UNIVERSITY OF PADUA
Department of comparative biomedicine and food science

GRADUATION THESIS IN VETERINARY MEDICINE

Tenogenic induction of equine mesenchymal stem cells by means of growth factors and low-level laser technology

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“Il vero viaggio di scoperta non consiste nel cercare nuove terre, ma nell’avere nuovi occhi”

Marcel Proust

INDEX

ABSTRACT

Tendon injuries are one of the most common orthopaedic problems in equine athletes but the avascularised nature, the hypocellularity and the low metabolic rate predispose tendons to slow healing with a high incidence of relapses.

Nowadays, the improvement of regenerative therapies, whose aim is the *restitution ad integrum* of the tissue, has shown promising results with the application of stem cell-based therapies, growth factors (GFs) and low-level laser therapy (LLLT).

The purpose of the present study was to develop a useful method to induce tenogenic differentiation of peripheral blood (PB)-derived mesenchymal stem cells (MSCs), through different combinations of basic Fibroblast growth factor (FGF), Transforming growth factor-β3 (TGF), Insulin-like growth factor-1 (IGF), Epidermal growth factor (EGF) and a low-level laser irradiation (LLLT) *in vitro*. The relative expression of the following genes was evaluated by means of Real time-PCR: Early Growth Response Protein-1 (EGR1), Tenascin C (TNC) and Decorin (DCN).

The results showed that the concomitant addition of FGF and TGF arrested cell proliferation and up-regulated the relative expression of EGR1 and DCN in both irradiated and non-irradiated flasks, whereas only the combination FGF + TGF + LLLT was able to increase significantly TNC levels. We concluded that the supplementation of expansion medium with FGF and TGF is a useful method to induce MSCs differentiation towards the tenogenic fate. Overall, more *in vitro* and *in vivo* study needs to be performed, in order to better understand the correlation between MSCs and their niche. Indeed, the development of standard protocols of differentiation is recommended, in order to routinely apply induced-MSCs for the treatment of tendinopathies in horses.

RIASSUNTO

Le lesioni tendinee rappresentano uno dei problemi ortopedici più frequenti nel cavallo sportivo. La scarsa vascolarizzazione e la bassa cellularità, accompagnata da un metabolismo strettamente anaerobico, predispongono il tessuto tendineo a una lenta guarigione e a un'elevata incidenza di ricadute, in seguito a traumi.

Al giorno d'oggi, lo sviluppo di terapie rigenerative, il cui l'obiettivo principale è la *restitution ad integrum* del tessuto danneggiato, ha mostrato risultati incoraggianti soprattutto con l'utilizzo di terapie cellulari, fattori di crescita e tecnologia laser a bassa energia.

Lo scopo del presente studio è quello di sviluppare un metodo efficace per indurre il differenziamento tenogenico, *in vitro,* di cellule staminali mesenchimali (MSCs) isolate dal sangue periferico (PB) di quattro cavalli adulti. Diverse combinazioni del fattore di crescita dei fibroblasti (FGF), fattore di crescita trasformante-β3 (TGF), fattore di crescita insulino-simile-1 (IGF) e fattore di crescita dell'epidermide (EGF), sono state testate insieme alla tecnologia laser a bassa energia (LLLT).

L'espressione relativa di geni coinvolti nello sviluppo tendineo è stata misurata tramite Real time-PCR. I geni che sono stati testati sono i seguenti: Early Growth Response Protein-1 (EGR1), Tenascina C (TNC) e Decorina (DCN).

I risultati hanno mostrato che l'aggiunta concomitante dell'FGF con il TGF ha causato un arresto della proliferazione cellulare e un aumento dell'espressione relativa dell'EGR1 e della DCN, sia nelle colture che non sono state trattate con il LLLT, che in quelle trattate. Invece, solo la combinazione FGF + TGF + LLLT ha provocato un significativo aumento nel livello di espressione della TNC.

Sulla base delle nostre osservazioni, possiamo dedurre che l'aggiunta di FGF e TGF nel medium di crescita è un metodo utile per incrementare il differenziamento tenogenico delle MSCs coltivate *in vitro*. Tuttavia, ulteriori studi in questo campo si rendono necessari per poter raggiungere una migliore conoscenza delle interazioni che si realizzano tra le MSCs e la loro nicchia. Infatti, la possibilità di sviluppare dei protocolli di differenziamento standard sarebbe auspicabile per utilizzare al meglio le MSCs nel trattamento delle tendinopatie nel cavallo.

INTRODUCTION

THE TENDON

1. Tendon anatomy

1.1 Macroscopic anatomy

Tendons are anatomical structures interposed between muscles and bones. Tendons allow the transmission of forces generated by muscles contraction, resulting in the movement of joints and the maintenance of body posture. Moreover, tendons work as an energy- storing system that during the lengthening period of locomotion stores energy, which will be used as an elastic recoil during the propulsive phase. This property improves movement and reduces the workload of the muscles. Tendons can tolerate very high tensions undergoing minimal deformations, thus muscle contraction is optimized, with the minimum loss of energy and the maximum performance (Dyce et al. 2009).

Healthy tendons are smooth, brilliant, and white in colour, with a fibro-elastic structure and a great resistance to mechanical loads. Tendons can be round cords, strap-like bands, or flatten ribbons. The shape and properties of tendons varies in function of the entire muscle-tendon complex behaviour. Muscles performing soft and delicate movements have long and thin tendons, instead, muscles creating powerful and resistant forces exert their action through short and thick cord- like tendons (Franchi et al. 2007; Sharma and Maffulli 2005).

In those areas subjected to increased mechanical stress, tendons are covered by specialized sheaths such as, fibrous sheaths (or retinaculae), reflection pulleys, synovial sheaths, peritendon sheaths (or paratenon), and tendon bursae (Kannus, 2000).

The histological knowledge of the tendon tissue is essential to understand its hierarchical structure and biomechanics.

1.2 Microscopic anatomy

A tendon is a tough band of dense regular connective tissue. It derives from mesenchyme, the embryonal connective tissue that takes origin from the mesoderm germ layer. Cells lie within an extracellular matrix (ECM), mainly composed by water (70%), fibres, proteoglycans (PGs) glycosaminoglycan (GAGs), and glycoproteins. ECM is a high-specialized dynamic structure, which not only offers a mechanical cell support, but mostly it regulates cell metabolism,

migration, proliferation, differentiation, apoptosis, and intercellular signalling. In turn, cells are responsible to secrete ECM components, thus conditioning ECM dynamics.

1.2.1 Cells

Tenoblasts and tenocytes constitute about 90-95% of the cellular elements of tendons. They are specialized fibroblast and fibrocytes that lie between collagen fibres. The remaining 5-10% of cellular elements consists of chondrocytes, synovial cells, and vascular elements, including endothelial cells and smooth muscle cells. Tenoblasts are immature tendon cells, with numerous long and thin cytoplasmic processes that extend into the matrix. They are high metabolic cells engaged in the synthesis of all matrix components. As they mature, they flatten and become very elongated, transforming into tenocytes.

Tenocytes are mature tendon cells with a low nucleus-to-cytoplasm ratio and longer and thinner cytoplasmic processes than tenoblasts. The cellularity in mature tendons is low and the metabolism is predominantly anaerobic. These characteristics are essential to maintain tendon tension and carry loads for long periods, but it predisposes the tendon to slow healing after injury (Kannus, 2000, Sharma and Maffulli 2005).

1.2.2 ECM: Fibres

Collagen type I is the main component of ECM, but also collagen type II and elastin are present. Collagen type II and elastin account for the 2% of the dry mass of the tendon, the former is particularly abundant at the osteo-tendinous junction, where high compressive loads are expressed and the latter is essential to give tendons the ability to cope with stretch and distension.

Collagen type I represent the 95% of the dry mass of the tendon. Histologically, collagen fibres show a compact and parallel arrangement, hence, the classification as a dense regular connective tissue. Collagen is responsible for the high tendon tensile strength and it is arranged in hierarchical levels of increasing complexity.

A collagen molecule is composed of three twisted polypeptide chains that form a right-handed triple helix, called tropocollagen. Soluble tropocollagen molecules align each other, head to tail, in overlapping rows, forming insoluble collagen fibrils.

At the electronic microscopy, collagen fibrils have a diameter of 10 to 300 nm and show a characteristic horizontal streak of 67 nm intervals. The fibril strength is due to covalent bonds between collagen molecules of adjacent rows. Cross-links between collagen fibrils are essential to form collagen fibres.

A collagen fibre is the smallest tendon unit that can be tested mechanically and it is visible under light microscopy. (Dellmann and Eurell 2000; Patruno, 2010; Sharma and Maffulli 2005). Collagen fibres are mainly oriented longitudinally, but fibres can also run transversally and horizontally, forming spirals and plaits. In general, there is a strong correlation between collagen orientation and functional requirements, in fact, the direction of fibres is always indicative of the prevalent tensile stress (Franchi et al. 2007). Fascicles of fibres then aggregate to form primary, secondary and tertiary bundles, which together constitute the tendon itself. Tendon fibres are bound together by the endotenon, a loose connective tissue layer that includes vessels and nerves. The epitenon is continuous with the endotenon and it surrounds the whole tendon.

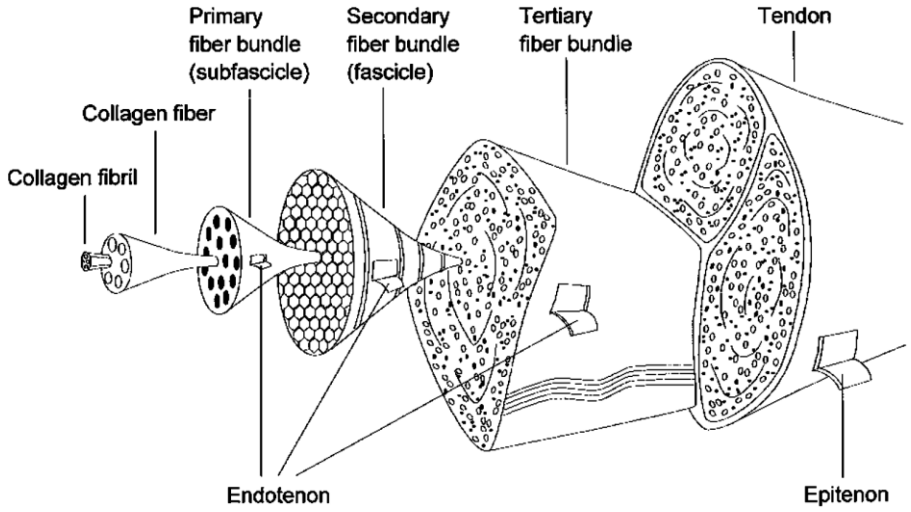

Figure 1: The hierarchical structure of the tendon (Kannus, 2000)

1.2.3 ECM: the ground substance

The ground substance occupies the space between fibres and cells. It represents the main constituent of the tendon and it is composed predominantly by water (70%), proteoglycans (PGs), hyaluronic acid (HA) and glycoproteins.

PGs are large macromolecules consisting of a core protein, to which many glycosaminoglycan (GAG) molecules are covalently attached. GAGs are short chains of polysaccharides made up of repeating disaccharide units, where one of the sugars is always a glucosamine. The main GAGs of ECM are chondroitin-sulphate, dermatan-sulphate, keratin-sulphate, heparin-sulphate and hyaluronic acid (HA). GAGs are poli-ionic acid molecules, highly negative

charged on the surface, because of the presence of sulphate, hydroxyl, and carboxyl groups, and thus highly hydrophilic. GAGs entrap water between fibres and cells forming a hydrated gel, which enables rapid diffusion of small water-soluble molecules and the migration of cells. (Dellmann, 2000; Patruno. 2010).

HA is an extremely long and rigid GAG that differently from the others, it does not bind to a core protein, but proteoglycans indirectly bind to HA via special linker proteins to form a giant macromolecule. The biochemical characteristics of GAG molecules are of fundamental importance for the maintenance of ECM structure and function.

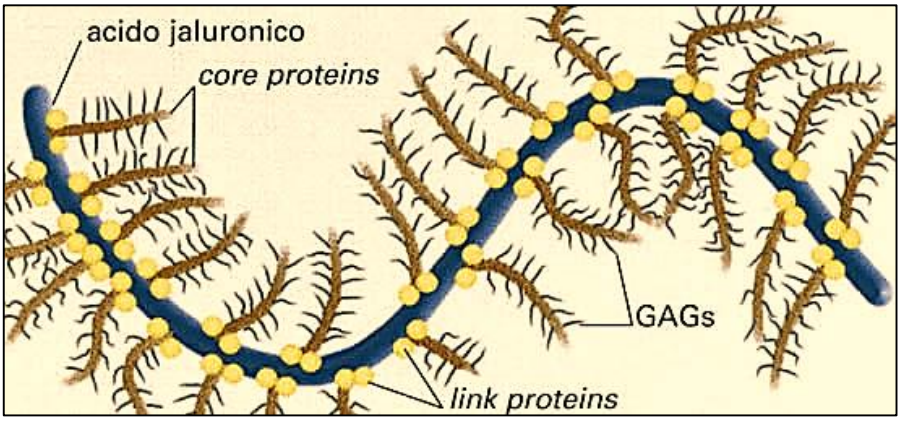

Figure 2: Schematic representation of a proteoglycan.

Two groups of PGs have been demonstrated in the tendon: the small leucine- rich PGs (SLRPs) and the modular PGs (or hyalectans).

SLRPs have a small core protein (~40 kDa) to which chondroitin-sulphate, dermatan-sulphate and keratan-sulphate molecules are attached. Decorin, biglycan, fibromodulin, and lumican belong to this group, they are able to bind to collagen fibrils, thus playing a key role in tendon collagen fibrillogenesis (Iozzo and Murdoch 1996; Yoon and Halper 2005). Collagen fibrillogenesis is a hierarchical process that depends on the unique structure and function of PGs. They promote the tip-to-tip collagen fibrils fusion and inhibits side-by-side interactions. Moreover, SLRPs interact with other ECM glycoproteins and growth factors, regulating cell proliferation (Graham et al. 2000).

On the other hand, hyalectans have a big core protein (~160 kDa) with multiple domains, which interact with HA. By virtue of GAGs high negative charges, hyalectans provide to collagen fibrils the capacity to resist high compressive forces, associated with loading and movement.

Decorin is the most abundant PG in tendon. Fibroblast, chondrocytes, endothelial cells and smooth muscle cells are all decorin-producing cells. The main function of decorin is to maintain collagen fibril structure, establishing inter fibrillary bridges, which contributes to keep the specific fibril parallel arrangement, thus preventing the lateral fusion (Iozzo et al. 1999). Some studies have demonstrated the functional role of this PG by generating decorin-deficient mice. Electron microscopic examination of decorin knockout mice skin showed that these animals presented skin fragility and a marked reduction in tensile strength. Compared with normal skin, the collagen network was loosely packed and collagen fibres had abnormal diameter (Danielson et al. 1997; Zhang et al. 2006).

Several glycoproteins can be found in amorphous ground substance of tendon tissue such as, cartilage oligomeric matrix proteins (COMP), fibronectin, thrombospondin, and Tenascin-C (TNC).

TNC has a very restricted expression in normal tissues, but it is up-regulated during embryogenesis and tissues particularly subjected to mechanical stress, as tendons. Those regions of tendons, particularly subjected to compressive loads, have a fibrocartilaginous structure and composition. Cells can maintain their spherical shape and minimize their deformation, by virtue of their reduced adhesion to the ECM. TNC has been described as an anti-adhesive molecule that can decrease cell-matrix connections, allowing the free movement of cells within the adjacent collagen fibres (Mehr et al. 2000). To assess the importance of mechanical stress on TNC expression, a study was conducted *in vivo* at the osteo-tendinous junction (OTJ) of rat quadriceps muscles. After three weeks of cast immobilization, the immunoreactivity of TNC was almost completely absent at the OTJ, but once the application of an increased mechanical stress, the immunoreactivity of TNC reached the level of the healthy contralateral limb (Järvinen et al. 1999). Moreover, TNC expression is up regulated by several growth factors such as, basic fibroblast growth factor and transforming growth factor (Tucker et al. 1993).

2. Etiology of tendon lesions in sport horses

2.1 Tendon injury

Tendon anatomical characteristics are essential to confer the appropriate stiffness and elasticity that tendons need to perform their tasks. However, the not-vascularized nature, the hypo-cellularity, and the low metabolic rate of tenocytes result in slow healing and an inefficient tendon regenerative potential.

Tendon injuries are one of the most common orthopaedic problems in equine athletes. Not only ageing and overloading, but also gender, morphology, foot conformation, ground conditioning, and training have been investigated as possible predisposing factors for tendinopathies. In thoroughbred horses, the prevalence of tendonitis is higher in the forelimbs and the superficial digital flexor tendon appears as the most involved structure, whereas, Standardbred racehorses show a higher incidence of desmitis at the suspensory ligament of the hindlimbs (Kasashima et al. 2004; Van den Belt et al. 1994).

Tendon injuries may result from an acute trauma (e.g deep wounds, infections, tendon laceration or rupture) or chronic overuse, which is related to tendon overstrain. In fact, tendons are high compliant structures, which stays under moderate tension thanks to the characteristic waved configuration of collagen fibres, called “crimps”. Under physiological conditions, load causes less than 2% increase in tendon length, resulting in the flattening of the crimp pattern. In those circumstances, the tendon will be in the elastic phase or “toe” region. If the strain increases, but it remains below 4%, collagen fibres deform in a linear way, as a result of the sliding between collagen fibrils. This is called the visco-elastic phase (or linear region) and the tendon can return to its original length, when unloaded. Strains above 4% result in irreversible changes in the ground substance, which cause poor fibrillary organization and may lead to complete tendon rupture, if the strain reaches the 8-12%. It has been recorded that during gallop, the equine superficial digital flexor tendon (SDFT) works close to its limit with a narrow safety margin, thus predisposing equine athletes to develop tendon injuries especially in the flexor compartment (Stephens et al. 1989).

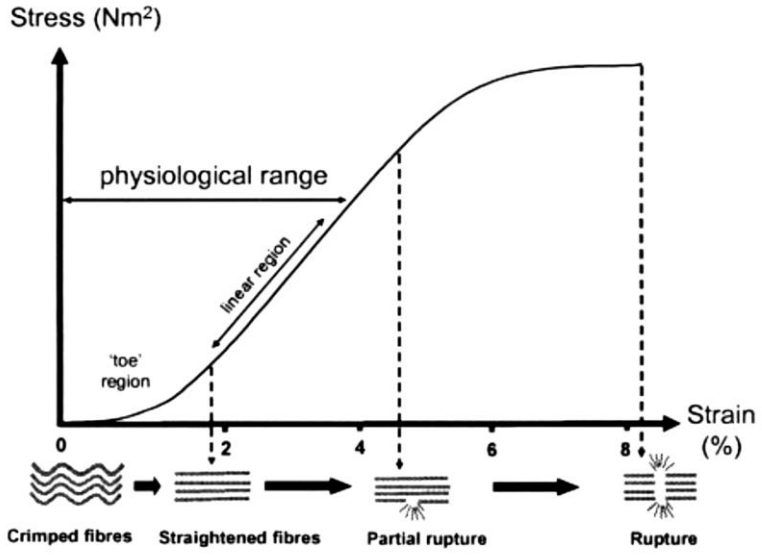

Figure 3: The curve shows the physiological relationship between stress and strain of a normal tendon (Riley 2004)

2.2 Tendon healing

The process of tendon healing represents an important paradigm for medical science. When tendons are damaged, they heal slowly, with the formation of scar tissue that is, almost always, mechanically inferior, and it cause a substantial loss in tendon original strength and elasticity.

After injury, the first reaction is an inflammatory phase that last from several days up to two weeks. The extent of the inflammatory response determines the level of pain experienced, even if, it is not always correlated to the extent of the damage.

Meanwhile, the repair phase starts and continue for several months. The repair phase is mainly characterized by angiogenesis and fibroplasia, with the deposition of random collagen type III fibres. As collagen type III tends to produce smaller and less organized fibrils, it will provide a structural (reparation) but not functional (regeneration) recovery after tendon lesion.

Finally, there is the remodelling phase. It may last several years and it is characterized of collagen type III replacement by oriented collagen type I fibres. Although the extent to recover the original structure, the diameter and the cross-linking of collagen fibrils often remain inferior after healing. This mechanically inferior repair tissue produces a substantial loss in strength, elasticity, and a reduced athletic performance with high incidence of relapse (Sharma and Maffulli 2005; Spaas et al. 2012).

TREATMENT OF TENDON INJURIES IN EQUINE ATHLETES

1. Conservative therapies

Many medical and surgical therapies have been advocated in the treatment of equine tendon injuries. However, there is little objective evidence that they have consistent and long-term beneficial effects.

The goals for the ideal treatment of tendinopathies are to decrease the local inflammation, reduce scar tissue formation, and improve tendon regeneration, in order to recover appropriate strength and elasticity.

Box rest, ice application, and bandaging have been the cornerstones in the treatment of equine tendon injuries, during acute stages. These actions are important to limit the action of proteolytic enzymes on the remaining intact tendon matrix and they decrease the amount of exudation.

In horses, it is possible to apply corrective shoeing, in order to progressively increase tendon strain and reduce the internal tension. However, this procedure may increase the damage in some circumstances, so its beneficial effect is controversial (Dowling et al. 2000).

Controlled exercise and controlled passive mobilization are recommended in the management of acute tendon injuries, in fact, there are several reports supporting the benefits of physiotherapy on rehabilitation of musculoskeletal tissues. The duration of controlled exercise programmes is critical, and since that standard protocols of loading and movement, have not been identified yet, the amount work and time for the owner can exceed the results (Buckwalter, 1996; Takai et al. 1991).

Besides controlled exercise is a valid method to improve tendon healing, pharmacological therapy is often necessary, especially during the acute phase of trauma.

The use of corticosteroids appears to be beneficial especially during the first 24 hours' post-injury, whereas, the delayed use of corticosteroids is not believed to be helpful, as they will inhibit the fibroblastic response, which is necessary for tendon healing. Moreover, the period of rest should be further prolonged, because they mask the clinical signs of pain and this could be dangerous, if inadequately managed. On the other hand, the use of nonsteroidal anti-inflammatory drugs is controversial and their application should be considered only to provide analgesia (Dowling et al. 2000).

The intra-lesional or peri-tendinous injection of HA has yielded conflicting results and it does not appear to show significant benefits.

The use of polyshulphated glycosaminoglycans and beta-aminoproprionitrile fumarate is potentially helpful, during the acute stages of tendinopathies. The beta-aminoproprionitrile fumarate is a toxic agent, found in the seeds of the plant *Lanthyrus odoratus*. It prevents the excessive fibrils cross-linking in the early stages of tendon repair, promoting the linearization of collagen fibres. However, it requires a strict and careful controlled exercises, and long-term data shows that only the 50% of treated horses return to their maximal athletic activity (Dowling et al. 2000).

Surgical therapies are invasive, expensive and sometimes dangerous, for all complications related to general anaesthesia and operation. The most commonly used techniques include: percutaneous tendon splitting, accessory ligament desmotomy, digital anular ligament desmotomy and the use of carbon fibres implants. Counter irritation was one of the most common methods employed in the horse for the treatment of chronic musculoskeletal injuries. By the time, it was demonstrated that firing has no benefits in tendon healing and it increase the incidence of peritendinous adhesions. It is now considered an inappropriate treatment, even if, there are still many supporters of it (Dowling et al. 2000, Spaas et al. 2012).

2. Innovative therapies

Since conservative therapies usually involve long period, with no significant improvement in tendon functionality, the use of regenerative therapies has been encouraged.

Regenerative therapy is an innovative medical field, whose aim is to stimulate the body's own repair mechanisms, in order to restore complete structure and function of damaged tissues and organs.

The broad field of regenerative medicine covers a variety of research areas, including stem cell therapies, growth factors, platelet-rich plasma, tissue engineering and low level laser irradiation.

2.1 Cell-based therapies

The limited ability of tendon to self-repair had encouraged researchers to develop cell-based strategies, in order to explore the natural endogenous system of tissue regeneration. Cell-based therapies are defined as the process of introducing new cells, in order to treat a disease

or regenerate damaged tissue, and it includes pluripotent stem cells, multipotent stem cells, and mature functional cells.

2.1.1 Definition of stem cells (SCs)

SCs are unique cells that have the capacity for self-renewal and to give rise different cell lineages that form mature adult tissues (Donovan and Gearhart 2001). SCs are able to form identical daughter cells, when performing symmetrical divisions, or more differentiated daughter cells, if asymmetrical division occurs (Blanpain and Fuchs 2009). Thanks to their proliferative nature, SCs are able to repopulate entire organs and regenerate tissues that normally heal with the formation of scar tissue, thus losing their original properties.

In order to better understand SCs origin and characteristics, a brief revision on embryology is advisable. After the fertilization of the oocyte, symmetrical divisions produce totipotent SCs, which are able to originate the embryo itself and the extra-embryonal tissues. Then, symmetrical and asymmetrical divisions form a blastocyst consisting of: i) the trophoblast and ii) an inner cell mass. The inner cell mass contains pluripotent embryonic SCs, which are able to proliferate, indefinitely, and differentiate into a wide variety of cell types, representative of the three embryonal germ layers: endoderm, mesoderm and ectoderm (De Vos et al. 2009; Donovan and Gearhart 2001; Li et al. 2006; Paris and Stout 2010). After gastrulation, multipotent stem cells develop from symmetrical and asymmetrical divisions. These cells have more specific tasks and a reduced plasticity than the pluripotent embryonic SCs, indeed, they are also called adult stem cells. The main function of the multipotent SCs is to regulate the inner body homeostasis, in order to replace those cells, which are damaged or death because of senescence or injury.

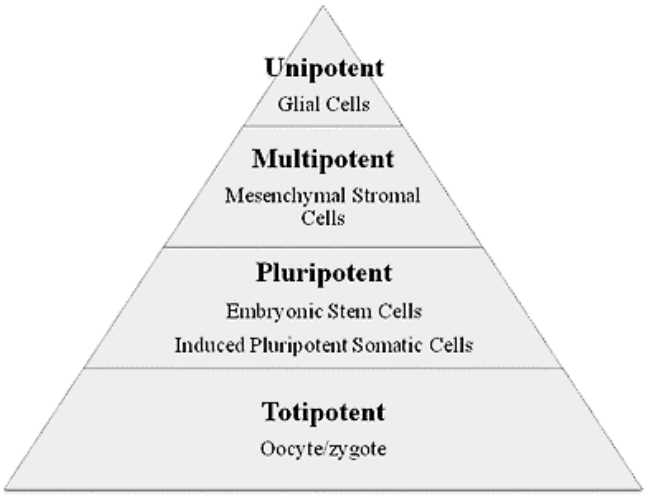

Figure 4: Stem cell hierarchy (Spencer et al. 2011)

2.1.2 Mesenchymal stem cells (MSCs)

Multipotent stem cells, which are able to differentiate into cells of the mesoderm germ layer, are called mesenchymal stem cells (MSCs). Since their discovery, their potential use in regenerative medicine has been studied with increasing interest. Actually, the use of adult multipotent MSCs is encouraged, because they do not show the collateral effect to form teratoma *in vivo*, differently from pluripotent embryonal SCs (Fong et al. 2010). In fact, MSCs do not elicit hypersensitivity reactions and they also have immunomodulatory effects (Ringdén et al. 2006). MSCs inhibit T-lymphocytes proliferation (Di Nicola et al. 2002), B-lymphocytes differentiation, immunoglobulin production (Corcione et al. 2006), macrophages activity (Ortiz et al. 2007), and dendritic cells maturation (Djouad et al. 2007). In the horse, the opportunity to use allogenic stem cells, without adverse reaction, has been described in several studies (Broeckx et al. 2014; Carrade et al. 2011a, Carrade et al. 2011b). MSCs have a fibroblast-like shape and they can be isolated from different sources such as, bone marrow (BM), adipose tissue, umbilical cord, umbilical cord blood, amniotic fluid, gingiva, periodontal ligament, and peripheral blood (PB). MSCs exhibit the ability to be committed into the cell lineages of the mesoderm layer and several studies have successfully proven their osteogenic, adipogenic, chondrogenic (Barberini et al. 2014; Spaas et al. 2013), myogenic (Martinello et al. 2010), and tenogenic differentiative potential (Kraus et al. 2013).

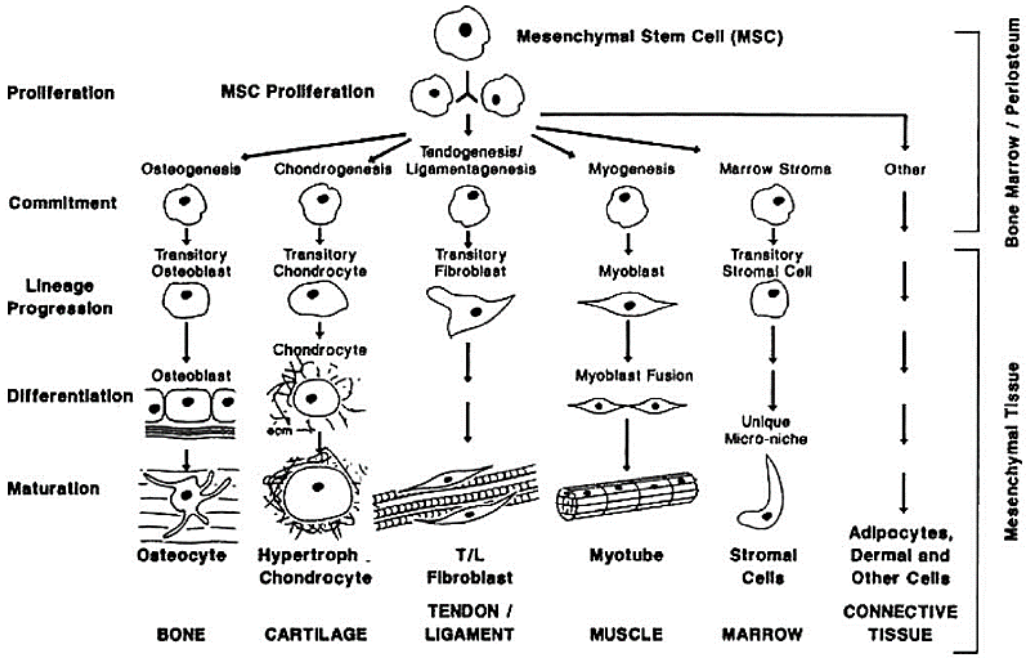

Figure 5: Schematic representation of differentiative potential of MSCs (Caplan, 2007)

In equine medicine, current MSCs therapies mainly use BM and PB-derived MSCs. Since the sample of BM-derived MSCs can be painful, risky and it requires a certain degree of competence, the use of PB-derived MSCs allows several advantages. In fact, the blood samples can be easily taken in a sterile manner, at the time of injury, and they provide a rapidly and a high accessible source of MSCs (Spaas et al. 2013).

Thanks to their great characteristics, PB-derived MSCs represent an innovative field of research, in the area of regenerative therapies for the treatment of musculoskeletal pathologies in horses.

2.1.3 Cluster of differentiation (CD)

The cluster of differentiation (CD) represents a system to identify molecules, mainly glycoproteins, which are expressed on the surface of the cell membrane. The CD is a method of cellular marking that usually is performed by means of flow cytometry. It permits cellular identification on the base of its membrane molecular expression, indeed, more than one CD marker is usually required, in order to identify a cell type.

In 2006, the International Society for Cellular Therapy (ISCT) has carefully determined the qualities that human cells must possess in order to be defined as MSCs. Human MSCs have to be positive for typical stem cell markers such as, CD73, CD90, CD105, and negative for adult blood cell markers like CD11b, CD14, CD34, CD45, CD79α, or CD19, MCH II and monocyte/macrophage (Mo/Ma) markers (Dominici et al. 2006).

No such guidelines have been described for equine MSCs, but several studies have been performed, in order to achieve a better characterisation of equine MSCs. In fact, considering the particular case of PB-derived MSCs, they were shown to be positive for CD13, CD29, CD44, CD51, CD90, CD105, CD117 and negative for CD34, CD45, CD79α, MCH II and Mo/Ma (Dhar et al. 2012; Martinello et al. 2010; Spaas et al. 2013).

2.1.4 The MSCs niche

The stem cell niche is defined as the micro-environment where SCs reside. It constitutes a basic unit of tissue physiology, it integrates signals and mediates a balanced response of stem cells with the needs of organism (Scadden 2006). This means that the specific interactions between MSCs and their micro-environment, determines whether or not, a SC remains quiescent, starts to proliferate, or differentiate into an adult cell (Kuhn and Tuan 2010). The niche is both physical and biological that is, factors such as, temperature, oxygen level,

relative humidity, surrounding cells, ECM, growth factors, and cytokines influence MSCs behaviour.

The understanding of that specific interactions, which exist between the niche and the residing MSCs, is fundamental to obtain the manipulation of the niche *in vitro*, in order to guide the further development of MSCs.

It has been described that MSCs can migrate at the injury site and release leukocyte chemokines important to attract immune cells and local MSCs, in order to heal the damaged tissue (Shi et al. 2010). The pro-inflammatory cytokines, which are present at the site of injury, influence MSCs behaviour, by increasing their proliferation and maintaining them at the undifferentiated state (Pricola et al. 2009).

Although various clinical applications of MSCs has been described, the exact *modus operandi* of SCs remains a question and their therapeutic effects are not well understood and characterized. It is generally accepted that the positive effects of SC therapy are due to the products they secrete, which enhance the metabolic function of other cells (Yang et al. 2013), even if, other studies reported a stable and uniform integration of MSCs in the injured site, indicating a sort of structural support (Martinello et al. 2012).

The possibility to understand, which factors alter MSCs behaviour *in vitro*, could be of vital importance to increase the efficacy of MSCs *in vivo*, in order to obtain the wanted commitment, improve the effectiveness of cell-based therapies, and increase the positive outcome of injured patients.

2.1.5 Use of MSCs for the treatment of equine tendinopathies

Once that the great *in vitro* potential of MSC has been discovered, the interest for regenerative medicine and in particular for cell-based therapy, increased exponentially. In horses, several *in vivo* studies have been performed, in order to better understand MSCs behaviour and effects after clinical application. The treatment of orthopaedic problems, in this specie, is one of the hottest topics, because of the large employ of these animals for sport competitions, their economic value, the high incidence of orthopaedic injuries and relapses.

The use of MSCs for the treatment of tendon injuries showed cheering results, compared to non-regenerative approaches, in several *in vivo* studies. The application of tenogenic induced MSCs for the treatment of desmitis of the equine accessory ligament, outcomes clinical and ultrasonographical positive results, with an improvement of about 80% on the ultrasound image, already 6 weeks after the intralesional injection (Beerts et al. 2013). SC therapy was applied also in a 13-year-old Belgian warmblood mare that showed lameness and swelling on

the right front leg. On ultrasound examination, heterogenic regions were noted in the mid-region of the suspensory ligament. Five weeks after the intralesional injection of SCs, the swelling had disappeared and the horse do not showed more signs of lameness. Moreover, the lesion was filled with tendon tissue, even if, at the edges was still noticeable some heterogeneity that disappeared after nine weeks. At six months' post-injection, this horse reached the original level of competition in show jumping, without more relapses (Spaas et al. 2011). Another case report demonstrated a positive evolution of proximal suspensory ligament desmitis, after the treatment with allogenic tenogenic-induced MSCs. The horse was submitted to two intralesional injections of tenogenic-induced MSCs and after 32 weeks, it went back to the previous performance level, with a total filling of the lesion on ultrasound (Vandenberghe et al. 2015). Smith et al. (2013) conducted a study on twelve career-ending horses, with naturally occurred SDFT injuries. The horses were treated with autologous BM-derived MSCs and after 6 months', the histological examination of tendons displayed an enhanced normalization in morphological and compositional parameters, than the untreated tendons (Smith et al. 2013). At last, the combined use of MSCs and platelet-rich plasma (PRP) was tested on experimentally injured deep digital flexor tendon (DDFT) of sheep. The results highlighted the predominant effect of MSCs on tendon healing, with enhanced tissue remodelling and improved structural organization, whereas, the combination of both treatments did not produce synergistic effects (Martinello et al. 2012).

All these studies represent only a little example of the great potential of MSCs. For all the aforementioned reasons, the improvement of regenerative medicine represents the key for the treatment of all that pathologies, which naturally do not heal with a *restitution ad integrum* of the tissue. Of course, more *in vitro* and *in vivo* studies must be performed, in order to better understand the correlation between MSCs and their niche, and be able to use regenerative therapies, routinely, for the treatment of tendinopathies in horses.

2.2 Growth factors (GFs)

The improvement of knowledge about tendon development and healing, has resulted in the investigation of various GFs as therapeutic agents in musculoskeletal healing. In fact, it is overall accepted that environmental factors are of vital important to control stem cell activity.

GFs are signalling peptides with various biological roles, including cell proliferation, differentiation, migration, and adhesion. They represent one of the most important molecules involved in healing processes, therefore, they can influence stem cells behaviour and

differentiation, with vital consequences for regenerative therapies progress. (Dahlgren et al. 2005).

Several growth factors have been investigated as playing a key role in tendon healing such as, basic fibroblast growth factor, transforming growth factor-beta, insulin-like growth factor-1, epidermal growth factor, vascular endothelial growth factor and platelet-derived growth factor (Tsubone et al. 2004). They can be produced by both intrinsic or extrinsic cells, they often have dose-dependent effects and work in synergy with other signalling molecules. In fact, a study demonstrated that the introduction of a single GF can modulate the expression of multiple GFs genes (Tang et al. 2014).

2.2.1 Basic fibroblast growth factor (FGF)

FGF is a signalling molecule required in the early stages of differentiation and an essential mediator for self-renewal in human stem cells.

Several studies have demonstrated the involvement of FGF in tendon healing by regulating cell growth and differentiation. In this regard, Chang et al. (1998) showed that tendons subjected to transection and repair, exhibited an increased mRNA expression of FGF, in both resident and migrating cells. FGF is able to stimulate cell proliferation and induce cell differentiation towards the tenogenic fate via the activation of the mitogen-activated protein kinase (MAPK), which includes ERK1 and ERK2 signalling pathways, both involved in tenogenic differentiation (Cai et al. 2013; Reed et al. 2014). In fact, the supplementation of FGF can increase the mRNA expression of TNC, collagen type I, collagen type III, fibronectin and alpha-smooth muscle actin. The microscopic evaluation of MSCs cultured with FGF supplementation resulted in a homogenous and dense ECM, with fibroblast-like and spindle shaped cells, supporting the key role of this GFs in tendon commitment (Chan et al. 2000; Gonçalves et al. 2013; Hankemeier et al. 2005; Molloy, 2003; Reed et al. 2014).

2.2.2 Transforming growth factor-β3 (TGF)

TGF is a well-known cytokine that regulates various cellular processes, including cell proliferation, differentiation, apoptosis, plasticity and migration (Zhao and Chen 2014).

TGF plays a key role during all phases of tendon healing, but its mRNA expression increases especially during the inflammatory phase (Fu et al. 2002; Molloy et al. 2003).

The supplementation of TGF in MSCs cultures *in vitro* is able to induce tenogenic differentiation by up-regulating the mRNA expression of TNC, Collagen type I, type III, COMP, tenomodulin and thrombospondin, which are all ECM components of tendon tissue

(Barsby et al. 2014; Dowling et al. 2000). The increased synthesis of this constituents is correlated with a concomitant decrease in cell number, via the cyclin-dependent kinase inhibitors, supporting the key role of TGF for tendon commitment (Klein et al. 2002; Molloy et al. 2003).

2.2.3 Insulin-like growth factor-1 (IGF)

IGF-1 is an important mediator in all phases of wound healing and it ensures a rapid response to injury especially during the inflammatory and repair stages. IGF is a versatile and widespread molecule with numerous tasks in tendon healing. It enhances tendon repair, promotes cell proliferation, and increases collagen type I and fibronectin synthesis. (Dahlgren 2005; Dowling et al. 2000; Hansen et al. 2013; Molloy 2003).

2.2.4 Epidermal growth factor (EGF)

EGF is a powerful mitogen. It participates in MSCs, fibroblast proliferation, and it is also involved in the initial phase of tendon healing. (Gonçalves et al. 2013). An *in vitro* study showed that EGF had a stimulatory effect on tenoblast migration and this might enhance tendon repair, by delivering immature cells at the site of injury (Jann et al. 1999).

2.2.5 Platelet-rich plasma (PRP)

The use of a single GF often may not be sufficient to improve the quality of tendon healing. Actually, one of the most explored GF-based therapy in equine medicine is PRP, an autologous concentrate of platelets in a small volume of plasma. PRP is used as a source of GFs, in fact, platelets α-granules are rich in GFs such as, TGFβ, IGF, platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF). These GFs may play a central role in tendon healing, reducing inflammation and scar tissue production. There are many experimental evidences that PRP can enhance angiogenesis, recruitment, proliferation and differentiation of cells involved in tissue regeneration (Anitua et al. 2015, Martinello et al. 2012). The use of PRP *in vitro,* resulted in increased cell proliferation and collagen deposition. In collagenase-induced SDFT lesions, studies showed successful results in the treatment of both acute and chronic injuries, with histological increase of fibroblastic activity and better organization of collagen fibres (Maia et al. 2009; Spaas et al. 2012). PRP was applied *in vivo* also in combination with MSCs, but the results are controversial. At present, the clinical efficacy of PRP is still debated because too many factors can influence the result

such as, the seriousness of the injury, the healing phase, the PRP preparation method, and of course the individual (Abate et al. 2012).

2.3 Low-level laser therapy (LLLT)

The clinical application of low levels of visible light for reducing pain, inflammation, and promoting tissue healing, has been known for almost forty years, since the invention of lasers. Despite many reports of positive outcomes, the clinical use of LLLT remains controversial. This is mainly due of two reasons: first, the biochemical mechanisms underlying the laser positive effects are incompletely understood, second the opportunity to change several parameters such as, wavelength, fluency, power density, and timing, enlarges the possibility to work in several different conditions and thus, to obtain a wide range of results.

It has been found that exists an optimal dose of light for each tissue and purpose, in fact doses lower, or more significantly higher, than this optimal value have a diminished therapeutic effect.

2.3.1 Physical mechanism:

According to quantum mechanical theory, light energy is composed of photons, or discrete packets of electromagnetic energy. The energy of an individual photon depends only on its wavelength, therefore the energy of a “light-dose” depends only on the number of photons and on their wavelength.

Photons delivered into living tissues can either be absorbed, or scattered. Scattered photons will escape from the tissue, whereas, the energy coming from absorbed photons will be conserved and internally converted into heat, fluorescence, or it can activate a broadly number of biochemical processes (Hamblin, 2006).

2.3.2 Biochemical mechanism:

The first law of photochemistry states that light must be absorbed, so that photochemistry can occur. This is a simple concept, but it is the basis for performing photo-biological experiments correctly. The optical properties of tissues are of fundamental importance because, both the absorption and the scattering of the light, are wavelength dependent. There is an ideal “optical window”, between 600 and 1200 nm of wavelength, in which laser radiations are absorbed by more tissue elements and this increases the opportunity for light to be absorbed (Hamblin, 2006).

It was suggested that the components of the respiratory chain were responsible for light absorption. Actually, it is known that cytochrome c oxidase is the primary photoacceptor, in fact, it absorbs light of wavelengths between 630 nm and 900 nm. The increased amount of energy in the respiratory chain leads to an acceleration of electron transfer, and thus ATP production. Moreover, changes in the redox state induce the activation of numerous intracellular signalling pathways and of course, cells at different state of development have distinct redox states (Hamblin, 2006).

2.3.3 *In vitro* and *in vivo* application of LLLT:

The effects of LLLT can vary considerably between cells, but in general, *in vitro* studies showed that the response after LLLT is an increased cell metabolism, proliferation, and synthesis of proteins. Sperandio et al. (2015) showed an enhanced *in vitro* proliferation and a faster *in vivo* maturation of human keratinocytes irradiated with a wavelength of 660 nm and 3, 6 and 12 J/cm^2 energy laser densities. Similar results have been reported in another study, in which laser energy densities of 5J/cm^2 were applied on wounded cells. The results showed an increased cell migration across the wound margin, fibroblast proliferation, and cell viability. Instead, higher doses of 10 and 16J/cm^2 energy densities, were characterized by a decrease in cell vitality and proliferation, because of a significant damage of the cell membrane and DNA (Hawkins and Abrahamse 2006). Several studies support the hypothesis that 5J/cm^2 is the optimal fluence to apply *in vitro*, in order to enhance cell proliferation and differentiation. In fact, it has been shown that an energy density of 5J/cm^2 dramatically enhances MSCs proliferation and differentiation, especially when cells are cultured in appropriate inducing medium (Hou et al. 2008). Similar results were obtained by de Villiers et al. (2011) who exposed human adipose tissue-derived MSCs to a 636 nm diode laser and a fluence of 5J/cm^2. Cell were induced to differentiate into smooth muscle cells by using retinoic acid. The results showed a significant increase of cell proliferation and viability after 72h post-irradiation, whereas a longer period of time (14 days) was necessary to induce MSCs differentiation.

Moreover, a recent study was performed on tenocytes *in vitro*. The application of LLLT (904 nm wavelength and 1J/cm^2 energy density) revealed a significant increase in cell proliferation and an up-regulation of collagen type I and TGF-β1 expression (Chen et al. 2015).

The use of LLLT has been proposed also in physical medicine, in order to accelerate recovery after musculoskeletal injuries and reduce the inflammatory phase. Studies showed that the application of LLLT on muscle, bone, and tendon cells can increase not only cell

proliferation, but also cell differentiation. The opportunity to increase cell differentiation, by the combined use of stem cell-based therapies and LLLT, can be of great help for the treatment of orthopaedic injuries, which have a reduced regenerative capacity such as, tendinopathies.

Since, in tendon injuries there is an increased concentration of pro-inflammatory cytokines, a study was conducted on forty-two male rats with collagenase-induced tendinitis. A LLLT was performed (wavelength of 780 nm; energy density of 7,7J/cm^2; 75s exposure) and the results showed a decrease in IL-6, COX-2, TGF-β, and TNF-α expression, in both acute and chronic inflammatory phases. These results suggest the therapeutical effect of LLLT and encourage the application of this protocol, for the management of pain during tendinopathies (Pires et al. 2011).

An interesting *in vivo* study was conducted on collagenase-induced tendinitis of the DDFT in sheep. The sheep was chosen as a model, since it shares a lot of similarities with the connective tissue of humans and equines flexor tendons. Sheep were divided into two groups and treated for 7 days with LLLT doses of 5J/cm^2 and 2,5J/cm^2 respectively. Clinical and histological evaluations demonstrated that the dose of 2,5J/cm^2 resulted in better improved of anti-inflammatory effects, significant decrease in fibroblasts number, low vascularity, better collagen fibre-alignment, and ECM organization than the 5J/cm^2 group (Iacopetti et al. 2015). This study further confirms that exists an optimal dose for each tissue and the application of lower, or higher doses, can produce null or even dangerous effects.

GENERAL AIM OF THE STUDY

The aim of the present study was to investigate the *in vitro* combined effect of growth factors and a low-level laser irradiation on peripheral blood (PB)-derived mesenchymal stem cells (MSCs).

Tendon injuries are one of the most common orthopaedic problems in equine athletes and they often compromise the return at the same performance level. Tendinopathies may result from an acute trauma or more often from chronic overstrain. In both cases, the tendon will heal with the formation of scar tissue, which results in a higher content of collagen type III, reduced strength and elasticity.

The *restitution ad integrum* of the damaged tissue is the goal for regenerative therapies, which includes stem cell based therapies, the use of growth factors and low-level laser therapy.

Moreover, a more recent approach is the tenogenic induction of MSCs before their clinical use, in order to increase the regenerative potential of MSCs.

This project focussed on the investigation of the *in vitro* combined effect of basic fibroblast growth factor, transforming growth factor-β3, insulin growth factor-1, epidermal growth factor, and low-level laser irradiation on equine PB-derived MSCs. Different combinations of these growth factors were tested, with and without the low-level laser irradiation, in order to evaluate the best culture conditions for tenogenic differentiation of MSCs. At the end of the culture, the relative expression of Early Growth Response Protein-1, Tenascin C and Decorin was measured by real-time PCR, since they are all crucial genes involved in tenogenesis and tendon healing.

MATHERIALS AND METHODS

1. Animal selection and management

The animals, chosen for the study, were four adult trotter mares of five, six and seven years old. The mares were coming from the same stable and were placed in a big paddock at EquiTom, Equine Clinic, in Belgium. The horses were healthy, vaccinated, and de-wormed. They were fed twice a day with hay, once with concentrate, and water always ad-libitum. The four donor horses were left in the paddock for one week, without stress or manipulations, in order to let them get used of the new environment.

Figure 1: The horses of the study, stabled at EquiTom Equine Clinic, in Belgium.

2. Isolation of the putative PB-derived MSCs

After one week of horses' acclimatization, twenty ml of blood were collected, from the external jugular vein, of each animal. The blood was taken in a sterile manner and placed into five sterile EDTA (Ethylene-diamine-tetraacetic acid) tubes of 4 ml. The blood samples were transported to the GST laboratory, at room temperature (RT), within four hours of sampling. Then, the blood was placed into two sterile 15 ml tubes and centrifuged at RT for 20 min and 1000g. The high G-force is important to separate the buffy coat, which contains white blood cells and stem cells.

The buffy coat was collected, diluted 1:2 in a phosphate buffered saline (PBS) solution, and gently laid on an equal amount of Percoll gradient (density 1.080 g/ml; GE Healtcare). Percoll is a colloidal silica particles suspension, which lets the higher isolation of stem cells from red blood cells, platelets, and plasma.

The solution with Percoll and buffy coat was centrifuged for 15 min at RT and 600g. After this process, it was possible to distinguish 4 phases: the upper containing plasma, an opaque interphase containing MSCs and mononuclear white blood cells, the Percoll solution, and at the bottom the pellet, consisting of red blood cells, granulocytes, and platelets.

The interphase containing the putative MSCs was collected and, in order to obtain a better isolation of cells from plasma, it was diluted 1:2 with PBS and centrifuged for 10 min at 200g. This passage was repeated 3 times. The upper part was thrown away, while the sediment re-suspended in an equal amount of PBS.

At last, the pellet was suspended in 12 ml of expansion medium prepared as follow: Dulbecco's Modified Eagle Medium (DMEM, Euroclone ECM0728L), 20% calf serum (FCS, Euroclone ECS0180L), 1% antibiotics/antimycotics (Euroclone ECB3001D) and 10^{-11} M dexamethasone (Sigma Aldrich).

3. Culture of the PB-derived MSCs

The putative MSCs were seeded in T75 flasks with the expansion medium previously described. The medium was refreshed twice a week and cells cultured at 37 °C with 5% of CO_2.

At 70% of confluency, the flask was changed for the next passage. First, the old medium was removed and the flask washed with 10 ml of PBS. In order to detach the cells from the flask, 6 ml of 0,25% Trypsin EDTA (Euroclone ECB3051D) were added and the flask was

incubated for a maximum of three minutes at 37 °C and 5% of CO_2. The incubation is important because trypsin is a protease and it needs culture conditions to perform its tasks.
Once that the cells were floating, 6 ml of expansion medium were added, in order to stop the action of trypsin by calf serum anti-proteases.
The suspension was centrifuged for 8 minutes at 300g. The supernatant was removed and the pellet, containing the cultured MSCs, re-suspended in 12 ml of new and fresh medium.
Finally, the cells were seeded in a new T75 flask and further cultured for the next passages. This procedure was repeated for each passage, from 0 to 10 ($P_{0 \rightarrow 10}$).
The expansion medium was supplemented with dexamethasone only at P_0, in order to enhance glucose metabolism and help the proliferation of cells at the beginning.

4. Population doubling time (PDT)

At each passage, cell count was performed, in order to seed the same amount of cells and calculate population doubling time (PDT).
PDT was calculated from P_1 to P_{10} with the following formula (Hoynowski et al. 2007):

- PDT= cell culture time (T)/cell doubling time (CDT)

T was expressed as days from N_i to N_f, where N_i is the initial number of cells and N_f is the final number of cells, for each passage. CDT was obtained with the following formula:

- $CDT= \ln(N_i/ N_f)/\ln(2)$

5. Flow cytometry

In order to achieve immunophenotypic characterization of undifferentiated PB-derived MSCs, the expression of several MSCs markers was evaluated by flow cytometry.
At passage P_5 and P_{10}, the putative PB-derived MSCs, cultured in expansion medium, were sent to GST-ANACURA laboratory and tested for stem cell Cluster of Differentiation (CD) markers CD29, CD44, CD90, and for adult blood cell (negative) markers CD45, major histocompatibility complex type II (MHC II), and monocyte/macrophage marker (Mo/Ma).

Per series, $2x10^5$ cells were labelled using the following panel of primary antibodies: CD29-APC (TS2/16, Biolegend), CD44-FITC (CVS18, AbD Serotec), CD90 (DH24A, VMRD), CD45-PeCy5.5 (F10-89-4, AbD Serotec), MCHII-PE (CVS20, AbD Serotec), and Mo/Ma marker-Alexa 488 (MAC387, AbD Serotec).

In general, cells were incubated for 15 min, in ice and dark with primary antibodies and then washed twice in DMEM. A second incubation, in ice and dark for 15 min, was perfomed only on CD90 positive cells, in order to label them with fluorocrome PE-linked antibody IgG1-PE-Cy7 (Biolegend).

Control isotypes were run for each marker, in order to define the threshold at 0-1% of positive signals. The samples were analysed on a FACSCanto II (BD Biosciences) instrument, equipped with a 488 nm solid state and a 633 nm HeNe laser. The optimal settings for the MSC were determined by Compbeads Plus beads. The performance of the flow cytometer was checked on a daily basis by Cytometer Setup & Tracking beads (BD Biosciences). A minimum of 10.000 MSCs were used for flow cytometric evaluation and the data were analyzed with the FacsDiva software.

Figure 2: The image shows FACSCanto II (BD Bioscences), the instrument used to perform flow cytometry.

6. Addition of growth factors (GFs)

In order to evaluate the effects of medium composition on MSCs tenogenic differentiation, several combinations of basic fibroblast growth factor (FGF), transforming growth factor-β3 (TGF), insulin-like growth factor-1 (IGF) and epidermal growth factor (EGF) were tested.

When PB-derived MSCs reached P_5, cells were collected, suspended in 6 ml of expansion medium, and seeded in T25 flasks, with a concentration of 1500 cell/cm^2.

The expansion medium was supplemented with the following combinations of GFs (Sigma-Aldrich): FGF, FGF + TGF, FGF + IGF, and FGF +EGF. All these GFs were added at the concentration of 10 ng/ml.

Since expansion medium (Exp) served as negative control, five culture conditions were tested for each horse, in duplicate, for a total of forty T25 flasks (Table 1).

Cells were cultured in T25 flasks for five days at 37 °C and 5% CO_2. The medium was refreshed at day three.

				
Exp	FGF	FGF + TGF	FGF + IGF	FGF + EGF

Table 1: Expansion medium was supplemented with several combinations of growth factors, obtaining five culture conditions, which were tested in duplicate, for each horse.

7. Low-level laser technology (LLLT)

The PB-derived MSCs, cultured in supplemented media as previously described, were divided in two equal groups of twenty T25 flasks for each, in order to test, in one group (Group 2), the combined effect of GFs and a low-level laser irradiation (LLLT) on MSCs proliferation, viability and tenogenic differentiation (Table 2).

The first irradiation was performed 24 hours after seeding. A dose of 5J/cm^2 was applied daily, for 2 min by the same operator, with continuous and pulsed emissions emitted by a single handpiece (ASAlaser, M6, handpiece). The radiation was produced by an InGa(Al)As

diode laser with 2 cm spot size. The wavelength of continuous and pulsed emission was 660 nm, and 905 nm, respectively.

The PB-derived MSCs, cultured in expansion and supplemented media, were irradiated from day two to five, after the seeding.

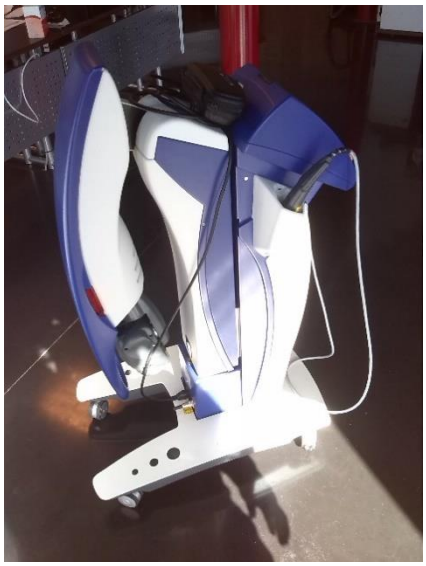

Figure 3: The picture shows the instrument used to irradiate the flasks (ASAlaser, M6).

Group 1: Non-irradiated flasks				
				
Exp	FGF	FGF + TGF	FGF + IGF	FGF + EGF

Group 2: Irradiated flasks				
				
Exp + LLLT	FGF + LLLT	FGF + TGF + LLLT	FGF + IGF + LLLT	FGF + EGF + LLLT

Table 2: Schematic representation of the treatments. Five culture conditions were tested for each horse, in duplicate, for a total of forty T25 flasks. The flasks were divided in two equal groups and one group was submitted to LLLT.

8. Cell count

Five days after seeding, the PB-derived MSCs cultured in T25 flasks, with expansion and supplemented media, and treated with LLLT, reached 70% confluency.

The old medium was removed and each flask was washed with PBS.

2,5 ml of 0,25% Trypsin EDTA (Euroclone ECB3051D) were added, in order to detach the cells from the flask, following the procedure descripted at point 3.

Then, cells were collected from each flask and counted in a Burker chamber. Alive and dead cells were distinguished by the use of Trypan Blue staining (Sigma B-7021). In fact, Trypan blue goes inside the cytoplasm of damaged cells and allow to establish the effects, of each treatment, on cell proliferation and viability.

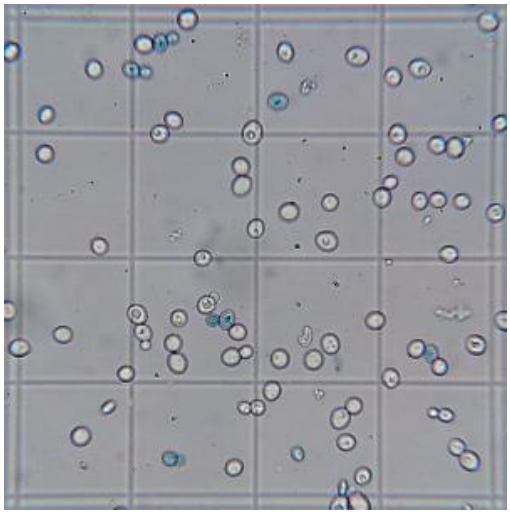

Figure 4: Appearance of MSCs when stained with trypan blue, in a burker chamber.

9. Cell freezing

After cell count, $5x10^6$ cells were collected from each flask and frozen at -80 °C, in order to stop cell proliferation and differentiation. The freezing medium was prepared with 90% expansion medium and 10% Dimethyl sulfoxide (DMSO). DMSO is a cryoprotective agent, which allows slow cooling rate and reduces ice crystal formation that can cause cell damage and death.

Once the samples, containing the PB-derived MSCs, cultured in several media conditions and treated with LLLT, were frozen, they were shipped to the department of Comparative Biomedicine and Food Science of the University of Padua for consecutive analysis.

10. RNA isolation

The RNA of the PB-derived MSCs was isolated, in order to evaluate the effects of GFs alone, or in combination with LLLT, on mRNA expression of genes involved for tenogenic differentiation.

In order to perform total RNA extraction, the samples were de-frozen and centrifuged at 800 rpm for 5 minutes. The supernatant containing the freezing medium was eliminated, while the pellet containing the PB-derived MSCs, was suspended in 1 ml of TRIzol reagent (Life Technologies). Trizol is a substance, which cause cell lysis and nuclear-protein complexes dissociation. The samples were incubated for 5 min, at RT, and after this period of time, the RNA was in solution with other organic molecules.

In order to isolate RNA, 0,2 ml of Chloroform (Sigma) were added. The solution was shuffled for 15 seconds and further incubated for 5 min at RT. Then, it was centrifuged at 12000g, for 15 min, at 4 °C, and after this passage, it was possible to distinguish a pellet, containing proteins and lipids, and interphase containing DNA molecules, and the upper phase containing the RNA.

The upper phase was collected and 0,5 ml of Isopropanol (Sigma) were added, in order to induce RNA precipitation. The samples were incubated for 10 min and then centrifuged at 1200g for 10 min.

At this point, the supernatant was eliminated and the pellet, containing the RNA of the MSCs, was re-suspended in 1 ml of Ethanol 70% (Sigma). Ethanol is important to wash RNA from isopropanol. The solution was centrifuged twice, at 7500g, for 5 min, and the supernatant gently removed from the pellet containing the purified RNA.

The samples were left for 1 hour at RT, in order to allow ethanol evaporation.

Finally, the total RNA was quantified. The pellet was re-suspended in 20 µl of distillate water and RNA quantified on a Nanodrop (Thermo Scientific) spectrophotometer.

11. cDNA retro-transcription

In order to perform real-time PCR, a complementary DNA (cDNA) was synthetized, starting from isolated mRNA of the PB-derived MSCs.

At the beginning, the RNA was purified from DNA residues, which could be present from the isolation process. Hence, from each sample, 2 µg of RNA were added to 1 µl of Buffer 10x (Life technologies), 1 µl of DNase (Life technologies) and the necessary amount of distilled

water to obtain 10 µl in total. Buffer 10x contains Mg^{2+} ions, which allows DNase to work correctly and degrade DNA.

The solution was incubated at RT and after 15 min, 1 µl of EDTA (Life technologies) was added, in order to chelate Mg^{2+} ions and stop DNase activity. Moreover, the solution was incubated at 65 °C for 10 min and then, for 1 min at 4 °C, with the purpose to create a thermal shock and stop definitely DNase activity.

At this point, the single strands of purified mRNA were ready to be copied in cDNA.

1µl of random primers and 1 µl of dNTP (Life technologies) were added to the solution, which was further incubated for 1 min at 65 °C and 1 min at 4 °C. Afterwards, 1 µl of Superscript (Life technologies), 1 µl of RNase OUT (Life technologies), 2 µl of DTT (Life technologies), and 4 µl of buffer 5x (Life technologies) were added to the samples. Hence, the solution was incubated for 10 min at RT and then, for 50 min at 42 °C. In this phase, Superscript (Reverse Transcriptase) synthetises the cDNA, starting from primers and then, it creates the helix using the four mixed deoxy-nucleotides (dNTP). Buffer 5x is important to allow superscript works correctly. RNase Out is a RNase inhibitor, which protects RNA from degradation and DTT (Dithiothreitol) works protecting RNase OUT from oxidation, because it is a reducing agent.

Since Superscript creates strands of cDNA, which are still linked to the original mRNA, the samples were incubated for 15 at 70 °C. This is the last passage for cDNA retro-transcription, but it is important to destroy the single strands of RNA, in order to obtain purified cDNA, which can be used for real time-PCR.

12. Real time-PCR

Real time-PCR (or Real time-Polymerase chain reaction) was performed on purified cDNA, after the treatment with several combinations of GFs and LLLT.

Real time-PCR is a laboratory technique of molecular biology, which allows the amplification and accumulation of cDNA *in vitro* and thus, the contemporary quantification of selected genes expression. In order to evaluate the effects of GFs and LLLT for tenogenic differentiation of PB-derived MSCs, the following genes were tested: Early growth response protein-1 (EGR1), Tenascin C (TNC) and Decorin (DCN). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene, with the purpose to obtain a relative quantification of the target genes.

Each sample was tested in triplicate. Untreated MSCs were used as calibrator sample and water as negative control. Real time-PCR was performed using the ABI 7500 Real Time-PCR system (Applied Biosystem), constituted of a thermocycler for a 96-well plate.

In order to test all samples, eight 96-well plates were arranged. For each well, a total volume of 30 µl was added. The solution was prepared as follow: 15 µl of Buffer (Life technologies), 10,2 µl of distilled water, 0,9 µl of primer Forward (diluted 1:10), 0,9 µl of primer Reverse (diluted 1:10), 3µl of cDNA (diluted 1:10) or 3 µl of distilled water for negative control.

PCR primers were designed using Primer Express 3.0 software (Applied Biosystem). Forward and Reverse sequences are shown in Table 3.

Table 3: Primers used for the quantitative Real time-PCR analysis

Gene	5' Forward primer 3'	5' Reverse primer 3'
GAPDH	GCATCGTGGAGGGACTCA	GCCACATCTTCCCAGAGG
EGR1	CGGACATGACAACAACCTTTTC	CCTTTGCCCTTTCCTTTAGCA
DCN	GAGAGCTGCGTGTCCATGAG	AGTGGGTTGGTGCCAAGTTC
TNC	CATCCACCATCATCCAGGAGTT	TGGCAAACACACGGATGAA

The possibility for Real time-PCR to detect and quantify the amplification of cDNA, is due to SYBR green, a fluorescent molecule included in the buffer. SYBR green is a DNA-binding dyes and it yields a proportional increase in the amount of amplified DNA and fluorescence. The cycle number, in which enough amplified product determines a detectable fluorescent signal is called Ct.

A $2^{-\Delta\Delta Ct}$ method was used to analyse the data and determine the relative expression of the target genes to GAPDH and calibrator sample.

The average number of Ct, for each gene and treatment, was normalized to Ct expression of GAPDH as follow:

- $\Delta Ct_{(test)} = Ct_{(target\ genes)} - Ct_{(GAPDH)}$
- $\Delta Ct_{(calibrator)} = Ct_{(target\ calibrator)} - Ct_{(GAPDH)}$

Finally, the relative expression of target genes was normalized to calibrator as follow:

- $\Delta\Delta Ct = \Delta Ct_{(test)} - \Delta Ct_{(calibrator)}$

13. Statistical analysis

Normally distributed data were expressed as the mean ± standard deviation. Normality of the data was confirmed using the Kolmogorov-Smirnov test (α=5%). Statistical analysis was performed using the paired Student t test (SPSS software, version 11.0, SPSS, IBM). The level of statistical significance was set a $P \leq 0.05$ for all analyses.

RESULTS

1. Flow cytometry analysis of isolated PB-derived MSCs

The cellular identification of the putative PB-derived MSCs was performed by using flow cytometry. Each sample, cultured in expansion medium, was tested at passage 5 (Figure 1A) and passage 10 (Figure 1B). The results showed a uniform positivity for stem cell markers CD29, CD44, CD90 and negativity for blood cell markers CD45, MHC II, Mo/Ma, at both passages.

Control isotypes were tested for each marker, in order to define the threshold.

The relative expression of positivity and negativity, for each CD tested, for each horse, is shown in table 1.

These results indicated a successful isolation of MSCs from peripheral blood of four healthy horses, thus increasing the validity of consecutive analysis.

	#1		#2		#3		#4		Average ± SD	
	P_5	**P_{10}**	**P_5**	**P_{10}**	**P_5**	**P_{10}**	**P_5**	**P_{10}**	**P_5**	**P_{10}**
CD44	87.8	99.3	98.4	99.8	89.7	99.7	92.2	98.5	92.0±4.0	99.3±0.5
CD90	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0±0.0	100.0±0.0
CD29	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0±0.0	100.0±0.0
Mo/Ma	0.8	4.8	0.3	0.6	0.9	0.0	1.1	0.1	0.8±0.3	1.4±2.0
MHCII	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0±0.0	0.0±0.0
CD45	2.2	0.7	0.8	1.2	13	1.3	2.4	1.6	1.7±0.6	1.2±0.3

Table 1: The table shows the relative expression of the CDs tested, for each sample, at P_5 and P_{10} and the average ± standard deviation (SD). Samples show a uniform positivity for antibodies against stem cell markers CD29, CD44, CD90, and negativity for blood cell markers CD45, MHC II and Mo/Ma.

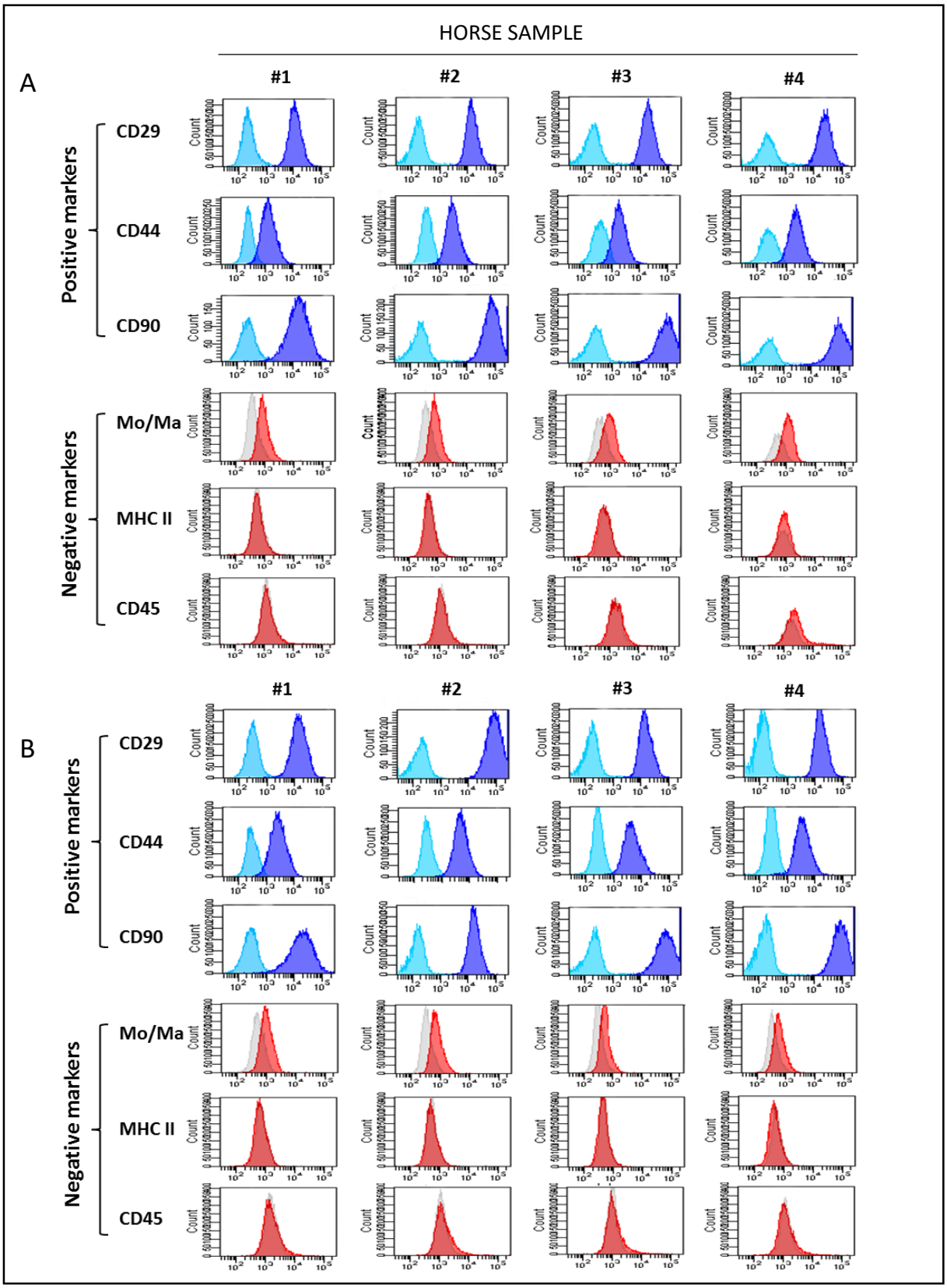

Figure 1: The histograms show the relative number of cells versus mean fluorescence intensity. Samples were tested at P_5 (A) and P_{10} (B) and at both passages, showed a positivity for stem cell markers CD29, CD44, CD90 *(in dark blue)*, and negativity for blood cell markers CD45, MHC II and Mo/Ma *(in red)*.

2. Population doubling time of isolated PB-derived MSCs

Population doubling time (PDT) was calculated, in days, for each sample, from passage 1 to 10 ($P_{1\rightarrow10}$), in order to determine the growth efficiency and proliferation rate of the PB-derived MSCs, cultured in expansion medium.

After an initial lag phase, MSCs divided rapidly at all further passages tested. PDT was relatively constant, starting from P_3 to P_{10} ($P_{3\rightarrow10}$) (Figure 2).

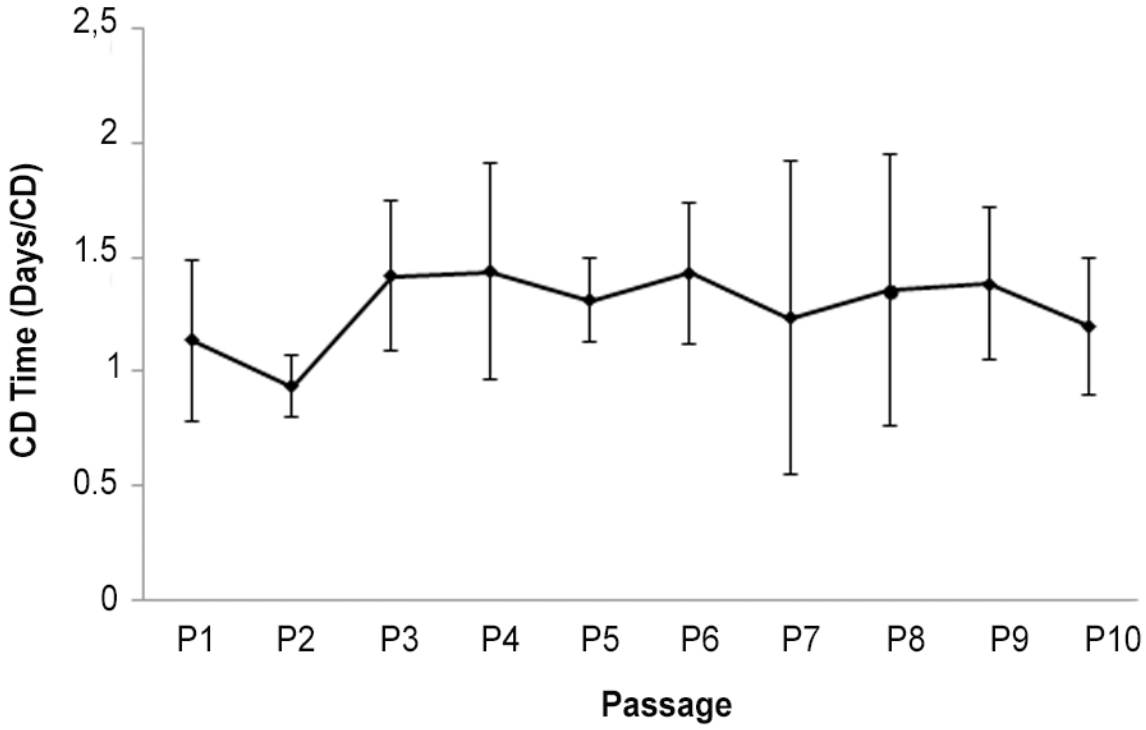

Figure 2: Population dubling time was calculated from P_1 to P_{10} of PB-derived MSC. The graphic shows the average cell kinetics of four horses. Error bars indicate ± standard deviation.

3. Cell proliferation and viability of PB-derived MSCs treated with GFs and LLLT

Figure 3 shows the average number ± standard deviation (SD) of alive and dead cells, which were counted at the end of each treatment with GFs and LLLT. It is shown that only the combination of FGF + TGF influenced cell proliferation, causing a significant decrease in cell number ($p \leq 0,05$), in both irradiated and non-irradiated flasks. On the other hand, the addition of FGF, FGF + IGF, or FGF + EGF, with and without LLLT, did not modify cell growth, compared to control.

All treatments showed the same cytotoxicity, which was rather enhanced than control medium, but not significantly high in none of the treatments.

These data indicate that TGF was the only growth factor able to cause a significant decrease in cell proliferation. However, it did not affect cell mortality, which remained rather constant among treatments.

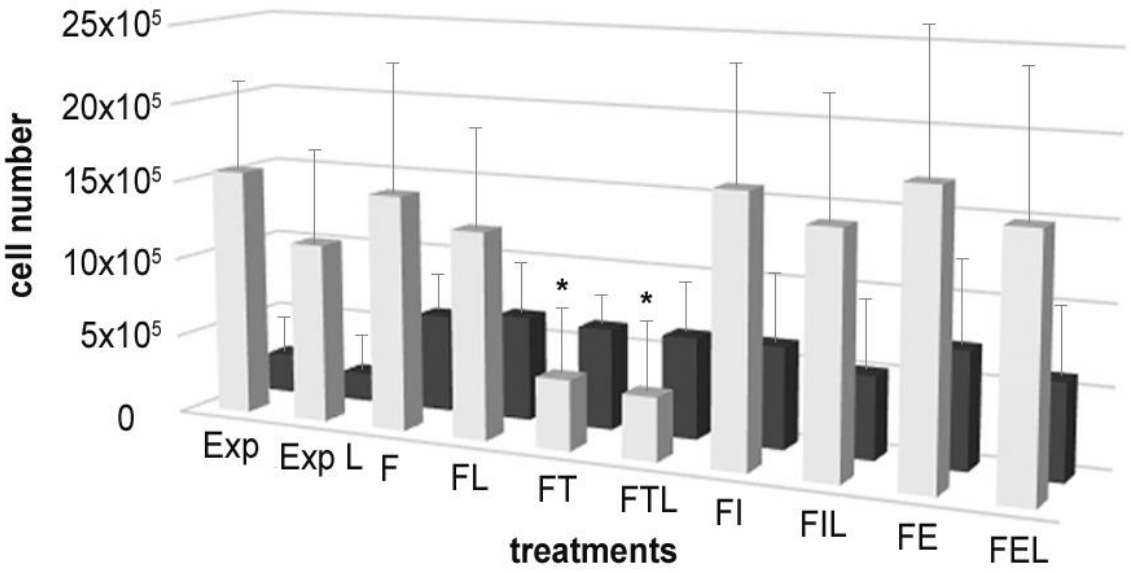

Figure 3: The histogram shows the average ± SD number of cells counted after each treatment. The number of alive cells is represented by light grey bars, whereas, the number of dead cells is indicated by dark grey bars. The treatments did not modify cell mortality but influenced cell proliferation, which was significantly decreased when cells were cultured with TGF, with and without LLLT.

4. mRNA expression of EGR1, TNC and DCN

In order to evaluate the best culture conditions for tenogenic differentiation, the relative gene expression of Early Growth Response Protein-1 (EGR1), Tenascin C (TNC) and Decorin (DCN) was measured by Real time-PCR, since they are all crucial genes involved in tenogenesis.

The relative expression of EGR1 and DCN increased significantly in presence of FGF + TGF, both with and without LLLT.

The relative expression of TNC increased when PB-derived MSCs were cultured in supplemented media with FGF +TGF, but the increase became statistically relevant ($P \leq 0,05$) only when this arrangement was combined with LLLT (Figure 4).

The addition of FGF alone, or in combination with IGF, or EGF, did not show significant increasing trends in comparison with the control (Exp). Cells treated with LLLT alone, or in combination with GFs, did not show a significant increase in the expression of target genes, except for TNC, which was up-regulated only when cells were cultured with FGF + TGF and treated with LLLT.

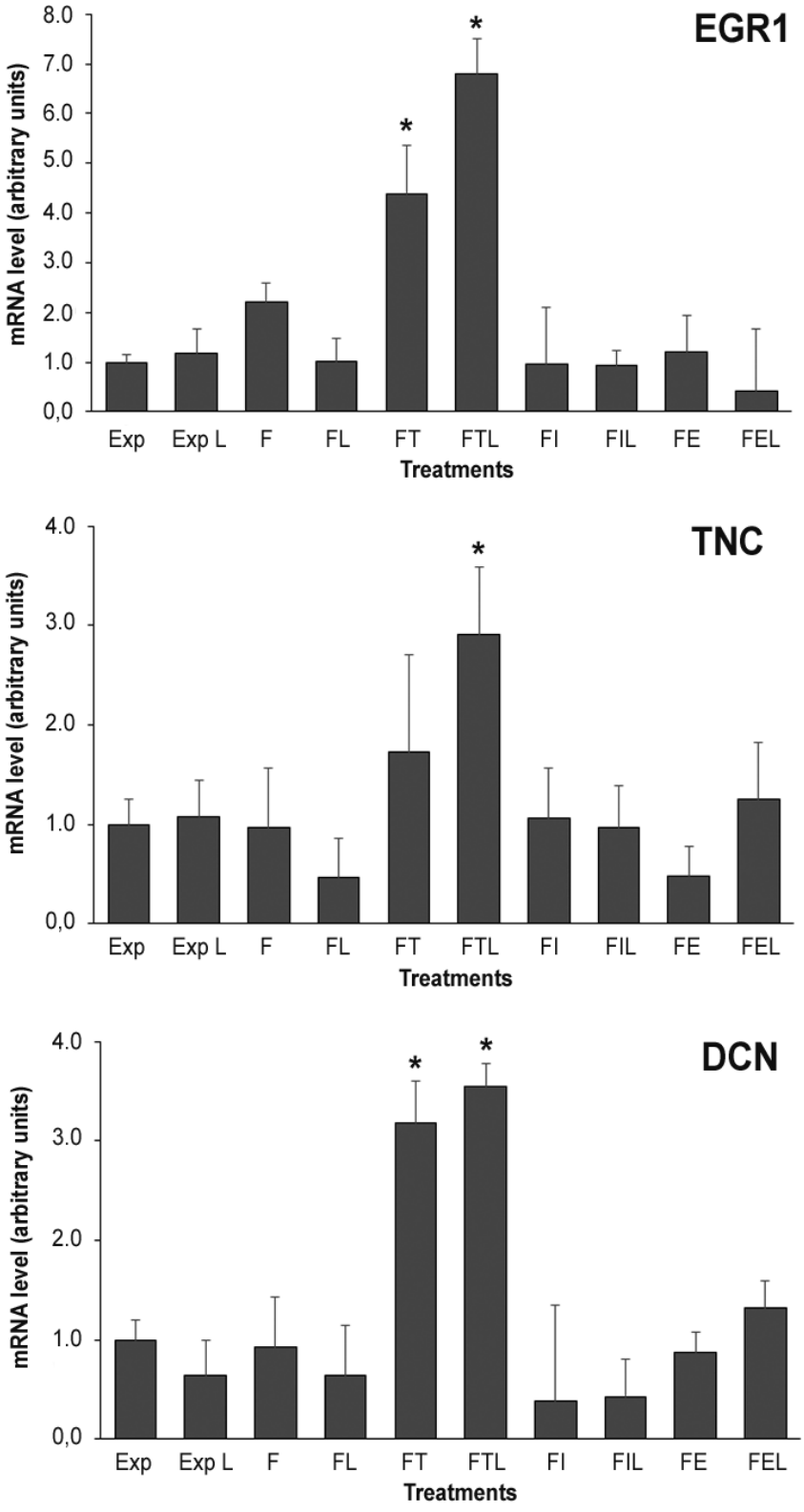

Figure 4: The histograms show the average mRNA expression of EGR1, TNC and DCN in PB-derived MSCs after five days of medium conditioning and four days of LLLT. The relative expression of the target genes was measured by Real time-PCR, GAPDH was used as housekeeping gene and cells cultured in expansion medium (Exp) served as calibrator. Asterisks indicate statistical treatments.

DISCUSSION

Tendon injuries are one of the most common orthopaedic problems in equine athletes. Besides, tendon anatomical characteristics are essential to confer the appropriate stiffness and elasticity, the avascularized nature, the hypocellularity, and the low metabolic rate predispose tendons to slow healing, after injury.

Tendon injury may result from an acute trauma, or more often, from chronic overstrain. Several predisposing factors have been investigated in the development of tendinopathies, but it has been recorded that during gallop, the equine flexor compartment works close to its physiological limit (Stephens et al. 1989).

Since tendons heal slowly, with the formation of scar tissue, which is always mechanically inferior, there is a substantial loss in strength, elasticity, and a reduced athletic performance, with a high incidence of relapses.

In fact, the higher amount of collagen type III tends to produce smaller and less organized fibrils, which will provide only a structural (repair), but not functional (regeneration) recovery.

Many medical and surgical therapies have been advocated in the treatment of equine tendon injuries, but without significant effects. Nowadays, the improvement of regenerative therapies has shown promising results with the application of stem cell-based therapies, growth factors (GFs) and low-level laser therapy (LLLT).

In this purpose, the hypothesis of the present study was to define, if a specific mixture of GFs, in combination with LLLT, could increase MSCs differentiation towards the tenogenic fate *in vitro.* In fact, it has been shown that the clinical administration of previously induced MSCs, can improve tendon regeneration and facilitate a more efficient recovery after injury (Pietschmann et al. 2013). However, the exact *modus operandi* of SCs is not completely understood and more *in vitro* studies must be performed, in order to better understand the correlation between MSCs and their niche.

MSCs were isolated from the peripheral blood of four healthy horses. It has been shown that peripheral blood is an excellent source of MSCs, in fact, samples can be easily taken in a sterile manner, at the time of injury. Therefore, peripheral blood is a high accessible source of MSCs for regenerative therapies, which is overall preferred than more invasive sources such as, bone marrow or adipose tissue (Spaas et al. 2013).

For quality control purposes, flow cytometry was performed on the putative PB-derived MSCs at passage 5 (P_5) and 10 (P_{10}). Flow cytometry works by detecting the fluorescent

signal delivered by the link between marked antibodies and glycoproteins, which are expressed on the surface of cell membrane, called Cluster of differentiation (CD). It is important to achieve the immunophenotypical characterization of the putative MSCs, in order to identify them objectively after isolation (Dominici et al. 2006). Passage 5 and 10 were chosen to generate multiple samples, because of insufficient cell yield at earlier stages. MSCs showed a positive expression for the stem cells markers CD 29, CD 44, CD90 and a negative expression for the blood cells markers CD45, MHC II, Mo/Ma, at both passages. These date confirmed a successful isolation of MSCs from peripheral blood and showed that cells did not lose their potential and self-renewal properties, by the expression of stem cell markers, in both passages tested.

Moreover, population doubling time (PDT) was calculated from P_1 to P_{10}, for each cell culture obtained from the four horses. PDT is an important parameter, which measures cell growth kinetics. It represents the time, which cells require to double in number. PDT is directly correlated with MSCs health and senescence, since it increases when cells start to differentiate, losing their potential. After an initial lag phase, PDT was relatively constant among passages, indicating that cells maintained their proliferative potential until P_{10}.

When MSCs reached P_5, they were seeded in T25 flasks and cultured in five different media conditions. More than expansion medium (Exp), which served as negative control, basic Fibroblast growth factor (FGF), Transforming growth factor-β3 (TGF), Insulin-like growth factor-1 (IGF) and Epidermal growth factor (EGF), were added to Exp, creating the following combinations: Exp, FGF, FGF + TGF, FGF + IGF, FGF + EGF.

All these GFs were added at the same concentrations (10 ng/ml), in order to standardize the effects. Each combination was tested in duplicate, for each horse.

Since several studies have confirmed the key role of FGF in tendon healing, cell proliferation and differentiation (Cai et al. 2013; Chan et al. 2000; Chang et al. 1998; Hankemeier et al. 2005), FGF was first tested alone, and then in combination with TGF, IGF and EGF. The purpose was to evaluate the synergistic effect of FGF and other GFs, which are involved in tendon healing and tenogenic differentiation.

In order to allow cell attachment and GFs conditioning, the first irradiation was performed after 24 hours from the seeding in T25 flasks. The flasks were divided in two equal groups and only one (Group 2) was submitted to LLLT. An energy density of $5J/cm^2$ was chosen, because several *in vitro* studies support the hypothesis that $5J/cm^2$ is the optimal fluence to apply, in order to enhance cell proliferation and differentiation (Hou et al. 2008; de Villiers et al. 2011). On the other hand, a recent *in vivo* study, conducted on collagenase-induced

tendinitis in sheep, showed that a dose of 5J/cm^2 was excessive for the treatment of acute tendinitis and the application of lower doses was recommended (Iacopetti et al. 2015). However, it is important to precise that *in vivo* studies include many variables, therefore the results could be really different among *in vivo* and *in vitro* studies.

After five days of media conditioning and four days of LLLT, the cells were counted, in order to evaluate the effects of the treatments on cell proliferation and viability.

The results showed that FGF, alone, was not able to increase significantly cell number, besides it is reported that FGF as a potent stimulator of cell proliferation (Cai et al. 2013; Chan et al. 2000; Chang et al. 1998; Hankemeier et al. 2005; Molloy et al. 2003). In fact, in both FGF and FGF + LLLT treatments, the total number of cells was not increased, compared to control, besides an appropriate dosage of FGF was used. It is known that an inappropriate amount of FGF may reduce its effects, but in this case, a concentration of 10 ng/ml was used, as reported by other studies, which have successfully tested it to increase MSCs growth and differentiation (Hagmann et al. 2013).

The combined addition of FGF + IGF and FGF + EGF caused a slight increase of cell number, in both irradiated and non-irradiated flasks, but the proliferation did not increase significantly. Beside the results were not significant, an addictive effect of IGF and EGF with FGF is supposed. In fact, both IGF and EGF are powerful mitogen factors, which increase especially after injury. In fact, IGF and EGF play a key role during the inflammatory phases, promoting cell proliferation and migration. In this regard, it is supposed that the increase observed after their addition is mainly due to these growth factors than FGF, which alone did not affect cell growth compared to control medium (Gonçalves et al. 2013; Molloy et al. 2003).

Only the combination of FGF + TGF caused a significant drop in cell number, confirming the anti-proliferative effect of TGF, which works through the activation of cyclin-dependent kinase pathway (Klein et al. 2002).

On the other hand, TGF did not influence cell death, which remained fairly constant among treatments. Since the addition of TGF stopped cell proliferation, the MSCs differentiation towards the tenogenic fate is supposed and this result is even more presumable, since cell death remained constant among treatments. In fact, several studies confirmed the ability of TGF to increase the mRNA expression of TNC, Collagen type I, collagen type III, COMP, tenomodulin, and thrombospondin, which are all components of the extracellular matrix (ECM) of tendon tissue (Barsby et al. 2014; Dowling et al. 2000; Klein et al. 2002). In addition, it is important to underline that TGF significantly decreased cell number in both

non-irradiated and irradiated flasks, despite several studies demonstrated the proliferative effect of LLLT, when it is applied with a fluence of 5J/cm^2. It has been shown that LLLT can enhance cell differentiation as well, when cells are cultured in appropriate inducing medium (de Villiers et al. 2011; Hou et al. 2008). In this regard, it is supposed that the application of a LLLT *in vitro* is useful to increases the effects of the medium where cells are cultured, but it is not able to produce conflicting effects. Moreover, the application of higher energy doses would not be useful to increase cell proliferation, because the cell damage would be excessive, thus increasing cell death (Hawkins and Abrahamse 2006).

After cell count, the MSCs were frozen and shipped to the department of Comparative Biomedicine and Food Science of the University of Padua, where Real time-PCR was performed.

For the first time, the relative expression of the following genes was evaluated in equine MSCs: Early Growth Response Protein-1 (EGR1), Tenascin C (TNC) and Decorin (DCN), since they are all crucial genes involved during tendon development.

The results showed that the combination FGF + TGF increased significantly the relative expression of EGR1 and DCN, in both irradiated and non-irradiated flasks, whereas only the combination FGF + TGF + LLLT was able to increase statistically the amount of TNC. Since the same treatments caused a significant decrease in cell proliferation, without affecting cell death, it is supposed that the MSCs started to differentiate towards the tenogenic fate. In fact, EGR1 is one of the first transcription factors involved in the tenogenic cascade (Lejard et al. 2011) and DCN is involved in tendon development as well. DCN participates in the organization of tendon hierarchical structure, by coordinating fibrillogenesis (Zhang et al. 2006). Since DCN is a biological ligand for the EGF receptor, we would expect a significant increase of its relative expression in the following combinations: FGF + EGF or FGF + EGF + LLLT. In fact, the link with the EGF receptor triggers a signalling cascade that leads to the activation of the MAPK pathway, which regulates cell proliferation and differentiation (Iozzo et al. 1999). However, the results showed that the relative expression of DCN was comparable to control medium, suggesting that the addition of EGF is not recommended to induce the tenogenic differentiation of MSCs.

The only combination, which increased statistically the relative expression of TNC was FGF + TGF + LLLT. TNC is a ECM glycoprotein, which plays a key role in collagen fibril organization, maintaining the interface between fibrils and the adjacent structures (Riley et al. 1996). TNC is normally up-regulated during embryogenesis, but it has been shown that its expression increases also in those areas subjected to mechanical stress. Moreover, TNC

expression may rise when cells are cultured with FGF or TGF. Pearson et al. (1988) showed that TGF may increase 4 times TNC secretion in chick embryo fibroblasts, whereas in another study FGF and TGF were first tested alone, and then in combination, resulting in addictive effect when used together (Tucker et al. 1993). Since in our study TCN increases only when the flasks supplemented with FGF and TGF were irradiated, it is supposed an enhancing effect by the LLLT. In fact, the application of an energy laser density of $5J/cm^2$ was showed to be optimal to increase cell proliferation, but differentiation was enhanced only when cells were cultured in appropriate inducing medium (de Villiers et al. 2011; Hou et al. 2008). Since LLLT alone is not able to induce cell differentiation, it is supposed that the irradiation of the flasks helped to increase the TNC content, to the point that it resulted statistically relevant. In fact, the cells were treated for only five days with supplemented media and four days with LLLT. In this regard, it is supposed that cells cultured with FGF and TGF were only induced towards the tenogenic line, but they did not differentiate in mature tenocytes. In fact, the relative expression of genes, which are normally up-regulated in tendon development is increased. In order to better define, if the supplementation of expansion medium with 10 ng/ml of FGF and TGF could be recommended to induce the tenogenic differentiation of MSCs, it would be interesting to culture the PB-derived MSCs for a longer period in this medium, with and without LLLT, and then evaluate the relative expression of genes, which are normally expressed in mature tendon such as, Collagen type I and cartilage oligomeric matrix proteins (COMP).

CONCLUSIONS

The effects of growth factors (GFs) on cell cultures are influenced by numerous variables such as: MSCs source, GF concentration, timing of incubation and GFs combinations.

We consider that the present study might provide a starting point for the development of an efficient approach for tenogenic induction of equine peripheral blood (PB)-derived mesenchymal stem cells (MSCs). In fact, the supplementation of expansion medium with 10 ng/ml of FGF and TGF, surely accelerate the differentiation of PB-derived MSCs towards the tenogenic fate. Overall, more *in vitro* and *in vivo* studies have to be performed, in order to better understand the correlation between MSCs and their niche. The possibility to induce MSCs differentiation *in vitro,* through the application of standard protocols of differentiation, would enhance the development of regenerative therapies. In the future, we do hope that it will be possible to apply MSCs routinely, not only for the treatment of tendinopathies, but also to restore complex structure of damaged tissues and organs.

RINGRAZIAMENTI

Mio padre mi ha sempre insegnato che nessuno nasce “imparato” e che l’apprendimento è un procedimento complesso che si sviluppa per tentativi ed errori. Ovviamente questo significa anche che per imparare molto, bisogna senza dubbio sbagliare altrettanto! È per questo motivo che non si deve mai avere paura di mettersi in gioco, perché solo provando e riprovando, ci si potrà scoprire in grado di fare cose, che fino a qualche tempo prima, si ritenevano impossibili. In questi anni mi sono resa conto che la forza di volontà è ciò che fa la differenza tra il riuscire e il non riuscire a raggiungere un obbiettivo, tuttavia, senza l’affetto e l’amore delle persone care qualunque risultato sarebbe solo fine a sé stesso.

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