunostaining was verified: 1) incubating sections with PBS instead of the specific primary antibody; 2) incubating sections with preimmune serum instead of primary antibody; 3) incubating sections with PBS instead of secondary antibodies. The results of these controls were negative (i.e. staining was not detected).

DNA analysis

To assess the total DNA content native tendons, decellularized and recellularized scaffolds with collagen were pulverized in liquid nitrogen and homogenized in 1 mL of DNA buffer (EDTA-Na 0,1M, Tris HCl 0,05M pH8, SDS 10%) and 20 μl of proteinase K 20mg/ml overnight at 55°C. The following day 300 μl of NaCl 5M solution was added and the samples were stirred for 20 min and centrifuged to 12,000 rpm for 30 min. The DNA was precipitated with isopropanol, washed with 75% ethanol and air-dried. The pellet was finally dissolved in ribonuclease-free water and stored at -20°C. The DNA content was qualitatively evaluated by amplification reaction using β-actin primers. PCR were conducted using specific primers designed by means of Primer Express software and purchased online (http://www.eurofinsdna.com). A β-actin fragment of 185 bp was amplified (forward primer: 5'–CCATCTACGAGGGGTACGCCC–3'; reverse primer: 5'–TGCTCGAAGTCCAGGGCGACGTA–3'). A negative sample control was also added in the same PCR reaction. Appropriate annealing temperature (60 °C) for 35 cycles was performed.

The DNA extraction and the PCR products were visualized on agarose gels by ultraviolet transillumination.

XTT assay

To determine the cellular viability and survival of cells in the scaffold, after collagen treatment, we used a cell proliferation kit II (XTT)-based (Roche, Milan, Italy) colorimetric assay after 7 days of incubation. Controls, decellularized and recellularized tendons (n=3 for all group) were incubated with 2 ml yellow XTT solution for 2 h. After incubation with XTT, the metabolically active cells developed an orange formazan product, 3x100 µl of each samples was quantified using an enzyme linked immunosorbent assay plate reader (Spectra Count; Perkin Elmer, Milan, Italy). The amount of orange formazan formed is directly correlated to the number of living cells.

Data are expressed as the mean ± standard deviation. Normality of the data was confirmed using the Kolmogorov-Smirnov test ($\alpha = 5\%$). Statistical analysis was performed using the paired Student t-test (SPSS software, version 11.0, SPSS Inc. Chicago, Illinois, USA). The level of statistical significance was set at $p \leq 0.05$.

RESULTS

Scaffold preparation

The human flexor digitorum tendon was harvested from different donors and de-cellularized with one unique cycle of the detergent-enzymatic method, which comprises one wash with EDTA in hypotonic Tris buffer to lyse cells and release intracellular contents, and one wash with SDS to remove cell membranes and cytoplasmatic proteins. In order to determine the optimum de-cellularization method, different size tendons were subjected to various SDS treatment times (data not shown); The optimal size was found to be 2 cm x 0,25 cm while 5h of SDS treatment was the most advantageous incubation period to obtain complete removal of cells and an integral matrix. Using H&E staining, the control tendon showed a normal distribution of collagen fibers (Fig. 1a). The tendon de-cellularized with 0,1% SDS exhibited a complete loss of cells with no substantial changes in ECM structure. Moreover, the histological features observed by H&E staining,

demonstrated that no cell fragments were retained within the tissue and no apparent disruption of

overall tissue histoarchitecture was noticed (Fig. 1b).

Scaffold seeding

The re-cellularization of acellularized scaffolds was performed using two slightly different methods

(one involving collagen gel and one without, see materials and methods section); in the seeding step

we used the same cell density (1×10^6 cells/scaffold). In figure 1 (c-d) it is shown a re-cellularizazion

protocol without the use of collagen in which the formation of an external layer of cells is clearly

evident. On the contrary, the use of collagen in recellularization of the scaffold dramatically

improved the penetration of injected cells (Fig. 1e-f); here it can be seen that together with rounded

mononuclear cells there are also flattened cells that align with collagen fibers (Fig. 1f, arrows).

Masson trichromic staining confirmed that the scaffold re-cellularization using collagen comprised

type I collagen and mesenchymal stem cells diffusing into the matrix (Fig. 1g-h).

Immunohistochemistry experiments showed a strong reaction for collagen type I especially in

elongated cells present at the border with collagen fibres (Fig. 2a-b) while the rounded cells did not

present any positive staining (Fig. 2b, asterisks). The anti-COMP antibody revealed that all injected

cells were positive and expressing this protein at the cytoplasmatic level (Fig. 2d).

Valuation of cell presence in the scaffold

The DNA assay verifies that, with respect to the native sample, DNA was removed by the de-

cellularization process (Fig. 3a) and the lack of amplification for the housekeeping gene β-actin

(Fig. 3b) confirmed that de-cellularized tendons were depleted of DNA. Figure 3 shows that cells

were still present following the seeding with collagen treatment after 7 days of culture; moreover

DNA was present and the β-actin gene was amplified by PCR.

To determine the effects of the seeding method with collagen on cell viability, the XTT assay was performed after 7 days of incubation. Figure 4 indicates the presence of proliferative cells in native and re-cellularized tendon. Moreover, re-cellularized tendons showed a greater viability respect to native tendons (Fig. 4) since the introduced cells presented higher proliferative capacity.

DISCUSSION

Biological scaffolds derived from de-cellularized tissues have been successfully used in the last decade in both pre-clinical animal studies and in human clinical applications (Ingram *et al.* 2007; Baiguera *et al.* 2010; Baiguera *et al.* 2011; Lu *et al.* 2010; Xu *et al.* 2008) . Removal of cells from native tissue offers many advantages with respect to artificial scaffolds including improved biocompatibility, enhanced ability for cellular repopulation and increased biomechanical strength (Liu *et al.* 2008). The use of acellularized tissues appears, therefore, an attractive approach for the resolution of tendon traumas that are frequent and debilitating. The unique biomechanical properties of tendon are attributed to the high degree of organization of extracellular matrix (ECM) and for this reason the goal of any de-cellularization protocol has always been to remove efficiently all cellular and nuclear material minimizing any adverse effects on the composition, biological activity, and mechanical integrity of the remaining ECM. Cartmell *et al.* 2000 and recently Deeken *et al.* 2011 demonstrated that tissues treated with TritonX-100 contained many disrupted cells, and there was also slight damage to the collagen architecture. Cartmell *et al.* 2000 also verified that the ionic detergent SDS was more effective in cell removal and did not cause significant denaturation of the rat tendon matrix demonstrating that the most effective de-cellularization protocol should include a combination of physical, chemical, and enzymatic approaches. Indeed, in our study the de-cellularization protocol commenced with the lysis of cell membranes using an hypotonic solution containing EDTA, and was followed by solubilization of cytoplasmatic and nuclear components using detergents (SDS 1%). Finally, the removal of cellular debris and the depletion of DNA was obtained by treating the tissue with nucleases. These steps were combined with mechanical

agitation to increase their effectiveness. Baiguera *et al.* 2010 and 2011 proposed bioengineered

human tracheas and larynx obtained from several de-cellularization cycles of natural tissues; this

demonstrates that to obtain the complete remove of residual cellular components without disrupting

the collagen architecture, it is essential to adapt the de-cellularization treatment according to the

starting tissue. For this reason our method was optimized for the size of the sample. Indeed, the

protocol presented here is optimal for a specific tendon size of 2cm x 0,25cm. Histological analysis

of the de-cellularized tendon revealed that cells and nuclear components were removed and the

integrity of matrix structure remained preserved. This result was confirmed also by DNA analysis.

Some authors have presented different methods for seeding proliferating cells in tissue scaffolds

stating that the success of tissue engineering products was based on the number of cells found in the

scaffold but not on the spreading of the cells within the matrix architecture. Ingram *et al.* 2007

indicated that cells formed a monolayer on the surface of the scaffold but after 7 days they were

able to penetrate to the centre although the matrix became disorganized, not resembling the native

tendon. In 2008 Thevenot *et al.* investigated cell distribution in a co-polymeric scaffold with an

elegant approach and obtained a three-dimensional image after seeding; these authors concluded

that cells tended to reside on the seeded surface and only few cells were present in the center of the

construct after injection seeding. The matter of seeding distribution still remains unsolved; indeed,

Pridgen *et al.* 2011 presented recently a succesfull decellularized biocompatible human tendon but

the cell attachment was observed only on the tendon surface. Our efforts had focused therefore on

developing and optimizing a more successful and functionally robust recellularization protocol for a

human tendon scaffold.

We obtained negative results with a re-cellularization protocol comprising injection together with a

dynamic seeding method. In this case the cells remained on the surface without penetration. To

achieve our aim we pre-treated the tendon scaffold with a collagen solution which at 37°C forms a

gel, and only after this we injected the cells. Our results demonstrated that the presence of a

collagen gel permits the penetration of injected cells. The latter presented a rounded morphology

and did not express collagen type I while in the gel, however, cells that spread towards the tendon matrix aligned with collagen fibers, showed an elongated morphology and expressed collagen type I. Both types of cells expressed COMP protein, an abundant non-collagenous pentameric glycoprotein of the tendon, particularly required where the axial alignment of type I collagen fibers is essential for tensile strength of tendons (Posey *et al.* 2008; Sodersten *et al.* 2005). Effective re-cellularization was also confirmed by the presence of genomic DNA and by the expression of the housekeeping gene β-actin.

Moreover, it is important to note that scaffolding materials for tendon tissue engineering use need some fundamental requirements including biocompatibility, before and after degradation, as well as bio-functionality or at least the ability to support cell proliferation. The scaffold presented in this study, and re-cellularized by a collagen gel, shows great biofunctionality tested by the XTT proliferation assay. The re-cellularized tendon also presented an increased absorbance value since adipose-derived stem cells, used in tendon injection, possess an higher proliferative capacity compared to tenocytes of the native tendon (Kryger *et al.* 2007).

Commonly, the cell types used in tendon tissue engineering studies are fibroblasts, tenocytes and stem cells of mesenchymal origin (Cao *et al.* 2002; Ouyang *et al.* 2003). In addition, Kryger et al. 2007 demonstrated that epitenon tenocytes, sheath fibroblasts, BMSCs and ASCs are all possible candidates to be used in tendon tissue engineering; however, these authors remarked that adipo-derived mesenchymal stem cells showed higher proliferation at late passages, when compared with epitenon tenocytes, and that they are easier to harvest. Other studies suggested that *in vitro*-cultured stem cells injected in tendon constructs and exposed to the appropriate environment may be driven towards tenocyte differentiation (Zhang *et al.* 2003; Ge *et al.* 2005); our data show that mesenchymal stem cells are able to produce collagen confirming their contribution to tendon matrix remodeling *in vivo*.

Our significant data indicate the feasibility of a new strategy for re-cellularizing scaffolds achieving, for the first time, an efficient infiltration into the core of the tendon; the possibility to

introduce cells into the tendon using a collagen gel, permitting the injected cells to migrate into

tendon matrix, to elongate and flatten and to express collagen I and COMP, represents in our

opinion a significant advance in our knowledge. This should open excellent new opportunities for

tendon scaffold engineering in hand reconstruction.

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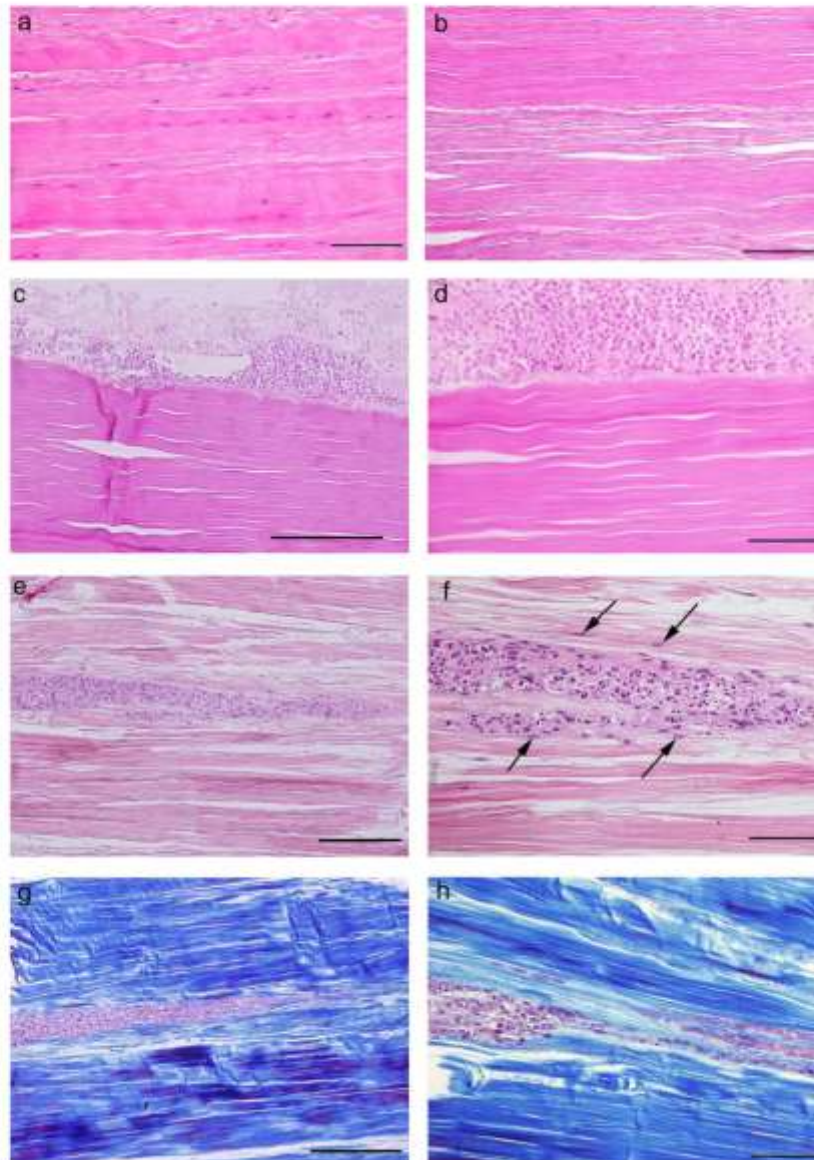Zhang AY, Chang J. 2003; Tissue engineering of flexor tendons. *Clin Plast Surg* **30**, 565-572.

Figure 1. Hematoxylin and eosin (a-f) and Masson trichromic (g-h) staining of untreated and treated tendons. The first method used provoked removal of cells while only the second method achieved cells to penetrate the scaffold. (a) Untreated tendon, scale bar = 100 μm; (b) acellularized tendon, scale bar = 100 μm; (c, d) tendon recellularized with method 1; cells form layers on the surface of the tendon at the level of peritenonium, scale bars: c = 500 μm, d = 100 μm; (e, f) tendon recellularized with method 2 using collagen; cells gather in the deep portion although some elongated cells (arrows) integrate in the matrix of the scaffold, scale bars: e = 500 μm, f = 100 μm; (g, h) Masson staining confirmed the presence of cells into the scaffold, scale bars: g = 500 μm, h = 100 μm. In H&E, blue staining indicates the nuclei of cells; in Masson trichromic, blue indicates collagen fibres while red indicates cells.
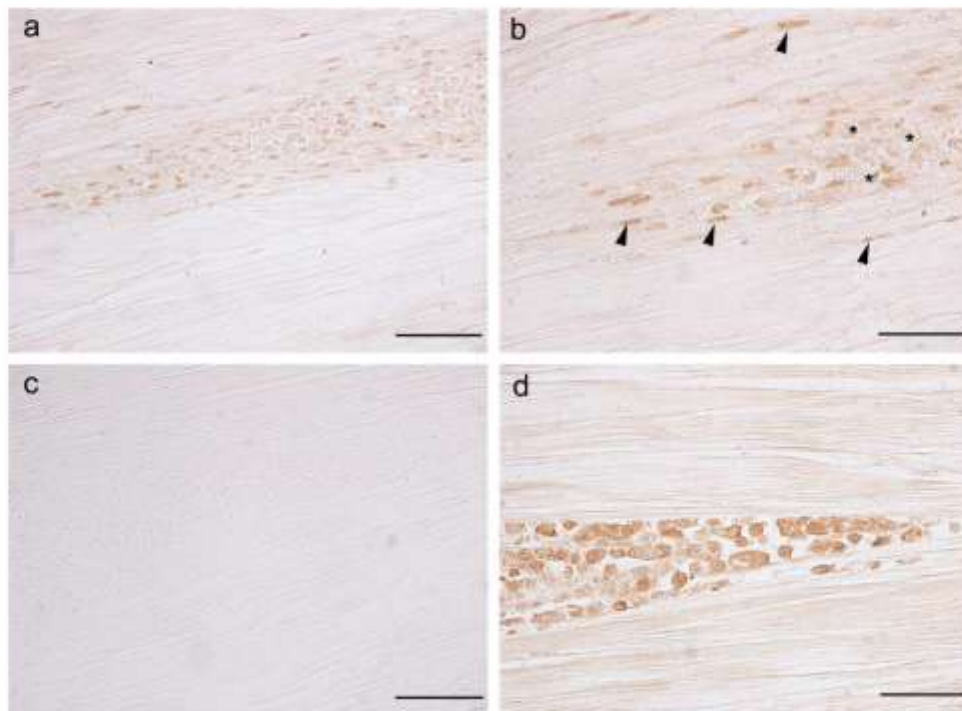
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Figure 2. Immunolocalization of collagen I and COMP proteins in recelullarized scaffolds. (a-b) Injected cells that presented an elongated shape were positive to the mAB anti collagen I (arrowheads) while rounded cells were negative (asterisks); scale bars: a = 100 μm, b = 50 μm. (c) Representative tendon used as a negative control (omitting the primary antibodies), scale bar: c = 100 μm. (d) the anti-COMP polyclonal antibody reacted with all injected cells; the positivity is well evident at the cytoplasm level, scale bar: d = 50 μm.
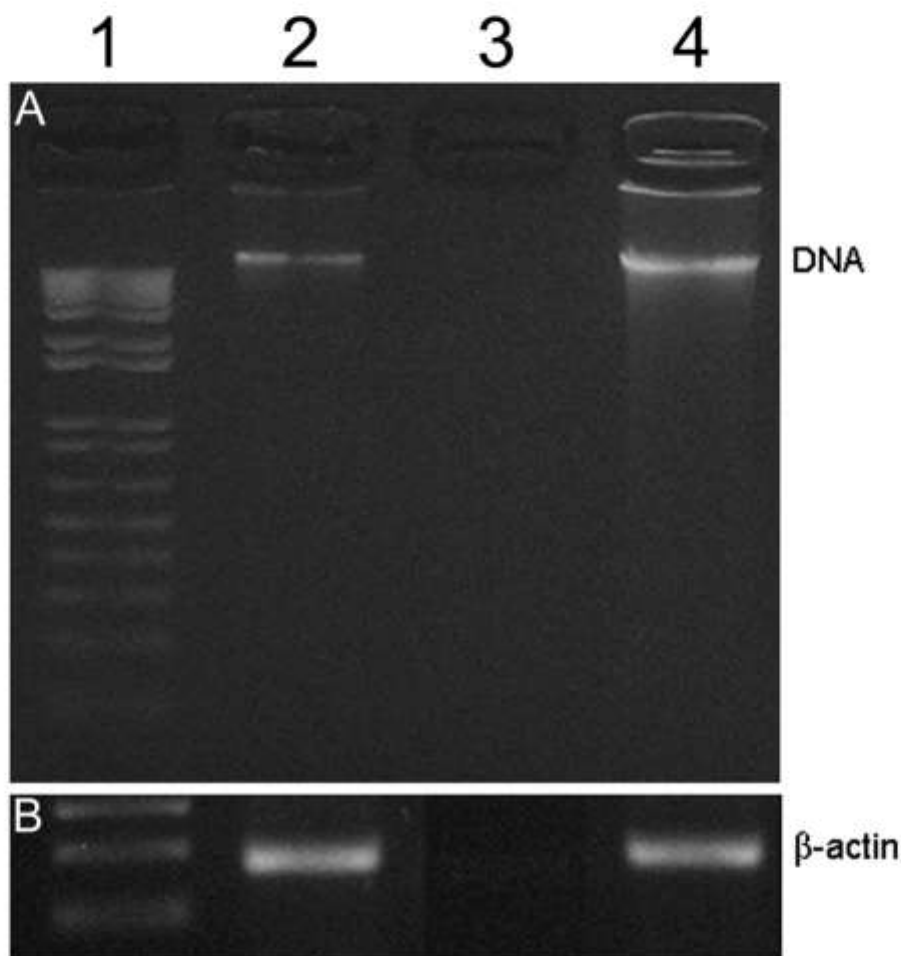
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Figure 3. DNA analysis by means of PCR of decellularized and recellularized (with the second method) tendons. (A) Genomic DNA content in native (lane 2), decellularized (lane 3) and recellularized (lane 4) tendons. (B) β-actin housekeeping gene expression in native tendon (lane 2), decellularized (lane 3) and recellularized tendons (lane 4). Lane 1 = Molecular size standard (1Kb Plus DNA Ladder, Invitrogen).
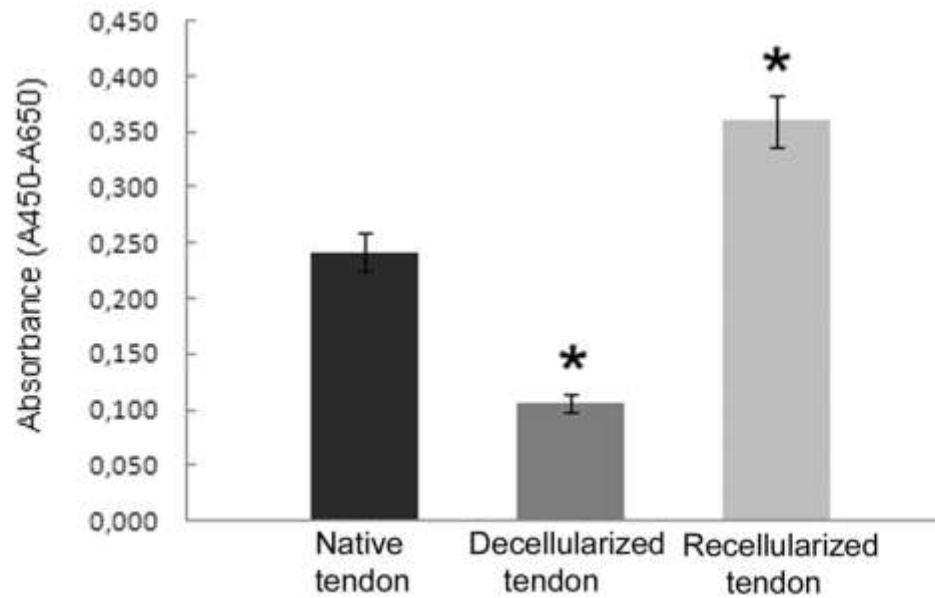
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Figure 4. Colorimetric assay to determine the survival of cells. The values shown are the average ± deviation standard of absorbance of native, decellularized and recellularized tendon. The Student's t-test t was used to set statistical significativity (*p<0.05).

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Effect of peripheral blood derived mesenchymal stromal cells (PB-MSCs), platlet rich plasma (PRP) or combination of both on deep digital flexor tendon repair of ovine experimental model.

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Abstract

Tendon injuries, degenerative tendinophaty and overuse tendinitis are common among professionals and amateur athletes. Novel therapies are aimed to restore tendon functionality through the effort of cell-based therapy, growth factor delivery and tissue engineering. This study examined the effect of mesenchymal stromal cell derived from ovine peripheral blood (sPB-MSCs), platlet rich plasma (PRP) and a combination of both, on experimental Collagenase 1A induced lesions on deep digital flexor tendons (DDFT) of Bergamasca sheep. The outcomes of these different treatment were evaluated after one month (30 days) and four months (120 days) for the lesion comparing of histologic characteristic and immunohistochemical expression of collagen types 1 and 3 , and cartilage oligomeric matrix protein (COMP). In both one and four months assay a significant difference in tendon morphology and ECM composition were found between treated groups and their corresponding control (lesioned not treated). After 4 months both ePB-MSC and ePB-MSC + PRP injected tendons resulted in improved tendon histological characteristics compared to controls. These results indicate a benefit on use of MSCs derived from peripheral blood alone or in association with PRP for the treatment of tendonitis.

Keywords: peripheral blood derived mesenchymal stromal cells (PB-MSC), platlet rich plasma (PRP), tendonitis, in vivo.

Introduction

Tendon injuries, degenerative tendinophaty and overuse tendinitis are common among professionals and amateur athletes (Dahlgren et al., 2005). In the veterinary field overstrain of weight bearing tendons are particularly frequent in races horses, because of the hyperextension of metacarpo-phalangeal joint during weight bearing. The superficial digital flexor tendon are the most injured among the equine palmar soft tissue, since it has been estimated that strain account for up to 46% of limb injuries in Thoroughbreds (Williams et al., 2001). Tendons are extraordinarily strong in resisting tensile load, given to their low metabolic rate and well-developed anaerobic energy generation capacity, tendons are able to carry loads and maintain tension for long periods. This property help in reducing the incidence of ischemia and necrosis, but on the other hand the slow metabolic rate entails slow and poor healing capacity after injury (Hoffmann and Gross, 2007). Usually the therapies for tendon injuries are conservative, consisting in prolonged period of rest, followed by controlled exercise for up to 12 months post injury, and often is associated with high re-injury rates accounting 56% for jumping horses and 66% for races horses (Dyson, 2004). Tissue engineering techniques had recently opened new treatment perspective for injured tendon, with the aim to restore functional tissue by introducing the use of cell-based and gene therapy as well as the local delivery of growth factors such as platelet rich plasma (PRP). Several studies have been reported on the use of mesenchymal stromal cells derived from bone marrow (BM-MSCs) in order to treat tendon lesions, and this method is now being used to treat many clinical cases (Godwin et al., 2011; Guest et al., 2008). MSCs, once inserted in the lesion, are supposed to act influencing the tissue regeneration by two mechanism: differentiating in mature tenocyte able to secret extracellular matrix (ECM), or secreting growth factor that can stimulate implanted or neighbor cells to produce ECM (Caplan et al., 2007; Murphy et al., 2003). With the same purpose PRP has been proposed as a novel treatment for tendon injures, and the main rationale for the use of PRP arise from the growth factor released from platlet α-granules, including platelet derived growth factor (PDGF), transforming growth factor-β (TGF-β), fibroblastic growth factor (FGF), vascular endothelial growth factor (VEGF) and insuline like growth factor (EGF) (Anitua et al., 2008; Schnabel et al., 2007; Everts et al., 2007; Nixon et al., 2008; Maia et al., 2009). Studies performed on use of BM-MSCs, showed positive results both for the tendon regeneration, and for the functionality observed, as verified by ultrasonography exam, by histology and immunohistochemistery (Crovace et al., 2007; Godwin et al., 2011; Koch et al., 2009). Despite these new outcomes, the therapy for tendon injuries do not provide fully satisfactory long-term results, and much studies have to be done in order to better understand the healing mechanism of cell based or growth factor therapy. In this

study 18 Bergamasca sheep have been used as an experimental model of tendon injury, with the aim to provide the opportunity to suggest a good experimental protocol adaptable for races horses. The regeneration of tendon tissue was compared using three different treatment: autologous MSCs derived from peripheral blood of sheep (sPB-MSCs) marked with green fluorescent protein (GFP), PRP, and PRP + sPB-MSC positive for GFP. The degree of wound healing produced by different treatment was verified after 1 month and after 4 months by ecographic, histochemical and immunoistochemical analysis. Even if studies on injured tendon using stromal cells or PRP have been reported, this work could interestingly point out some important novel aspects of the influence of MSCs or PRP on the tendon healing process: I. the analysis were performed after four months from the experimental lesion, II. the cells source is innovative, III. no previous studies focused on clinical use of MSCs derived from peripheral blood in musculoskeletal injures, IV. the sPB-MSC were marked with GFP and consequently it has been possible to monitor the MSCs fate.

Materials and Methods

sPB-MSCs collection:

Peripheral blood samples were obtained from 12 female Bergamasca sheep homogeneous for size and age. To isolate mononuclear stromal cells, peripheral blood was diluted 1:1 with phosphate-buffered saline (PBS) and carefully placed on Ficoll-paque solution (Amersham Biosciences). After density centrifugation (R.C.F. Meter4233R; A.L.C International S.r.l.) at 1600 g for 20 min, cells were removed from the interphase and washed twice with PBS. Cells obtained from each 100mL of blood were plated in $25cm^2$ culture flasks (Falcon; BD Biosciences) in non inductive medium consisting of Dulbecco's modified Eagle's medium (DMEM D5671; Sigma-Aldrich S.r.l.) with 20% fetal bovine serum (FBS; ECS0180-L; Euroclone), penicillin and streptomycin (Sigma-Aldrich S.r.l.), and glutamine (Sigma-Aldrich S.r.l.), and incubated in an atmosphere of humidified air and 5% CO2 at 37°C. After overnight incubation, nonadherent cells were removed and fresh medium was added to the flasks. After 2–3 weeks, the developed colony-forming units of adherent cells were recovered and replated for amplification, and the medium was changed every 3 days. Isolated cells close to confluence were quantified and subcultured in 10% FBS.

Transfection of sPB-MSC:

The stable tranfection of sPB-MSC was performed only for the 6 sheep euthanized after one month from the collagenase 1A lesion. The plasmid pEGFP (kindly provided by Professor Negro, University of Padova) was purified using Qiagen Plasmid Midi kit, according to the manufacturer's

instructions. For each sheep a total of $1X10^6$ cells for sheep were transfected. sPB-MSCs of each sheep were transfected in culture dish at $1,5X10^5$ cells/dish, 2μg of DNA were mixed with 300μl of medium (Opti-mem, Gibco-BRL Gaithesburg MD USA) and combined with 300μl of Lipofectamine reagent solution (Invitrogen Life Technologies) for 20 min. Then solution were applied to the cells and removed after 5 hrs. After 24 hrs of culture, selection was started using 1μg/ml of G418 (Neomicin). sPB-MSCs of each sheep, once transfected, were cryopreserved in medium containing 90% FCS and 10% DMSO and stored until the treatment.

Experimental lesion on DDFT:

18 female Bergamasca sheep were used in this study. Sheep were rested in a box 4 weeks prior to beginning the experimental study. Any previous lesion was assessed by clinical and ultrasound examination. Sheep were tranquilized with 0,2 mg/Kg of Metadone and 5μg Medetomidine (Domosedan), and positioned in lateral decubitus. 500 IU of filter sterilized bacterial Collagenase type 1A (C-9891, Sigma, Milan) was injected in each DDFT of two limbs (left and right hind) under ultrasonographic guidance. A 23G needle was utilized, and inserted at 15 cm, in proximal-distal direction, from the calcaneal bone. During the procedure the limb was maintained flexed 90°. To each lesioned limb a suture point was applied where needle was inserted. A dose of 5mg/kg of phenilbutazone was administrated, and antibiotics for 5 days.

All of the work described was performed with ethical approval under University Ethic Department for Animal Experimentation (CEASA) license and approved by the National Health Department on 17 May 2010 (DM n° 97/2010-B).

Obtaining Platelet-Rich Plasma

18 ml of jugular peripheral blood, collected from 12 sheep, was draw in sterile tubes containing 2 ml of anti-coagulant (ACD). PRP was obtained after centrifugation with SmartPReP2 centrifuge (Harvest®). 3-5 ml of PRP product were obtained with a platelet concentration 4-5 times higher respect to the basal level. 1 ml of PRP containing meanly $970X10^3$ platelet μl^{-1} was used for each treatment.

Treatment:

The 18 sheep group was divided and treated as described in diagram 1 after 7 days from the collagenase injection, since it has been observed that between 5 and 7 days after lesion the collagenase are effective and the inflammation reduced (Dehghan at al. 2007). For each sheep of group 1-6 left hind DDFT received the tree different treatment, while right hind DDFT was only

injected with 1ml of saline solution (PBS), being the internal control (lesioned, not treated). All the treatment injections were performed under ultrasound guide and under sedation with 0,2 mg/Kg of Metadone and 5μg Medetomidine (Domosedan).

Group 1 and 4 received $1X10^6$ sPB-MSCs conveyed in 1 ml of hyaluronic acid, used as a vehicle; 1 ml of PRP (containing meanly $970X10^3$ platelet μl^{-1}) and 1ml containing $1X10^6$ sPB-MSCs in PBS were injected in group 2 and 5. sPB-MSCs injected in group 1 and 2 were previously isolated from each sheep and transfected with GFP as previously described. Finally, 1 ml of PRP containing meanly $970X10^3$ platelet μl^{-1} was injected in tendon of group 3 and 6.

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9 Sheep euthanized after 1 month (30 days):
- sPB-MSC positive for GFP (group 1; n=3)
- sPB-MSC positive for GFP + PRP (group 2; n=3)
- PRP (group 3; n=3)

9 Sheep euthanized after 4 months (120 days):
- sPB-MSC (group 4; n=3)
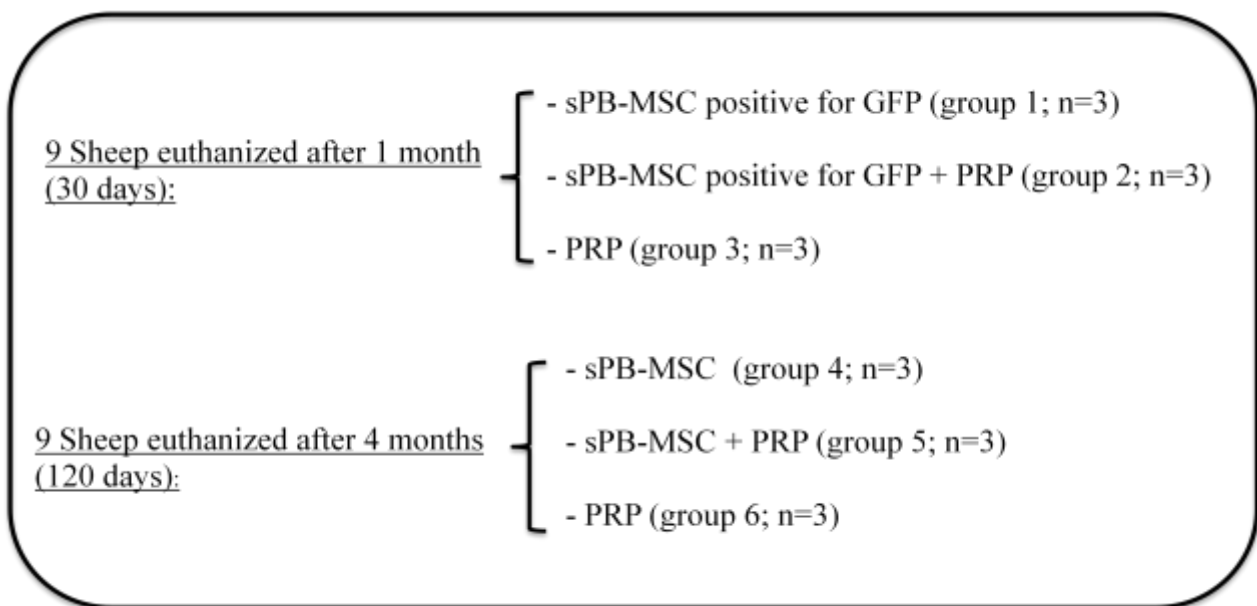- sPB-MSC + PRP (group 5; n=3)
- PRP (group 6; n=3)

Diagram 1: schematic description of the treatment distribution.

Clinic and Ultrasound follow up:

Sheep of all treated group were monitored clinically and by ultrasound examination weekly for the first month; then at 2th , 3th months and before euthanasia (4th month). Sheep were euthanized, according to DR 97/2010-B guidelines, sedation was performed with 0,2 mg/Kg of Metadone and 5μg Medetomidine (Domosedan), induction with 4 mg/Kg of Propofol, and euthanasia with Tanax. DDFT of both hind limbs were harvested for the immunochemical and histological analysis, after 1 month and after 4 months from each different treatment. DDFT of one forelimb was harvested as a positive control.

Histological analysis:

After euthanasia, harvested DDFT were cut in size of 1 cm, marking the proximal-distal direction; then paraformaldehyde-fixed tissue sections for histology were dehydratated, cleared in xylene (Shandon Cittadel), embedded in paraffin, sectioned at 5 μm, and mounted in microscope slides. Hematoxylin and eosin (HE), Masson Trichromic and Mallory staining were performed in order to highlight the collagen fibers and glycosamminoglycan contenent (GAG) of the treated tendon tissue. Section were observed and photographed with light microscope (Olympus Vanox photomicroscope, Japan) and carefully analyzed for organization of ECM, collagen fiber orientation, cellularity, inflammatory infiltration and neovascularization. Specifically for each different parameter analyzed a score was assigned (Table 1).

Tendon Parameter	Score
Cell density	0 = normal
	1 = slightly increased
	2 = mildly increased
	3 = highly increased
Cell shape	0 = normal
	1 = slightly increased
	2 = mildly increased
	3 = highly increased (plump cell)
Neovascolarization	0 = normal
	1 = slightly increased
	2 = mildly increased
	3 = highly increased
ECM organization	0 = normal
	1 = slightly damaged
	2 = mildly damaged
	3 = highly damaged
Tissue characteristics	Evaluation of collagen fiber disposition

Table 1: Each parameter analyzed was scored from 1 (normal) to 3 (most damaged).

For each group were evaluated 3 representative sections and in the latter 10 random field were examined. Group 1-6 were scored and a mean value was derived on qualitative observations basis.

Detection of marked GFP sPB-MSC:

1 month sheep tendon samples, which received the treatment with sPB-MSC marked with GFP (group 1,2), were dissected and snap frozen in isopentane previously cooled with liquid nitrogen. Sections were cut with a cryostat, 7μm thick; then fixed in 100% acetone for 10 min and stored at -20° until required. For immunohistochemistry, primary antibodies, mouse anti-GFP (Invitrogen, A6455) was incubated 4°C overnight in a humidified chamber, after which the peroxidase ABC detection system (Vector laboratories, Inc. Burlingame CA), containing a biotinylated anti-mouse secondary antibody, and DAB substrate was applied according to the manufacturer instructions.

Immunohistochemistry analysis:

For immunohistochemistry, the tendon sample were fixed in 4% parformaldehyde (PFA), paraffin embedded and sectioned with a thickness of 5 μm. Section were deparaffinized in xylene, rehydrated through a graded ethanol series, treated with 0,3% TritonX solution for 10 min, and incubated with 5% normal fetal serum (FCS) in phosphate buffered saline (PBS) for 60 min. Sections were then incubated with the following primary antibodies: mouse monoclonal anti-Collagen 1, (C2456 Sigma-Aldrich®); mouse monoclonal anti-Collagen 3 (C7805 Sigma-Aldrich®); rabbit polyclonal anti-COMP (ab74524 Abcam®). All antibodies were utilized 1:100. Incubation was performed at 4°C overnight in a humidified chamber, then the samples were PBS washed and incubated with biotin-conjugated anti-mouse secondary Ab (Vector) for collagen 1 and 3, and with anti-rabbit secondary Ab (Vector) for COMP. Slides were incubated with the secondary antibodies for 1 hour. After further PBS washes, slides were incubated 30 min with ABC reagent, finally the immuno-reactive site were visualized with 3.3'-diamminobenzidine tetrahydrocloride (DAB) kit (Vector laboratories, Inc. Burlingame CA) for 5 min, dehydrated, and mounted.

Results

Isolation of sPB-MSCs and PRP production:

sPB-MSCs were successfully isolated from peripheral blood of 12 sheep (group 1,2 and 4,5), using the standardized protocol previously used for isolation of MSCs from peripheral blood of horses (Martinello et al., 2010). Cells obtained from group 1 and 2 were efficiently (approximately 60%) transfected with GFP. From 12 sheep of group 2, 3 and group 5, 6 was successfully obtained PRP, with meanly 970×10^3 platelet μl^{-1}

Clinical evaluation following collagenase treatment:

After collagenase 1A injection, all sheep showed an inflammatory reaction, with a mild localized thickening of DDFT and peritendonous tissue. Pain by palpation remained for first five days from the Collagenase 1A treatment as well as a slightly lameness and a increased local temperature observed during 3 days. The lesions were detectable in DDFT by ultrasonography 7 days after their creation, allowing a good environment for PRP and sPB-MSCs implantation (fig. 1A).

Clinical examination at 1 month:

7 days after collagenase injection all sheep were treated as reported in diagram 1. No inflammatory reaction were detected in neither group after treatment, and generally, for all sheep the treated left DDFT showed faster reduction of respect to the right DDFT used as a control, lesioned but no treated. An almost absent swelling was observed in all animals at 30th day. Ultrasounds examination were performed in Group 1, 2, 3 and no significant difference parameters were found between the three different treatment, all group present the same degree of lost of the echogenicity and the collagen fiber alignment (fig. 1).

Localization of GFP-positive sPB-MSC:

The anti-GFP antibody staining reveled presence of positive cells within the lesions, and in the adjacent healthy tendon (fig. 2). The distribution of the cells in the ECM appear to be uniform and, even if an high number of cells were found, the majority of them appear to be longitudinally aligned and integrated in the tendons matrix. Immunohistochemical controls were negative in both group 1 and 2.

Histological results at 1 month:

All DDFT of the different groups of treated sheep (1, 2, 3,) were stained for morphological evaluation with standard HE (fig. 3), Masson tricromic (fig. 4) and Mallory staining (fig. 5). After 30 days right hind DDFT (internal control, lesioned not treated) of all group showed a not-organized extracellular matrix, with high cellularization, and fibroblast-like cells with plump nuclei; an high vascularization was detected associated with a mild inflammatory response. All treated groups showed an increased cellularization; in particular the group 1 and 2 showed cells with an elongated morphology respect to group 3. Moreover, in the group 1 and 2 the cells resulted more ordered and aligned inside the ECM; the latter resulted more preserved with a slight increased vascularization in the group 1 and 2 while in the control tendon and group 3 was highly vascularised. Mean score assigned to each group are listed in table 2.

Tendon parameter	Score Group 1 (MSC)	Score Group 2 (MSC+PRP)	Score Group 3 (PRP)	Score Internal Control tendon
Cell density	3	3	3	3
Cell shape	1-1,5	1-1,5	2	3
Neovascularization	1,75	2	2,5	3
ECM organization	1,5	1,5	2	3

Table 2: Mean score of the different treated group compared with the control group.

Immunohistochemical results at 1 month:

The immunohistochemical analysis obtained after 30 days, showed that Collagen 1 (fig. 6) was well expressed in cells of group 1, 2 and 3; the internal control (lesioned, not treated) showed an higher expression of Collagen 1 respect to the three treated groups. Collagen 3 (fig. 7) was well present in the internal control whereas resulted faintly expressed in groups 1 and 2 : the group 3 (PRP) showed more positive cells respect to groups 1 and 2. The COMP expression (fig. 8) was elevated in all treated groups and in the internal control . The healthy adult sheep tendon (HT control) showed a faint staining for the anti-collagen 1 and anti-COMP antibodies while no reaction was detected for the anti-Collagen 3 .

Clinical examination after 4 month:

No apparent clinical differences in the degree of swelling between the three different treatment was observed neither in comparation to lesion tendon. Moreover ultrasound examination confirm the presence of uniform tendon, in all groups, concerning shape, echogenicity and the collagen fiber alignment (fig. 9).

Histological results after 4 months:

In the histological evaluation at 4 month after treatment were found the most interesting observation. After 120 days, right hind DDFT (internal control, lesioned not treated) of all groups still presented a disorganized ECM, with presence of intensive vascularization and high cellularity if compared with treated animals (group 4, 5, and 6) (HE stain; fig. 10); however, the tendon architecture was significantly better organized if compared with the right hind DDFT (internal control, lesioned not treated) after one month from the lesion. Group 4 (MSC) showed a lower cellularization, vascularization and an improved ECM organization, respect to   group 5 (MSC+PRP) and 6 (PRP). In the group 1 (MSC-one month), the majority of fields observed presented elongated cells, well integrated in the ECM; in the group 4 (MSC-4 months), a significant increase in all parameters evaluated was found respect to group 1. Group 5 and 6 showed a similar cell density , in both cases lower if compared with group 2 and 3 . Particularly in group 6, cells

appeared poor aligned in the extracellular matrix respect to group 4 and 5 . All treated left hind DDFT of 4 months study generally resulted in a better ECM organization and less neo-vascularization respect to the 1 month treated DDFT. (Masson tricromic fig. 11 and Mallory staining fig 12)

Tendon parameter	Score Group 4 (MSC)	Score Group 5 (MSC+PRP)	Score Group 6 (PRP)	Score Internal Control tendon
Cell density	1	1,5	1,75	2,5
Cell shape	1	1-1,5	1,5	2,1
Neovascularization	1	1,5	2	2
ECM organization	1,5	1,75	1,75	2

Immunohistochemical results after 4 months:

The immunohistochemical analysis obtained after 4months, showed that Collagen 1 (fig. 13) was still expressed in some areas of the internal control (lesioned, not treated) while in the treated groups only few scattered cells were positive; in particular, the group 6 showed an higher number of positive cells compared to groups 4 and 5. Collagen 3 (fig. 14) was still expressed in some cells of the internal control whereas was not in all treated groups. The COMP expression (fig. 15) was present in some areas of the internal control and only in some cells of treated groups.

Discussion

The aim of this work was to compare the effect on tendon healing, of three different protocols of treatment, estimating the obtained results after 1 month and after 4 months after an experimental lesion injection, performed with bacterial collagenase 1A. Bergamasca sheep were chosen as model for this study, since it has been supposed, that among the experimental animals, they represent a good template to study tendon regeneration for horses. Peripheral blood has been used as a stromal cells source, due to the easiness of its collection and the less tenderness respect to the bone marrow harvesting procedure. PRP is a biological source enriched in growth factors, which have been recognized able to accelerate the healing process, and recently proposed, as a gelatinous or liquid matrix, to be used in association with MSCs; the idea was that growth factors released from active platelet, should promote MSCs differentiation and migration in the injured site (Everts et al., 2007). It has been chosen to use collagenase 1A injection to create experimental tendon lesions because, although were not intended to represent a true model of naturally occurring injury, some features were reproduced (Dehghan et al., 2007). Differently from what is reported in other studies, that

employed bacterial collagenase 1A to create tendon lesions, we did not obtain a real core lesion, but in the majority of cases, we found a lesion with non-defined edges and with infiltrative characteristics. This fact might be due to the differences between horse and sheep DDFT thickness and the consequent difficulties in reproduce an experimental lesion using an enzymatic procedure. Clinical examination of the sheep after the collagenase 1A injections, highlighted a moderate lameness and pain in the insertion site, which persisted 2-3 days; no swallowing, lameness or pain were detected after treatment (one week after the collagenase injection) with sPB-MSCs, sPB-MSCs+PRP and PRP alone, neither when sPB-MSC were transfected with GFP in group 1 and 2. Over the course of the present study an ultrasonographic follow up has been performed monitoring dimensions and changes in morphology or echogenity of the lesions during time. Thirty days after the collagenase 1A injection, the lesions appeared to increase in size, like previously reported in lesions created surgically (Van Schie et al., 2009), indicative of acute matrix destruction; after this time it has been observed a progressive improvement of all treated groups regarding the echogenity of the lesion and the fibers disposition. These observations are confirmed also from previous studies focused on tendons healing after mostly 30-40 days (Nixon et al., 2008; Arguelles et al., 2008; Crovace et al., 2010). The ultrasonographyc follow up in the sheep euthanized after 4 months, confirmed this improving trend as regard to the decreasing of the CSA (cross sectional area), and the increasing echogenicity. No significantly differences were found in ultrasonographic parameters among the group 1, 2, 3 and 4, 5, 6-treated tendon and their corresponding controls (lesioned, no treated), despite the significant improvement in histologic evaluation between left DDFTs (treated) and right DDFTs (lesioned) tendons of both one and four months studies; this evidence probably confirm the insensitivity of ultrasound to provide fine structural details, as reported in other studies, (Shnabel et al., 2009; Nixon et al., 2008). The immunohistochemical analysis performed to found labelled GFP positive sPB-MSCs in group 1 and 2 (1 month study), proved the presence of MSCs inside the treated tendons, and this result confirm that MSCs survived for at least 30 days without showing any relevant differences in number or without eliciting a detectable cell-mediated immune response. Although Guest et al., (2008) reported a lower percentage of survival of GFP positive MSCs injected in a tendon lesion, it should be taken into account that cells gradually loss GFP protein during cell division, if not maintained under selection condition (Neomicin selection).

The histological examination demonstrated that, for both 1 months and 4 months treated DDFT, the morphological appearance of the lesions improved for all parameters evaluated, and they became more similar to not lesioned, healthy tendon; these pattern was particularly evident after 4 months. The Masson's trichrome and Mallory staining highlited an increased neo-formation of ECM (collagen and GAG) and organized fibrous structures, in treated 1 month group (1, 2, 3), while the

corresponding controls (lesioned, not treated) were positive for the trichromic staining, but with a higher cellularization and poor ECM organization. The immunohistochemical analysis of one month study showed no significant difference in Collagen 1 expression among group 1, 2, 3, while an higher expression of collagen 1 in control tendons (lesioned, not treated) were found, corresponding to the higher cellularization present in the control group. Group 1 and 2 showed a lower expression of Collagen 3 if compared with group 3 and internal control (lesioned, not treated), suggesting the sPB-MSCs or the sPB-MSCs+PRP higher capacity of promote Collagen 1 production after 30 days. Treated tendons after 120 days from collagenase lesions injections (group 4, 5, 6) resulted more similar to the HT control, as observed from both istological and immunohistochemical results, respect to internal control (lesioned, not treated). The trichrome staining (Masson and Mallory) showed less positive (blue) staining of the ECM component in both treated group (4, 5) and HT control respect to group 6 and 1 months treated group (1, 2, 3); these outcomes were further confirmed by the immunohistochemical analysis for Collagen 1, 3 and COMP, where a decrease in cells positive for these molecules were found in treated group (4, 5, 6) and HT control respect to internal control (lesioned, not treated) and 1 month treated group (1, 2, 3). In a previous study (Dahlgren et al., 2005) immediate and sustained increases in collagen 1 and 3 expression were found to be present until week 8 in equine healing tendon after collagenase lesion, and levels continued to be higher if compared with a healthy tendon after 24 weeks. In the present study it has been found that after 120 days the DDFT of group 4 (ePB-MSCs) and 5 (sPB-MSC+PRP) presented morphological characteristic more similar to the control HT respect to group 6. These results derived from the observation of a significant lower number of cells present in the lesion site, their well defined spindle-shape morphology, and for the ECM architecture and its histological and immunohistochemical features, if compared with the group 6 and 1, 2, 3. Concluding all these data suggest the predominant effect of MSCs on tendon healing, through better contribution on structural organization and reduced cell number. Several histologic parameters including cell density and shape, neo-vascularization, and ECM organization, all trended lower in treated left hind DDFT respect to the internal control (lesioned, not treated) in both 1 month and 4 months study, differently from how reported in the study of Guest et al., 2010, where no significant difference were found between MSC and serum-injected sites. Even if in this study the injections of MSCs has been performed only after one week, and this does not reflect the normal clinical situation for horses, where delay results from the necessary culture and expansion of autologous MSCs, it has been shown that MSCs implantation should bring positive outcomes in tendon healing, like in other experimental situation (Hu et al., 2007). Outcomes derived from this study does not address any convenience in the concomitant clinical application of MSCs and PRP, since

no relevant differences were found between two different treatment after 4 months. Indeed, results obtained highlights how the use of MSCs, from a different source respect to the most common used bone marrow, contribute to a better tendon restoration after injuries.

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Fig. 1 longitudinal ultrasonographic images of representative DDFT lesioned with collagenase and no treated (lane A), treated with MSCs (lane B), MSCs + PRP (lane C) and PRP (lane D). A, B, C, D before lesion; A2, B2, C2, D2: T0, at 7 days after lesion and just treated, A3, B3, C3, D3: T7, at 7 days from the treatment; A4, B4, C4, D4: T14 ; A5, B5, C5, D5: T21; A6, B6, C6, D6: T28.
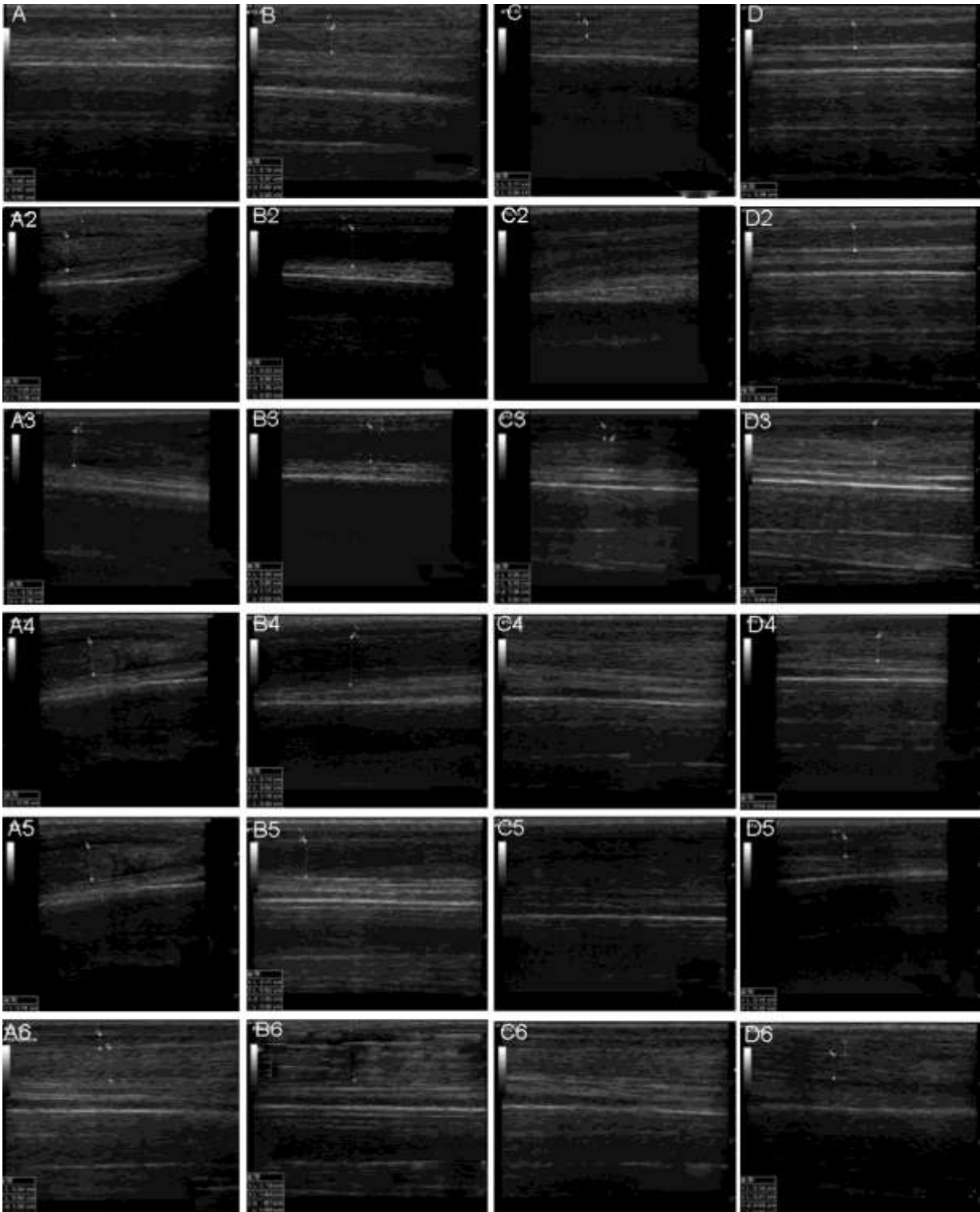
Fig 2 Presence of GFP positive cells within the lesions (B) in a representative section of DDFT treated with trasfected MSCs and analyzed at 30 days from treatment; slide A: immunohistochemical negative control.
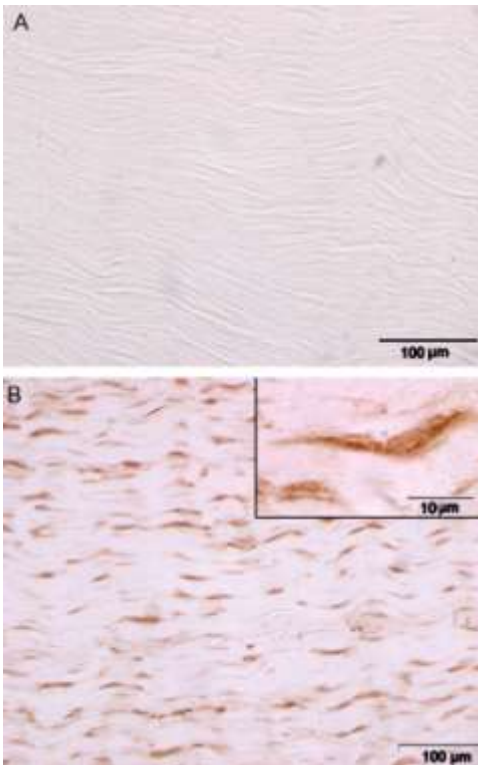

Fig. 3 Representative HE staining of sections from DDFT at 1 month after the lesion. (A) Not treated; (B) treated with MSCs; (C) treated with MSCs and PRP; (D) treated with PRP.
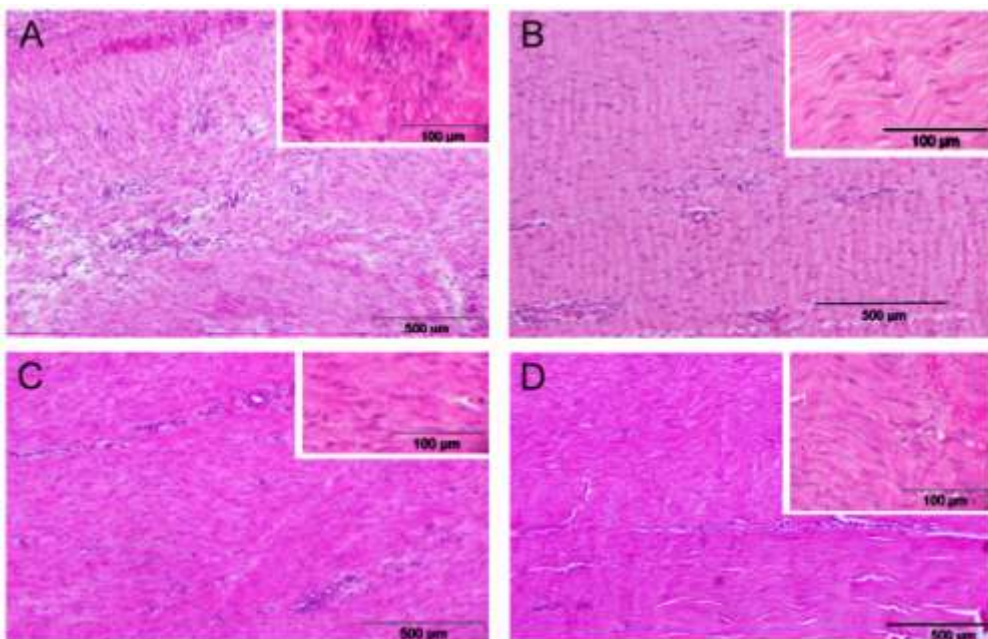
Fig. 4 Representative Masson tricromic staining of sections from DDFT at 1 month after the lesion.
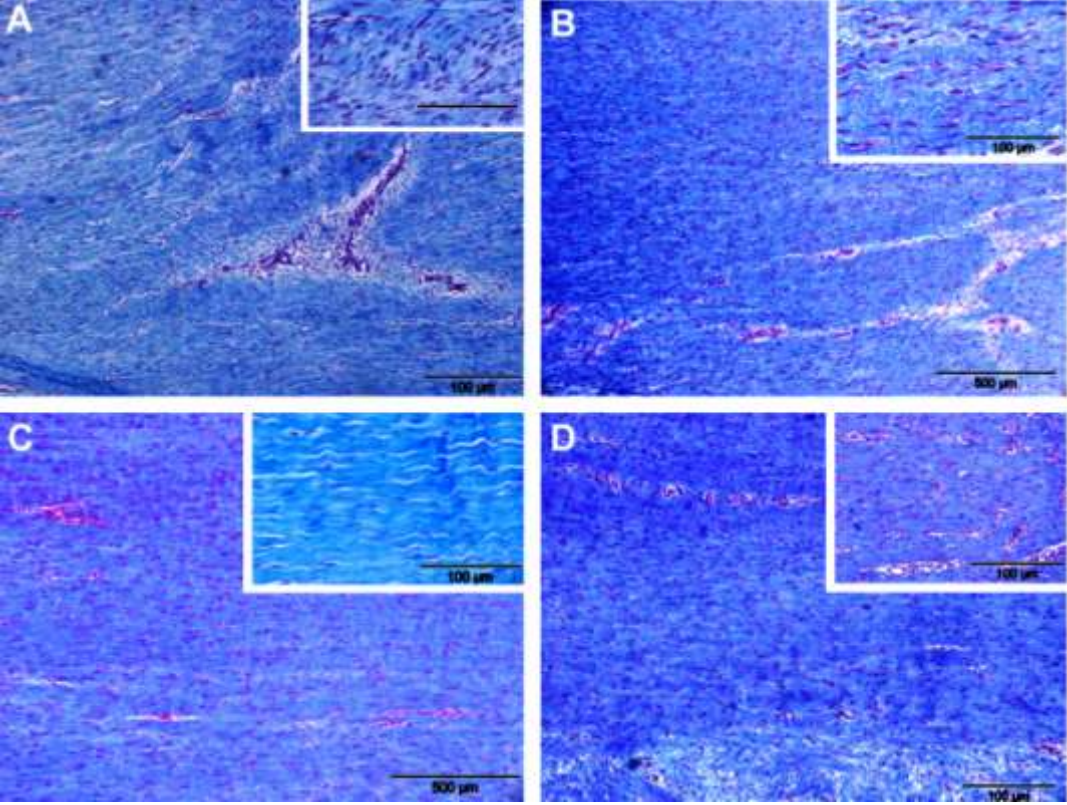(A) Not treated; (B) treated with MSCs; (C) treated with MSCs and PRP; (D) treated with PRP.

Fig. 5 Representative Mallory staining of sections from DDFT at 1 month after the lesion. (A) Not treated; (B) treated with MSCs; (C) treated with MSCs and PRP; (D) treated with PRP.
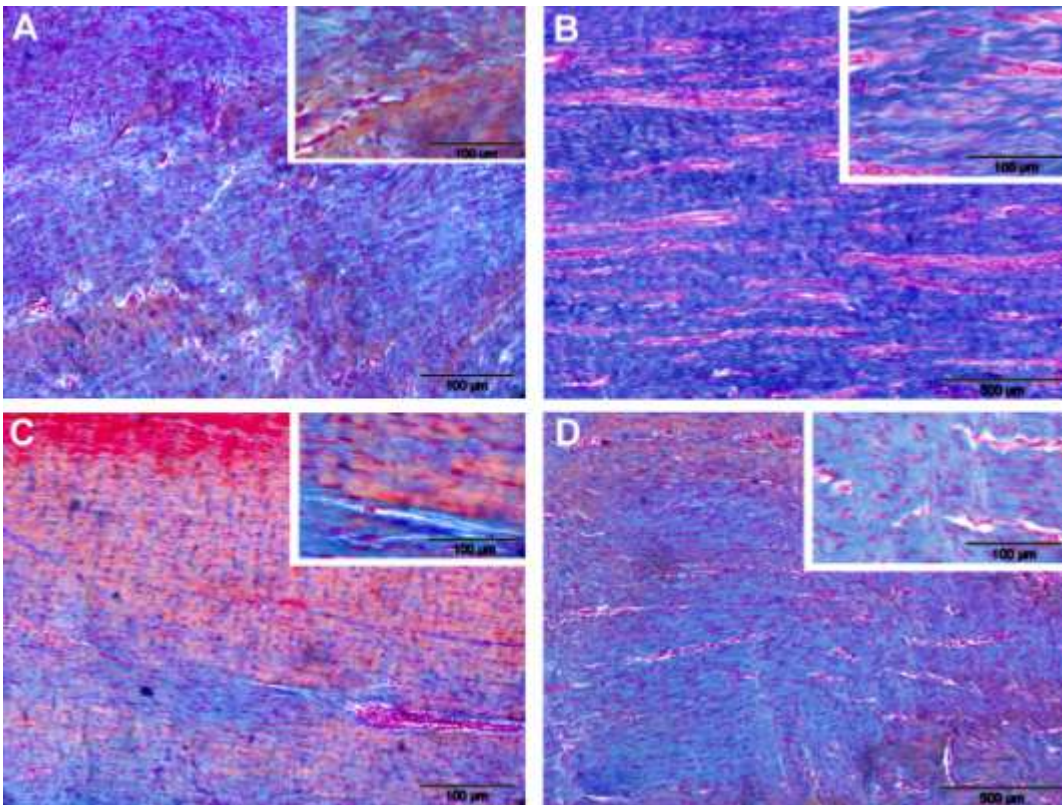

Fig. 6 Representative sections of immunohistochemical staining for anti-collagen type 1 of DDFT sections at 1 month from treatment. Lesioned not treated (A); treated with MSCs (B); treated with MSCs + PRP (C); treated with PRP (D); Sections are counterstained with hematoxylin.
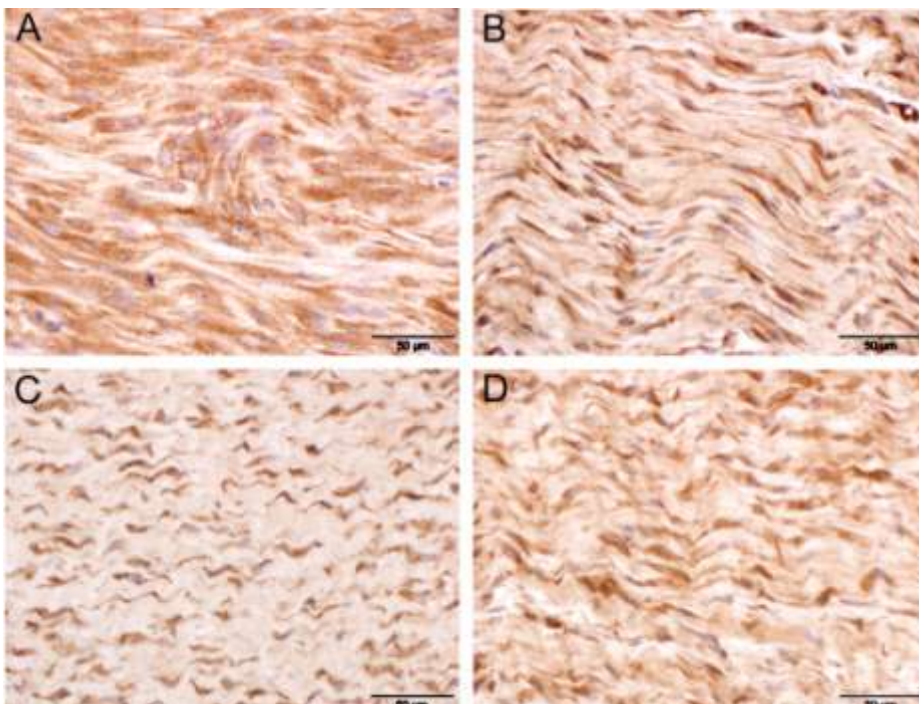
Fig. 7 Representative sections of immunohistochemical staining for anti-collagen type 3 of DDFT sections at 1 month from treatment. Lesioned not treated (A); treated with MSCs (B); treated with MSCs + PRP (C); treated with PRP (D); Sections are counterstained with hematoxylin. Inset of panel A, scale bar 10 μm.
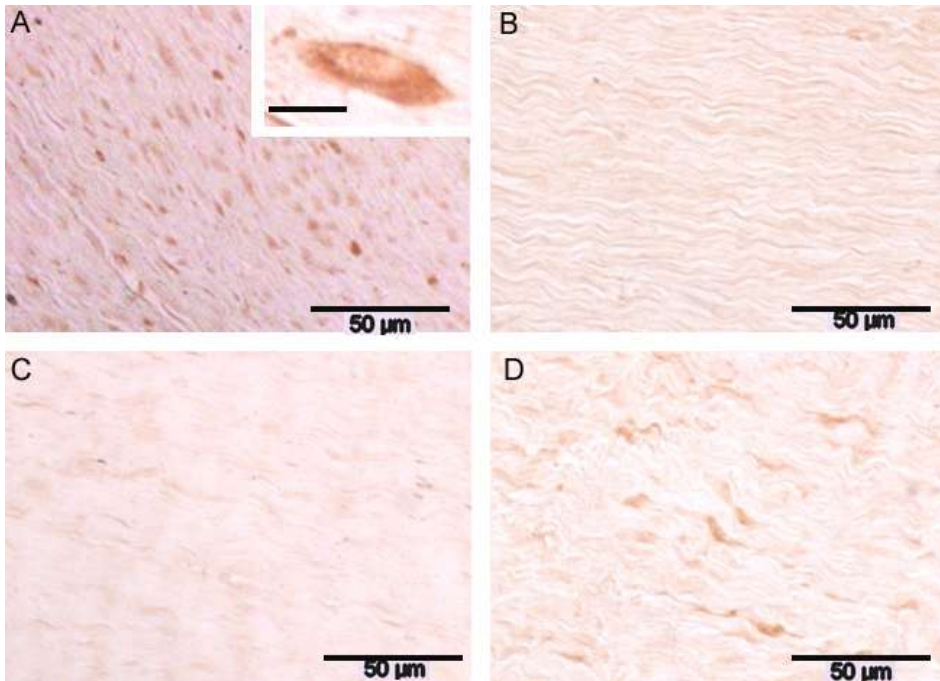

Fig. 8 Representative sections of immunohistochemical staining for COMP of DDFT sections at 1 month from treatment. Lesioned not treated (A); treated with MSCs (B); treated with MSCs + PRP (C); treated with PRP (D); Sections are counterstained with hematoxylin.
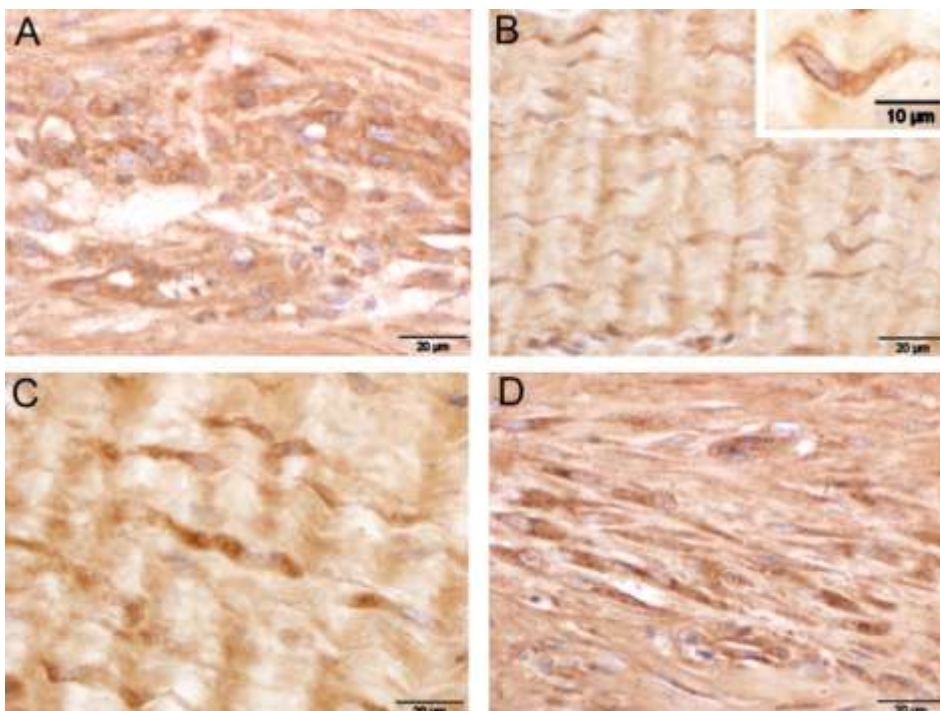
Fig. 9 Longitudinal ultrasonographic images of representative DDFT lesioned with collagenase (lane A), treated with MSCs (lane B), MSCs + PRP (lane C) and PRP (lane D). A, B, C, D before lesion; A1, B1, C1, D1: T0, 7 days after lesion and just treated, A2, B2, C2, D2: T30, 30 days from the treatment; A3, B3, C3, D3: T60 at 60 days; A4, B4, C4, D4: T90 at 90 days; A5, B5, C5, D5: T120 at 120 days.
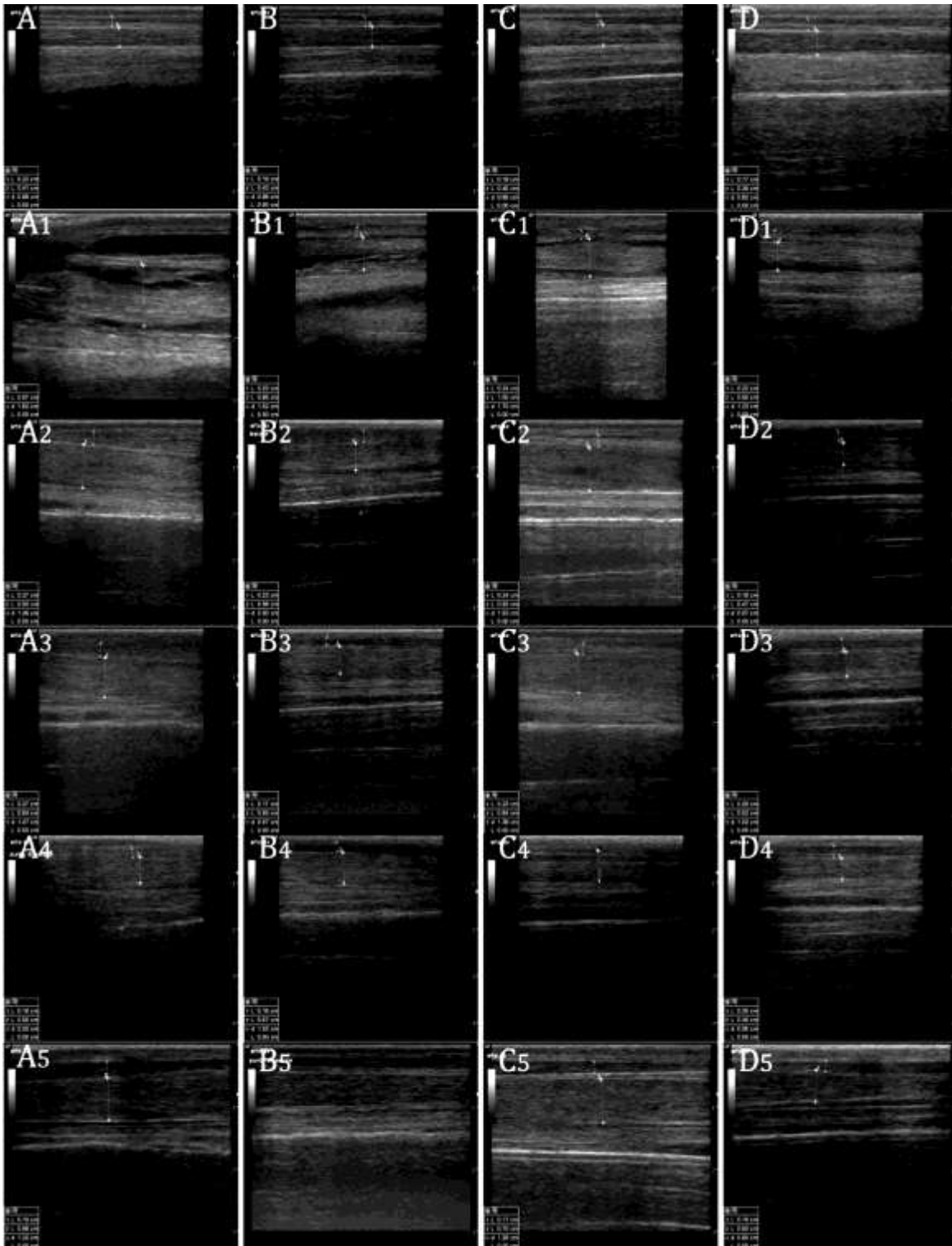
Fig. 10 Representative HE staining of sections from DDFT at 4 month after the lesion. (A) Not treated; (B) treated with MSCs; (C) treated with MSCs and PRP; (D) treated with PRP.

Fig. 11 Representative Masson tricromic staining of sections from DDFT at 4 month after the lesion. (A) Not treated; (B) treated with MSCs; (C) treated with MSCs and PRP; (D) treated with PRP.

Fig. 12 Representative Mallory staining of sections from DDFT at 4 month after the lesion. (A) Not treated; (B) treated with MSCs; (C) treated with MSCs and PRP; (D) treated with PRP.

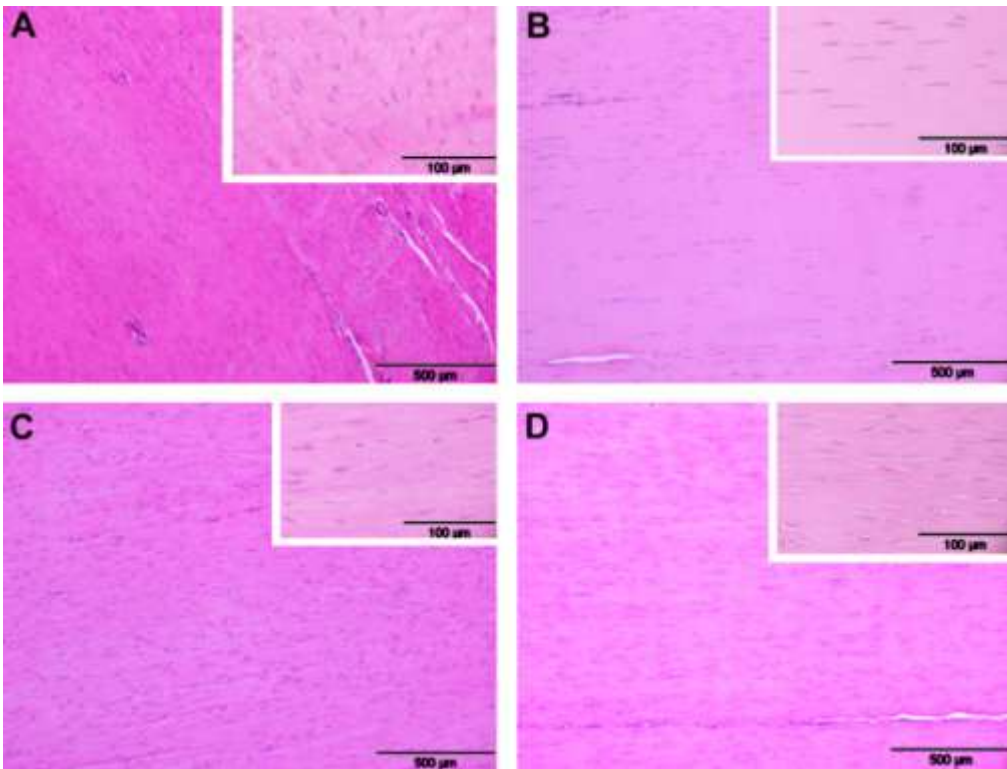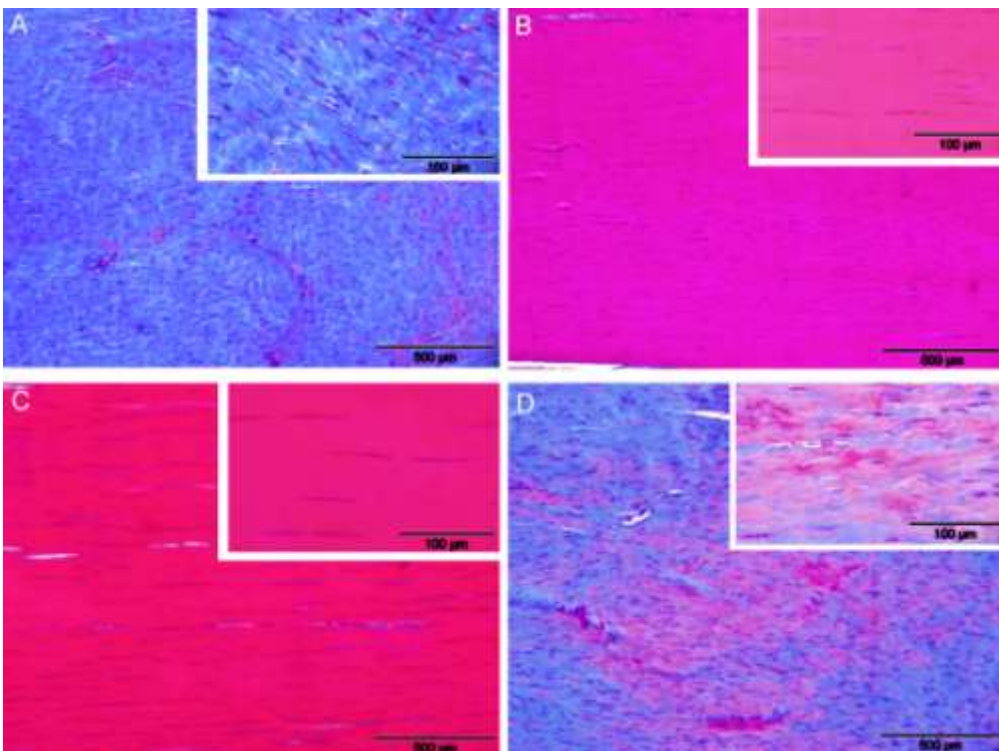Fig 13 Representative sections of immunohistochemical staining for anti-collagen type 1 of DDFT sections at 4 month from treatment. Lesioned not treated (A); treated with MSCs (B); treated with MSCs + PRP (C); treated with PRP (D); Sections are counterstained with hematoxylin.
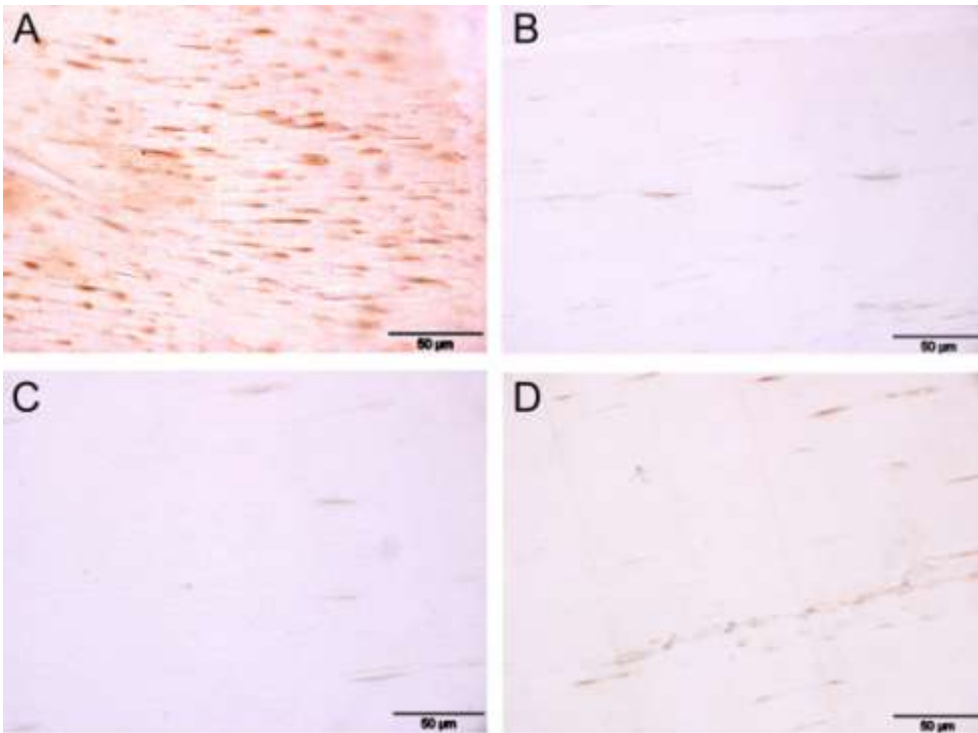
Fig. 14 Representative sections of immunohistochemical staining for anti-collagen type 3 of DDFT sections at 4 month from treatment. (A) Lesioned not treated; (B, C, D) high magnification of A in which is well evident the cytoplasmic staining. (E) representative image of all treated samples that are negative for the anti-collagen3 antibody. Sections are counterstained with hematoxylin.

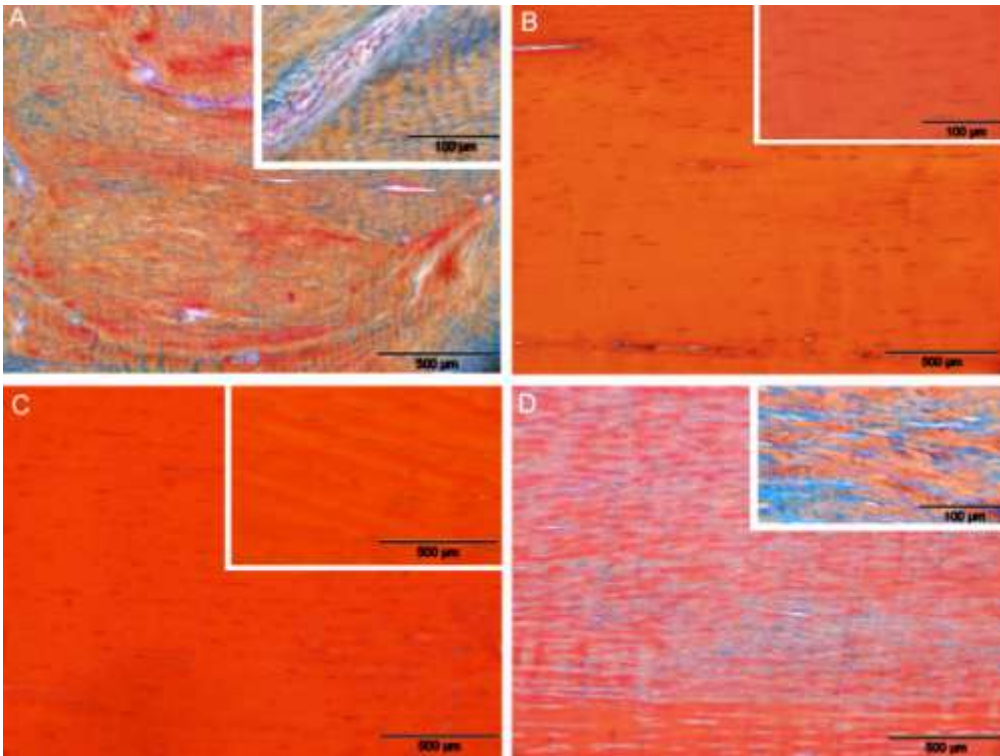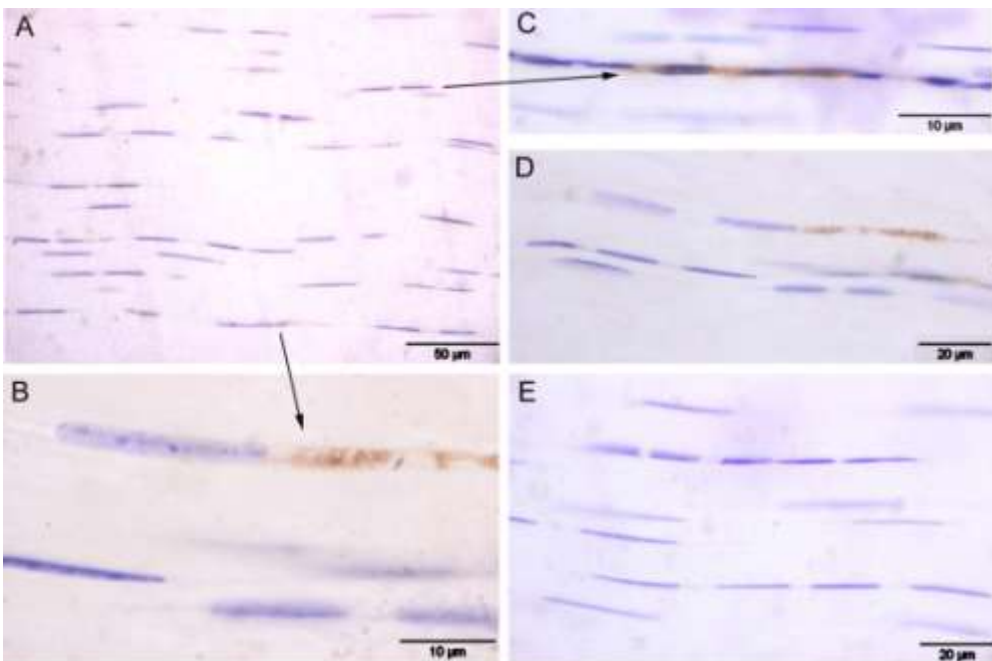Fig. 15 Representative sections of immunohistochemical staining for COMP of DDFT sections at 4 month from treatment. Lesioned not treated (A); treated with MSCs (B); treated with MSCs + PRP (C); treated with PRP (D); Sections are counterstained counterstained with hematoxylin.
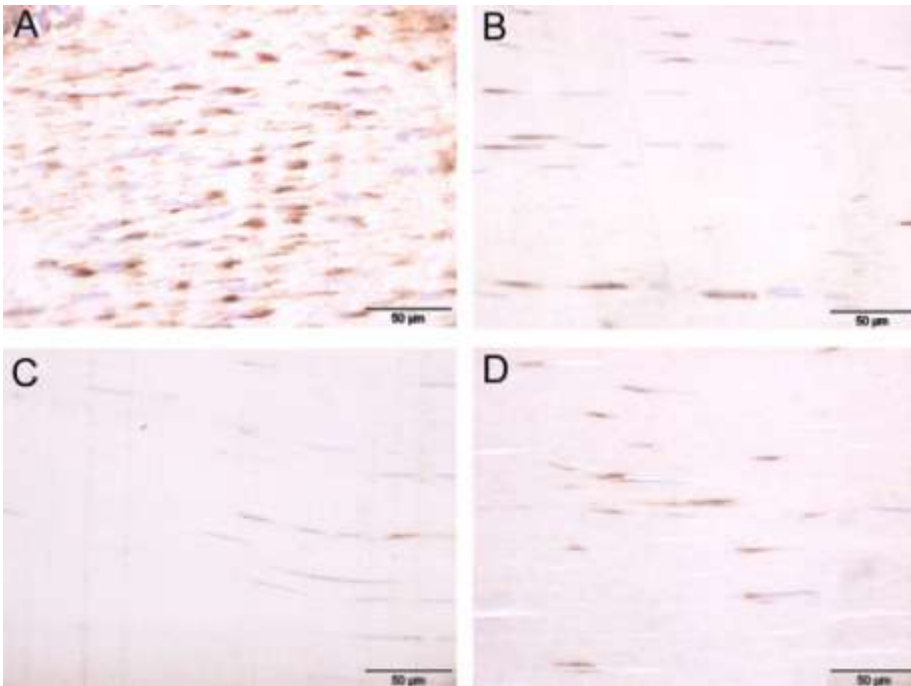
Fig. 16 Representative sections of health tendon stained with (A) HE and Mallory (scale bar 100 µm); (B) stained for anti-collagen type 1; (C) stained for anti-collagen type 3; (C) stained for anti-COMP. Only few cells were found positive for collagen type 1 and COMP, while any reaction were observed for collagen 3.
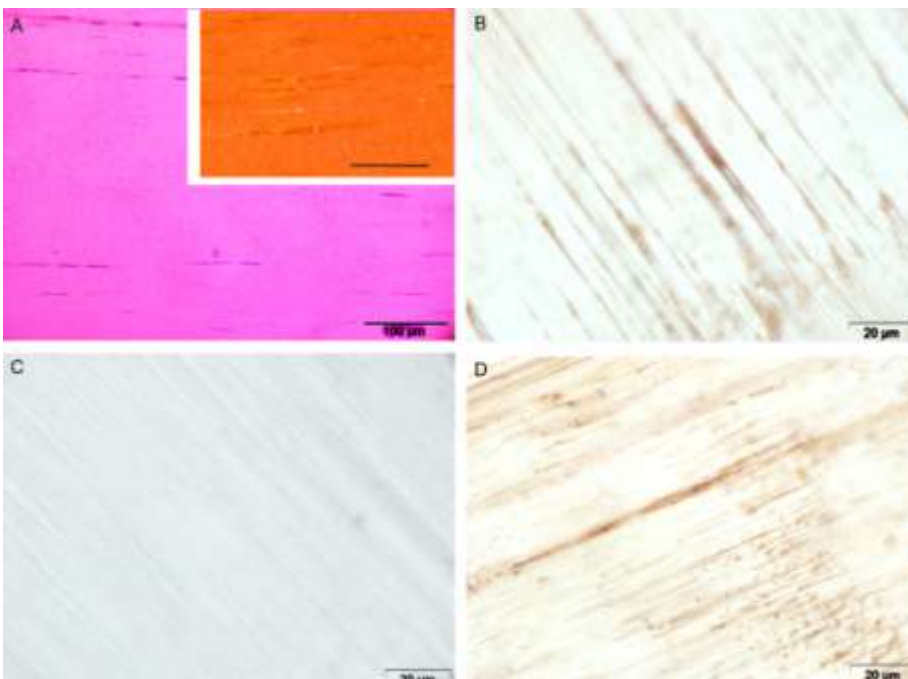
DISCUSSION

The present work has been focused on two major subjects: the first part focused on the *in vitro* study of isolation, characterization and plasticity of stromal cells derived from tissue of mesenchymal origin, their possible cryopreservation and their delivery optimization in the short term period; the second part focused on the study of *in vivo* application of MSCs in tendon regeneration, with the main goal to better define the putative action of MSCs during tendon regeneration.

Adult stem cells have been isolated from several tissues and their potential application is still under investigation in both human and veterinary medicine. Indeed, MSCs offer great promise in treating previous incurable disease for several reasons: i) they lack the ethical controversies that are associated to embryonic stem cells (ES) since are derived from adult tissues and do not require manipulation or destruction of embryo, ii) do not posses the risk of tumorigenicity if implanted *in vivo*. It has generally demonstrated that MSCs posses the ability to undergo toward different lineages of differentiation, and recently their immunomodulation capacity has been investigated. Despite the great advances made on isolation, expanding and defining stromal cells population, several challenges remain open to full understand the MSCs behavior of *in vivo* and *in vitro*.

The first part of this work was aimed to investigate stromal cells derived from peripheral blood of horses (hPB-MSCs) and adipose tissue of dog (cA-MSCs) and to fully define their characterization, and the differentiation potential before and after 1 year of cryopreservation. Indeed, in order to develop cell-based therapies a crucial requirement is to obtain a sufficient number of MSCs, and for this reason the possibility to cryopreserve these cells in order to stock a high amount of quickly available cells will play an important role for the success of these treatment (Attarian H et al., 1996; Park YB et al., 2008; Malpique R et al., 2009). Therefore, it is fundamental to verify that the cryopreservation does not alter the stemness characteristic and the differentiative potential of isolated MSC (Martinello T et al., 2009). Bone marrow derived MSCs is the main source of stromal cells for horses nowadays; however, in this work MSCs derived from peripheral blood of horses (ePB-MSC) were investigated as an alternative sources of stromal cells, for their easier and less painful harvesting procedure compared with bone marrow. Previous works reported isolation and differentiation potential into three different lineages of ePB-MSCs but without providing a full characterization of these cells (Giovannini JM et al., 2008; Koerner J et al., 2006). Our study tried to fulfill this gap. Moreover, also the isolation and a full characterization of cA-MSC was

investigated since adipose tissue constitute an attractive source of stromal cells because of its abundance and easy accessibility; this is particularly true for dogs due to size limitation. The comparison between fresh and one year cryopreserved hPB-MSCs and cA-MSCs demonstrated that the stemness characteristics of these cells are not changed after cryopreservation, for all parameters evaluated, such as the alkaline phosphatase activity (AP), the population doubling time (PDT), telomerase activity and CD expression, and the differentiation potential towards the adipose, osteo and muscular fates. Overall, these studies demonstrated that both ePB-MSCs and cA-MSCs constitute an accessible and effective source of adult stromal cells, and our data strongly support the use of these cells for clinical purposes.

The continuous progress made in the study of MSCs prompt their use in clinical field; however, for a safe and effective use of stromal cells there is a need of scientific investigations, especially into the preparation and maintenance of these cells as well as rigorous regulation regarding their production and use in veterinary medicine., Cells are usually delivered frozen or at 4°C for clinical purposes but while cryopreservation is the well-studied and best choice for long-term storage of MSCs (Haack-Sørensen M and Kastrup J et al., 2011) no available data were present in literature on MSCs delivery in not frozen state. Moreover, of considerable concern it is the fact that MSC maintained in suspension do not survive for a long period (Gorodetsky R., et al., 2011). For these reason a study aimed to provide an analysis of the influence of relevant parameters, during short-term shipment of both ePB-MSCs and cA-MSCs, was carried out. Specifically, the impact of different medium, time and temperature applied to MSCs maintained in suspension was investigated. The aim here was to evaluate the influence of different experimental shipping conditions on survival of ePB-MSC and cA-MSC, and to quantify the extent of cell death by determining the number of cells that are viable during and after our shipping model. Moreover, important parameters such as the CD expression, the presence of apoptosis resistance and the beta-galactosidase positivity were verified on cells that maintained the higher value of survival after exposure to experimental shipping conditions. Since the delivery of stem cells for clinical usage is becoming increasingly important in veterinary field, the novel data provided by this study lead to the conclusion that both ePB-MSC and cA-MSC should be shipped and used within 9-12 hrs of their preparation and that they should be maintained in PBS at room temperature during shipment. These data are important for setting up recommendations and standard guidelines for the shipment of MSC for clinical veterinary purposes.

The second part of this work has been focused on application of stromal cells of mesenchymal origin in tendon repair. Tendons injuries are common and money-wasting pathologies in both

human and veterinary medicine. Recently, in order to improve tendon healing, enhanced biological approaches have been used such as platelet rich plasma, bone marrow aspirate, growth factor supplements and cell- and gene-modified therapy. Also tissue engineering techniques employing scaffolds as a support to cell-therapies have been utilized. Taking advantages of the *in vitro* studies on characterization of mesenchymal stromal cells derived from peripheral blood and from adipose tissue, the second goal of this work was to investigate the capacity of these cells sources to contribute to the tendon repair process.

The first work focused on study of capability of MSCs derived from human adipose tissue (lipoaspirate) to integrate and re-cellularize a human biocompatible scaffold that could be potentially used in full thickness hand flexor tendon lesion. Biological scaffold derived from de-cellularized tissues have been successfully used in the last decade in both pre-clinical animal studies and in human clinical applications (Ingram JH et al., 2007; Xu H et al., 2009; Baiguera S et al., 2011). Indeed, removal of cells from the native tissues offers many advantages with respect to artificial scaffold, including higher biocompatibility, enhanced ability for cellular repopulation and increased biomechanical strength (Liu Y et al., 2008). In this work we obtained a successful de-cellularized tendon scaffold, adapting previous de-cellularization protocols (Cartmell JS et al., 2000; Baiguera S et al., 2010 and 2011) for a specific tendon size of 2cm x 0,25cm.

Secondly our efforts had focused therefore on developing and optimizing a functionally robust re-cellularization protocol for human tendon scaffold, since no previous works achieved this goal (Ingram JH et al., 2007; Thevenot P et al., 2008; Pridgen BC et al., 2011). The re-cellularization was established by the support of a collagen matrix gel, that allowed a good penetration of MSCs inside the tendon ECM, with cells assuming a spindle-shape morphology and expressing collagen type 1 and COMP protein. These significant data suggest the feasibility of a new strategy for re-cellularizing scaffolds, achieving an efficient infiltration into the core of the tendon. The possibility to introduce cells into the tendon using a collagen gel represents a significant advance in the field of scaffolding materials, opening excellent new opportunities for tendon scaffold engineering in hand reconstruction.

The second work focused on studying the application of MSCs derived from peripheral blood of ovine species (sPB-MSCs), alone or in combination with (platelet rich plasma) PRP, in the treatment of experimental collagenase 1A induced lesions in the short and long term period. Among different species of veterinary interest, the horse is more prompted to develop tendon injuries because of the hyperextension of metacarpo-phalangeal joint during weight bearing, and the consequent excessive load resulting to these structures. Ovine species have been chosen in this study because, among different experimental animals, they represent a good template to study

tendon regeneration for horses. Some innovative aspects of this study were working on a different source of MSCs, like peripheral blood derived MSCs, and use them for the first time *in vivo,* with the aim to verify their capacity to contribute to tendon regeneration. Moreover, introduce in an *in vivo* model and in the same experimentation, a comparison among the effects on tendon healing of MSCs and PRP both alone or in association; even if previous studies attempt to monitor the fate of MSCs injected in tendon lesions *in vivo*, marking them with GFP protein, this is the first report of GFP positive peripheral blood derived mesenchymal stromal cells traced in tendons. Finally, the three different treatments were evaluated after 120 days, differently from the majority of studies, that analyzed the benefit of cell-based or growth factor delivery therapies in the short time period. Treated and control tendons were evaluated both with histological and immunohistochemical techniques, and compared among each other and between 1 and 4 months studies. Results obtained demonstrated that the morphological appearance of the lesions improved for all histological parameters evaluated in the left hind treated tendons; particularly after 4 months, treated tendons appeared to be more similar to the not lesioned, healthy tendons. The immunohistochemical analysis at one month showed no significant difference in Collagen 1 expression among the three different treated groups, while an higher expression of collagen 1 in control tendons (lesioned, not treated) were found, probably due to the higher cellularization present in the control group. Groups that received the sPB-MSCs showed a lower expression of Collagen 3 if compared with PRP treated group and internal control (lesioned, not treated), demonstrating the higher capacity of sPB-MSCs and sPB-MSCs+PRP to promote Collagen 1 production after 30 days. Treated tendons after 120 days from the collagenase lesion resulted more similar to the healthy tendon respect to the internal control (lesioned, not treated tendon). All our data suggest the predominant effect of MSCs on tendon healing, evidencing a better contribution on structural organization and a reduced cell number . Several histologic parameters including cell density and shape, neo-vascularization, and ECM organization, all trended lower in treated left hind DDFT respect to the internal control (lesioned, not treated) in both 1 month and 4 months study, differently from what reported in the study of Guest DJ et al., 2010, where no significant differences were found between MSC and serum-injected sites. Outcomes derived from this study do not address any convenience in the concomitant clinical application of MSCs and PRP, since no relevant differences were found between the two different treatments after 4 months. Indeed, results obtained highlights how the use of MSCs, isolated from a different source respect to the most common used bone marrow, contributes to a better tendon restoration after injuries.

Overall, and on the basis of collected data during this research project, it should briefly point out some final conclusions:

- Among different sources of adult stromal cells, peripheral blood derived and adipose tissue derived MSCs represent valid alternative cells respect to the more utilized bone marrow derived MSCs.

- The possibility to cryopreserve both ePB-MSCs and cA-MSC for long-term period, open the possibility to have large amount of cells available to be used in clinical trials, ensuring the maintenance of their phenotype and differentiation potential.

- The study performed in order to verify the possibility to deliver both ePB-MSCs and cA-MSC in the short-term period, avoiding cryopreservation, has led to the conclusion that the best way to ship these cells is maintaining them in PBS, at room temperature and for no more than 9-12 hrs. This delivery options are undoubtedly straightforward, and inexpensive, and for this reason could represent a valid alternative way to make available and ready to use MSCs for clinical purposes.

- The work performed using adipose tissue derived MSCs for re-cellularization of human biocompatible scaffold, has led to the conclusions that mesenchymal stromal cells derived from adipose tissue are a potentially good source to reseed a previously de-cellularized tendon with cells that are able to integrate in the ECM and contribute to production of protein like collagen 1 and COMP, essential for the tendon structure and functionality. These results open the possibility to use biocompatible human tendon scaffold recellularized with autologous mesenchymal stromal cells for an improved functionality restore of full thickness hand flexor tendon lesions.

- MSCs derived from peripheral blood of ovine sheep, demonstrated the potentiality to significantly contribute to tendon healing processes, if compared with the use of PRP alone. Results obtained from for this experimental study demonstrated that the ovine species is a good animal model for the easy management and its affinity to the horse species. Moreover, the results obtained open the possibility to further investigations about the use of MSCs for the *in vivo* treatment of tendon lesions in horses, adapting analogous protocols used in this study.

Over the past decade, there have been a large number of publications on MSCs from several tissue sources. Moreover, a number of animal models for human diseases have shown encouraging results for the use of MSCs in terms of repair and restoration of functional tissue, as well as a growing

number of human clinical trials. There are no specific phenotypic MSCs markers that could be used, so the exact clinical effects of such sorted cells may be uncertain, as the cell populations used may differ. In addition, clinical outcomes are variable, and generally show small improvements, but to date, only few studies have reported a long period of observation, the outcomes of more than one MSCs infusion, or whether MSCs survive engraftment (Otto WR. 2011). This research project aimed to improve the understanding of characterization and behavior of MSCs of two different sources *in vitro* and providing models for an *in vivo* study of their potentiality in promoting tendon regeneration. Future perspective will be direct to further analyze the use of MSCs in biocompatible scaffold in *in vivo* models, testing their mechanical properties and functionality and apply MSCs derived from peripheral blood in a larger number of clinical cases, to confirm the outcomes derived from this study.

ACRONYMS

ADSCs	adipose tissue derived stem cells
ACL	anterior crucial ligament
AECs	epithelial stem cells
BM	bone marrow
BMSCs	bone marrow stromal cells
cA-MSCs	canine adipose derived mesenchymal stem cells
CD	cluster of differentiation
CESCs	corneal epithelial stem cells
CFU-Fs	fibroblast like colonies forming units
CSA	cross sectional area
CSCs	cardiac stem cells
DCs	dendritic cells
DDFT	deep digital flexor tendon
DMEM	dulbecco's modified eagle medium
ECM	extracellular matrix
EGF	epithelial growth factor
EGFR	epithelial growth factor receptor
eNCSCs	epidermal neural crest stem cells
ePB-MSCs	equine peripheral blood derived mesenchymal stem cells
EPCs	endhotelial progenitor cells
ES/ESCs	embryonic stem cells
FBS	fetal bovine serum
FGF	fibroblast growth factor
GFP	green fluorescent protein
GMP	good manufacturing practice
GvHD	graft versus host disease
hAECs	human amniotic epithelial cells
HGF	hepatocyte growth factor
hMSCs	human mesenchymal stromal cells
HSCs	hematipoietic stem cells

IGF-1	insuline like growth factor
ISCT	International society for cellular therapy
MAPCs	multipotent adult progenitor cells
MDSCs	muscle derived stem cells
MIAMI	marrow-isolated adult multilineare inducible cells
MSCs	mesenchymal stromal cells
MTJ	myotendineus junction
NSCs	neural stem cells
OTJ	osteo tendineus junction
PAX7	paired box gene 7
PBS	phosphate buffer saline
PDGF	platlet derived growth factor
PDMSCs	placenta derived multipotent stem cell
PRP	platlet rich plasma
PSCs	pancreatic stem cells
RSCs	retinal stem cells
SCF	stem cell factor
SCX	basic-helix-loop-helix transcription factor
SDFT	superficial digital flexor tendon
sPB-MSCs	sheep peripheral blood derived mesenchymal stem cells
TGF	transforming growth factor
TNFR	tumor necrosis factor receptor
UCB-MSCs	umbilical cord blood derived mesenchymal stem cells
VEGF	vascular endothelial growth factor
VEGFR-2	vascular endothelial growth factor recepror-2
VSELs	very small embryonic-like stem cells

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