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Novel treatments in equine medicine: differences in the expression of inflammatory molecules

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ABBREVIATIONS

AAEP = American Association of Equine Practitioners

AD-MSCs = Adipose-Derived Mesenchymal Stem Cells

ALDDFT = Desmopathy of the Accessory Ligament of the Deep Digital Flexor Tendon

AOPP = Advanced Oxidises Protein Products

bFGF = basic Fibroblast Growth Factor

BID = twice a day

CT = carbonyl groups

DALSDFT = Desmotomy Accessory Ligament Superficial Digital Flexor Tendon

DMEM = Dulbecco's Modified Eagle Medium

DMSO = dimethyl sulfoxide

DMSO = dimethyl sulphoxide

ECM = Extracellular Matrix

ECSWT = ExtraCorporeal Shock Wave Therapy

FBS = Fetal Bovine Serum

GAG = glycosaminoglycan

HA = Hyaluronic Acid

IGF-I = Insuline-like Growth Factor I

MCP = metacarpophalangeal

MHC = Major Histocompatibility Complex

MMPs = Matrix MetalloProteinases

MTJ = Miotendinous Junction

MTP = metatarsophalangeal

NSAIDs = Non-Steroidal Anty-Inflammatory Drugs

OTJ = Osteotendinous Junction

PBS = Phosphate Buffer Saline

PDGF = Platelet Derived Growth Factor

PPP = Platelet Poor Plasma

PRP = Platelet Rich Plasma

PSGAGs = polysulfatet glycosaminoglycans

PSLD = Proximal Suspensory Ligament Desmopathy

PT = Total Protein

ROM = Range Of Motion

SID = single dose

SVF = Stromal Vascular Fraction

T-BARs = ThioBarbituric Acid Reactive substances

TGF-β1 = Transforming Growth Factor β1

VEGF = Vascular Endothelial Growth Factor

ABSTRACT

In equine practice, lameness due to musculoskeletal disease is the most common diagnosis, with ligament and tendon injuries resulting as the most frequent lesions. These kinds of injuries have a significant impact on the horse's athletic performance as well as their quality of life; furthermore, the economic cost for the required treatments is very high.

Currently, the most common therapy is surgery, that is usually associated to stall rest and pharmacological management of the inflammatory stage that follows the lesion. These therapies aim to repair the lesion, but the newformed cellular material does not have the same biological and biomechanical properties of the native tissue. In fact, tendons and ligaments are poorly vascularized tissues consisting of few cells lying in abundant extracellular matrix, the healing process is slow and leads to the formation of scar tissue, and often, to high reinjury rates that can reach over the 80% of the cases.

The aim of regenerative medicine is not only to provide wound healing, but to repair damaged tissues too, leading to the restoration of the normal function of the injured tissue. In the case of tendon and ligament lesions, regenerative medicine aims to restore both the structure of collagen fibers and their biomechanical proprieties, so that after the lesion has healed, tendon's structure and function is the most possible like the original tissue; this achievement should allow the horse to get back to the same activities it was used to and, moreover, to the same performance level with minimum risks of reinjury.

In this study, a double-injection of adipose derived mesenchymal stem cells associated to platelet rich plasma was performed on two horses affected of tendonitis of the superficial digital flexor tendon of one forelimb. Clinical assessments were performed every 2 weeks starting from the day of the first injection; general condition, pain, heat and swelling at the site of the injury, grade of lameness and the horse keeper's evaluation were the considered parameters. Ultrasonographic evaluations of the metacarpal region of both forelimbs were performed by means of longitudinal and transverse scans. The obtained images were evaluated and scored for i) the lesion echogenicity and ii) the lesion longitudinal fiber alignment; the contralateral healthy limb was used as comparison. Horse plasma was also analyzed in both horses at baseline, one week and two weeks post-injection, and in subject n°2 also one week prior to treatment and one month after treatment, in order to assess different oxidative stress molecules' levels: total protein, advanced oxidation protein products, carbonyl group and malondialdehyde. Two interleukins important in the inflammatory process were also estimated: interleukin 1 and interleukin 10. Platelet derived growth factor, insulin-like growth factor 1 and transforming growth factor β1 values were also determined.

The results of this study suggest a beneficial effect of the performed combined treatment that demonstrated to be safe and effective; importantly, no adverse reactions were observed, as confirmed my biochemical parameters, and the horses were able to get back to competition. These evidences might therefore encourage the combined application of mesenchymal stem cells and platelet rich plasma for the treatment of tendon injuries in equine clinical practice.

RIASSUNTO

Nella medicina equina, la zoppia conseguente a patologia muscoloscheletrica rappresenta la diagnosi più comune, di cui il 50% è dato da lesioni tendinee o legamentose che risultano quindi le patologie più comuni. Queste lesioni hanno un impatto significativo sulle performance atletiche dei cavalli ma anche sulla loro qualità di vita, e la spesa economica per i trattamenti necessari è molto alta.

Al momento la terapia più comune è la chirurgia, che viene generalmente associata a riposo scuderizzato e ad un trattamento farmacologico per la gestione dello stato infiammatorio che segue la lesione. Queste terapie mirano a riparare la lesione, ma il tessuto neoformato non ha le stesse caratteristiche biologiche e biomeccaniche del tessuto originale. Infatti, tendini e legamenti sono tessuti scarsamente vascolarizzati costituiti da poche cellule sparse in abbondante matrice extracellulare, per cui il precesso di guarigione è lento e porta alla formazione di tessuto cicatriziale, che determina alti tassi di recidiva che possono raggiungere l'80% dei casi.

Lo scopo della medicina rigenerativa non è solo di portare alla guarigione della ferita, ma anche di riparare i tessuti danneggiati portando alla rigenerazione delle normali funzioni del tessuto lesionato. Nel caso delle lesioni tendinee e legamentose, la medicina rigenerativa mira al recupero sia della struttura delle fibre di collagene che delle loro proprietà biomeccaniche in modo che, una volta guarita le lesione, la struttura e la funzionalità del tendine siano il più possibile simile a quella del tessuto originale; questo permette al cavallo di tornare alle attività precedenti ed allo stesso livello di performance con il minimo rischio di recidiva.

In questo studio, una doppia iniezione di cellule staminali derivate da tessuto adiposo associate a concentrato piastrinico è stata eseguita su due cavalli affetti da tendinite del tendine flessore superficiale di un arto anteriore. Valutazioni cliniche sono state eseguite ogni due settimane a partire dal giorno della prima iniezione; i parametri valutati sono stati condizione generale del soggetto, dolore, calore ed edema a livello della lesione, il grado di zoppia e la valutazione del proprietario. Valutazioni ecografiche della regione del metacarpo di entrambi gli arti anteriori sono state eseguite in scansione longitudinale e trasversale. Le immagini ottenute sono state valutate e vi è stato assegnato un punteggio in base all'ecogenicità ed all'allineamento longitudinale delle fibre; l'arto controlaterale sano è stato utilizzato come paragone. È stato inoltre analizzato il plasma dei cavalli al momento della prima terapia ed una e due settimane dopo l'iniezione e, nel soggetto n°2, anche una settimana prima del trattamento ed un mese dopo, in modo da stimare i livelli di diverse molecole implicate nello stress ossidativo: proteine totali, prodotti dell'ossidazione avanzata delle proteine, gruppi carbonili e malonaldeide. Sono stati determinati anche i valori di due interleuchine importanti nei processi infiammatori: l'interleuchina 1 e l'interleuchina 10. Sono stati determinati anche i valori

del fattore di crescita derivato dalle piastrine, del fattore di crescita insulino simile e del fattore di crescita tumorale β1.

I risultati di questo studio suggeriscono un effetto benefico del trattamento combinato eseguito che si è dimostrato essere sicuro ed efficace dato che nessuna reazione collaterale è stata riscontrata; inoltre, i cavalli sono stati in grado di tornare a gareggiare. Questi risultati potrebbero quindi incoraggiare l'applicazione delle cellule mesenchimali staminali e del concentrato piastrinico per il trattamento delle lesioni tendinee nella pratica ippiatrica.

CHAPTER 1: TENDON AND LIGAMENTS ANATOMY

1.1 Macroscopical and microscopical anatomy

Tendons are those structures that connect muscles to bone via myotendinous (MTJ) and osteotendinous (OTJ) junction, while ligaments connect bone to bone, allowing the transmission of mechanical forces induced by muscle contraction in order to achieve movement (1, 2). As they have an elastic component, some tendons also have the ability to store and release energy (3, 4, 5).
When healthy they appear brilliant white and have a smooth surface, but in largest tendons a longitudinally striated aspect can be seen due to the thickness of the fasciculi (6, 7). As ligaments have a higher elastic component, they appear more yellowish (8).
Usually, tendons are shaped as cords or straps of round or oval cross-section, or they can be flattened ribbons (6, 9). Tendon's macroscopical shape is strictly related to its function: the more precise and subtle a movement must be, the more the tendon will be long and thin, while tendons that are required to be strong and resistant are thick and short.
Adjacent tendon's fibers, of the same tendon or of near ones, can form cords or bridges.

Microscopically, tendons are a poorly cellularized tissue composed of tenoblasts and tenocytes lying within a network of extracellular matrix (ECM) (6, 7).
Tendons can be defined as a dense regular connective tissue as they are mainly composed by thick collagenous fibers aligned in a parallel arrangement (8); fibers that run transversely and horizontally can also be found, forming spirals and plaits (6, 10). Collagen fibers' parallel arrangement allows tendons to resist tension so that the energy produced by muscle contraction doesn't get lost during load transmission (11, 12). Tendons and ligaments are primarily composed of parallel collagen fibers surrounded by a net of few elastic fibers; collagen fibers work in opposite way respect to the traction force (8).
Tenoblasts are immature spindle-shaped tendon cells; they are characterized by shape and the high presence of cytoplasmic organelles that reflect their high metabolic activity. As they age, tenoblasts become elongated, reduce their nucleus/cytoplasm ratio and differentiate into tenocytes; the metabolic activity also decreases (6, 7).
Based on their nuclear morphology, three different types of tenocytes have been identified: type 1 tenocytes are characterized by a spindle-shaped nucleus, type 2 tenocytes have a more cigar-shaped nucleus, and type 3 tenocytes are found in wraparound regions showing a more chondrogenic

phenotype. Three dimensionally, nuclei of type 1 and 2 tenocytes appear as slightly flattened ovoids, with type 2 cells showing a greater range of nuclear width (13, 14, 15).
As the horse ages, an increase in the proportion of type 1 tenocytes and in nuclear lengths can be measured, along with the reduction of type 2 tenocytes and overall cellularity (13).

Together, tenoblasts and tenocytes account for 90-95% of tendons' cellular elements; the remaining 5-10% consists of chondrocytes at the bone attachment and insertion sites, synovial cells of the tendon sheath and vascular cells, including capillary endothelial cells and smooth muscle cells of arterioles (6, 7, 16, 17). The dry mass of human tendons is approximately 30% of the total mass, with the remaining 70% consisting of water (18, 19, 20).
Tenocytes synthesize both collagen and all the components of the ECM.

Collagen fibers represent the most common connective tissue fiber type; they are flexible and characterized by a high tensile strength (21).
Collagen is arranged in hierarchical levels of increasing complexity, starting with tropocollagen, the structural unit of a collagen fiber. Tropocollagen is made up of three polypeptide chains that are twisted together into a right-handed triple-helix called alpha chain. Except for the ends of the chain, every third amino acid is a glycine, generally preceded by a hydroxyproline and followed by a proline. Sugar groups are associated to the triple helix, therefore collagen should be properly called a glycoprotein (21).
As there are differences between the alpha chains that form a helix, sixteen different types of collagen have been identified; they are classified by Roman numbers on the basis of the chronology of discovery. The most common fiber is type I collagen, which constitutes about 90% of total body collagen as it can be found in the dermis of the skin, bone, organ capsules, and tendons; type II collagen can be found in cartilage and is composed of finer fibers in comparison to type I; type IV collagen is found in the basal lamina (21).
Collagen type I is the most important protein of tendons (95%) (5, 13), as it defines their high tensile strength and structure; type III collagen can be found in small amounts between fascicles and in the endotenon, whereas type II collagen can be seen in fibrocartilaginous tendon regions subjected to compressive forces as the wrap around bony prominences (13).
In collagen I fibers, tropocollagen molecules consist of a triple helix made of two alpha-1 chains and one alpha-2 chain that spontaneously assemble after secretion into collagen micro fibrils; each fibril is made up of collagen molecules that are aligned, head to tail, in overlapping rows. Within each row

there is a gap between the tail of one molecule and the head of the next. Covalent bonds are present between collagen molecules and these bonds are the ones that confer fibrils their strength (21).
A graphical representation of this structure can be seen in *Figure 1.1*
Tendon's hierarchical structure has microfibrils unite into fibrils that arrange themselves into larger units called fibers; the collagen fiber is considered to be the smallest tendon unit visible on light microscopy and mechanically testable. Fibers gather then into primary fiber bundles, also called subfascicles; fiber bundles form fascicles, also called secondary bundles, then tertiary bundles and, in the end, the tendon itself (22).

While the collagen fiber is considered to be the smallest tendon unit, fascicles are the largest ones, with a diameter of approximately 1 mm and a variable cross-sectional polygonal shape; an age-related reduction in the cross-sectional area can be seen (13, 23). Fascicles are divided by a small amount of loose connective tissue, named endotenon, which contains blood, lymphatic vessels and nerves; blood vessels do note penetrate into the fascicle under normal circumstances. Fascicles appear to move independently (13, 24, 25).
The endotenon is continuous with the epitenon that covers the whole tendon.
In addition, the epitenon is surrounded by the paratenon, a loose connective tissue that allows movements within the surrounding tissue. Type I and type III collagen fibrils and some elastic fibrils form the paratenon, and in areas subjected to increased mechanical stress, where efficient lubrification is required, synovial cells can be found (26). Epitenon and paratenon together constitute the peritenon (27).
A schematic view of tendon's hierarchical structure and its connective tissue is shown in *Figure 1.2*

The extracellular matrix is composed of fibers and the ground substance; in the connective tissue the ECM component predominates over the cellular elements.
The ground substance is called that way as, in specially prepared sections, it has an amorphous gel-like appearance; it fills the space between cells and fibers of the connective tissue. The ground substance has a high-water content and allows the diffusion of gases and metabolites; its composition influences tissues' characteristics.
Ground substance is primarily composed of proteoglycans and hyaluronic acid.
Proteoglycans are large organic macromolecules in which many glycosaminoglycan (GAG) molecules are covalently attached to a core protein. GAGs are formed by the association of long-chained polysaccharides made up of repeating disaccharide units, in which one of the sugars is a

glycosamine (or hexosamine), thus the name GAG; the disaccharide units are generally composed by derivatives of glucose and galactose.
In these molecules there is a variable content of sulfate and carboxyl groups, that give them a high negative charge. The high density of negative charges attracts water forming a hydrated gel that allows a fast diffusion of water-soluble molecules but inhibits the movement of macromolecules and bacteria.
The most common GAGs are chondroitin sulfate, dermatosulfate and keratosulfate.
Hyaluronic acid (HA) is a GAG but has unique characteristics as it is non-sulfated and extremely long: it consists of a chain of several thousand sugars (about 20.000 disaccharide unit with an average of 7 million Da per molecule), while the other GAGs are composed of several hundreds sugars (3-4 million Da).
Another difference is the absence of the core protein: proteoglycans indirectly bind to HA via special linker proteins forming giant macromolecules (8, 21).

Tendons connect muscle to bone via MTJ and OTJ; these are tendon's weakest points.
At the MTJ, myocytes form deep recesses in which collagen fibrils are inserted in order to transmit to the collagen fibers the tension generated by the muscle fibers. This complex structural architecture allows a reduction of the tensile stress exerted on the tendon during muscle contraction (28).
The OTJ too has a specialized structure to prevent collagen fibers from bending, fraying, and failure (29). Four different zones can be identified: bone, mineralized fibrocartilage, fibrocartilage and a dense tendon zone (30).

Connective tissue cells can also secrete reticular fibers, made of type III collagen fibrils that provide a supporting framework for the cellular elements of different tissues and organs, and elastic fibers.
Elastic fibers are interwoven with collagen fibers in order to give tissues the ability to cope with stretch and distension preventing excessive distensibility and tearing.
These fibers are thinner than the collagen ones and are arranged in a branching pattern to form a 3D network. Elastic fibers are composed of elastin and microfibrils.
Elastin is a collagen related protein composed of many molecules of tropoelastin connected together; these molecules are characterized by a particular polypeptide backbone that causes a random coiling; therefore, the configuration of one molecule's coiling is not permanent but oscillates from one shape to another.
The peculiarity of this fiber is that the coiled elastin can be stretched, but when the force causing the elongation is withdrawn, the molecule recoils back to its former state.

Certain ligaments contain elastin molecules and are therefore named elastic ligaments (21).

Histological images of a longitudinal and a transverse section of a collagen fibre bundle can be seen in *Figure 1.3*

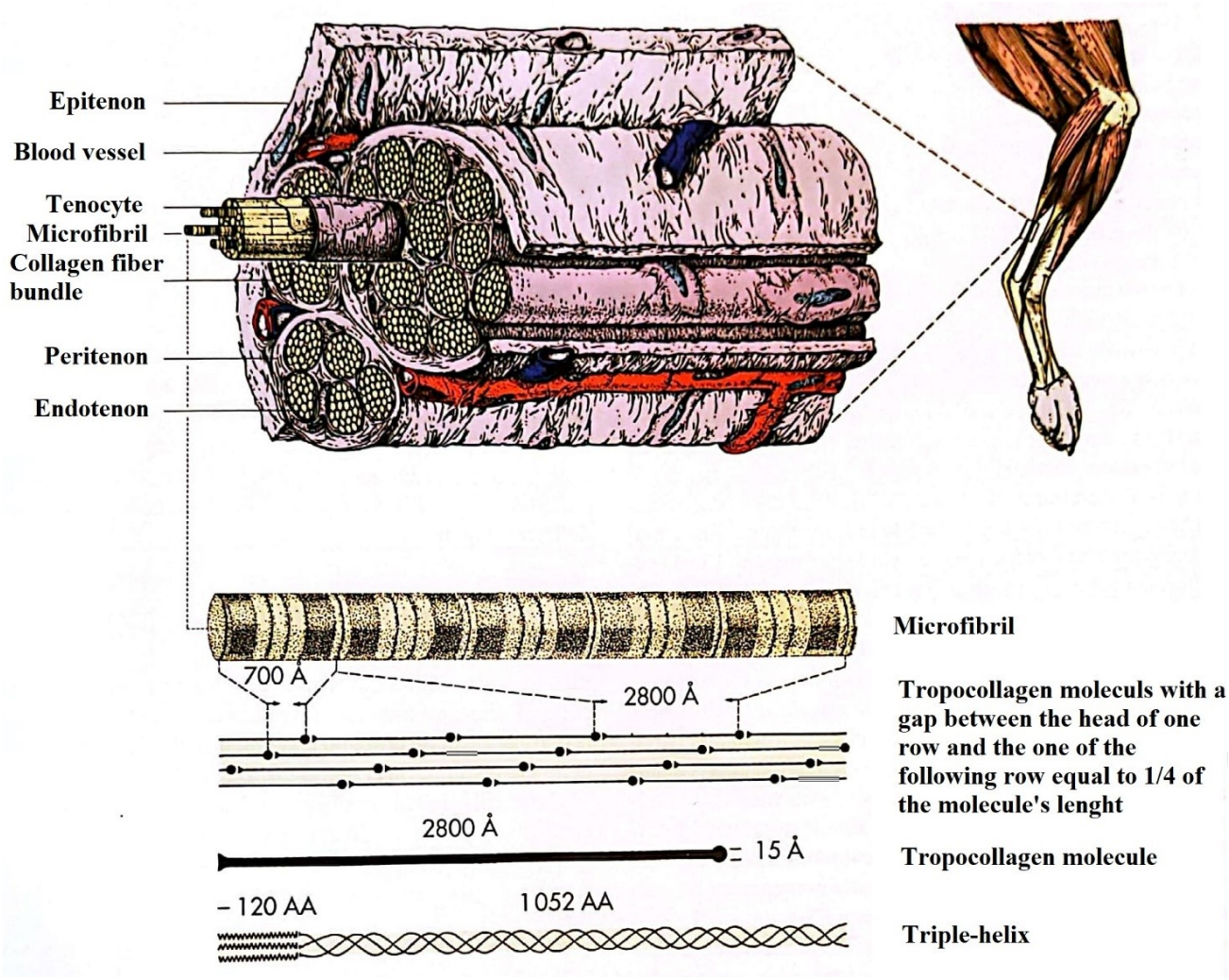

Figure 1.1: Schematic of a multi-unit hierarchical structure of the tendon.

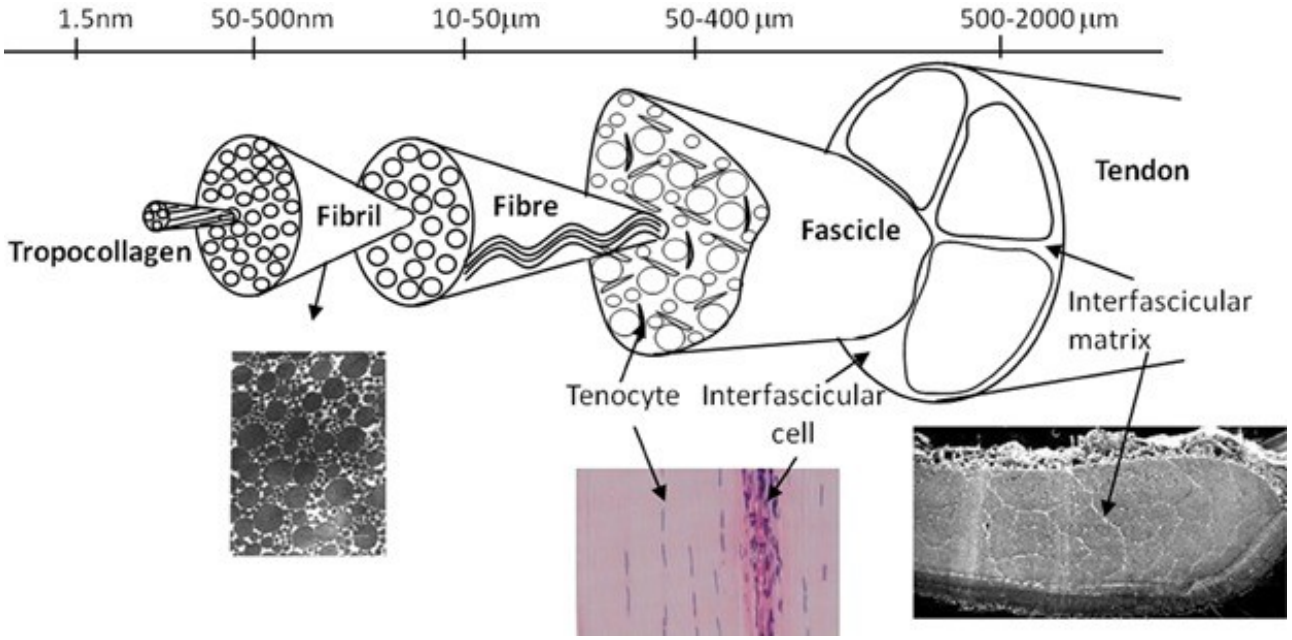

Figure 1.2: Tendon's fascicles, fibres and fibrils, made of tropocollagen molecules of dense regular connective tissue (tendon) and relative surrounding connective tissue.

The tendon has a multi-unit hierarchical structure composed of collagen molecules, fibrils, fibre bundles, fascicles and tendon units that run parallel to the tendon's long axis. This hierarchical structure contributes to the mechanical competence of the tendon.

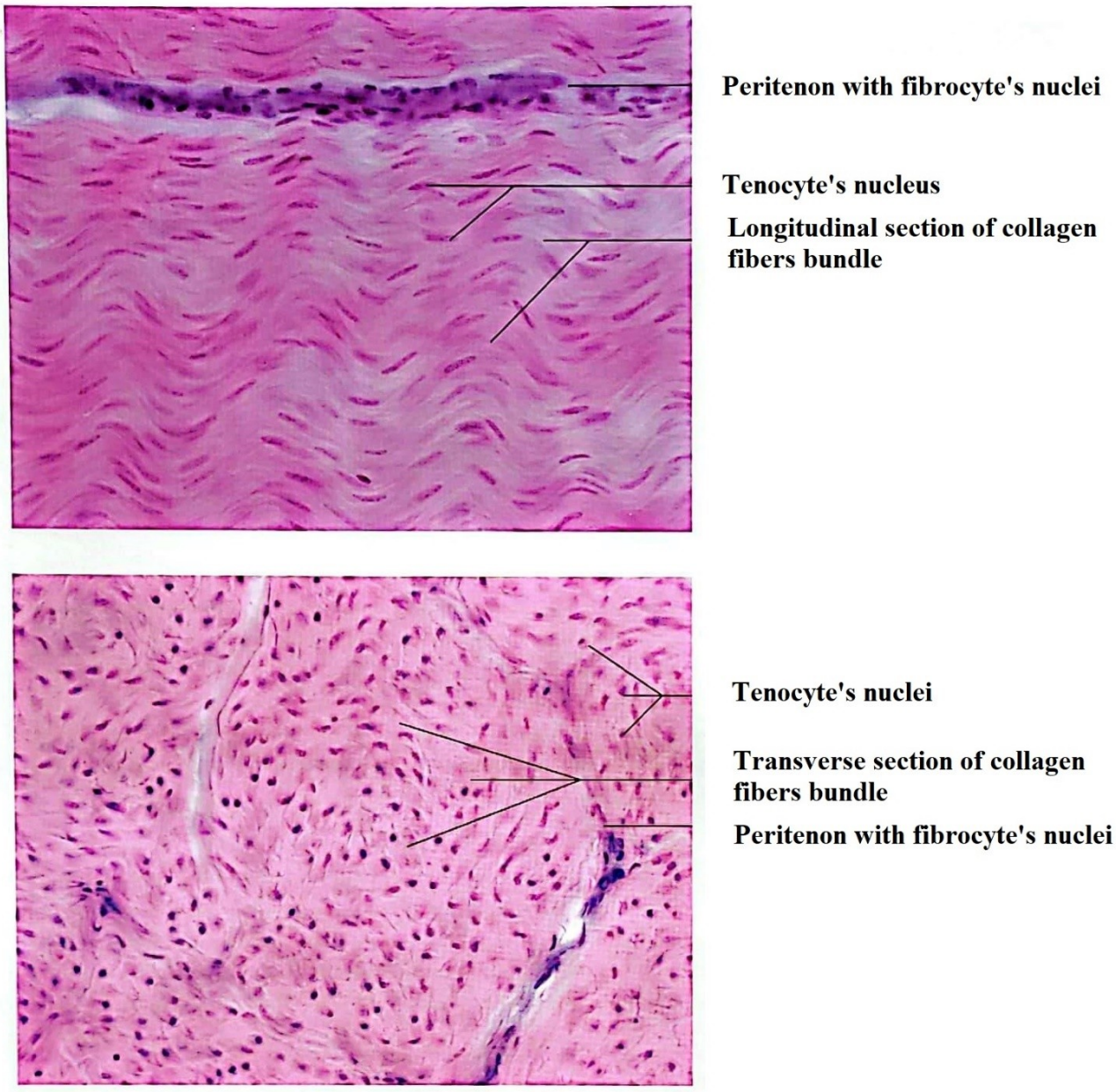

Figure 1.3: Histological longitudinal and transverse section of a collagen fiber bundle (Istologia e anatomia microscopica dei mammiferi domestici e degli uccelli, Hans-Georg Liebich, 2012).
Coloured with hematoxylin and eosin stain.
Magnification respectively 480 × and 300 ×.

1.2 Blood supply

Tendons' blood supply is granted by an intrinsic and an extrinsic system, with differences in the ratio of blood supplied by the two between tendons (31, 32).

The intrinsic system sees perimyseal vessels from the muscle continuing between the fascicles of the tendon through the MTJ; blood vessels coming from the muscle are however unlikely to continue beyond the proximal third of the tendon (31).

The blood supply from the OTJ is limited to the insertion zone, even though some periosteal vessels communicate with the extrinsic system (31).

The extrinsic system provides blood via the paratenon or the synovial sheath (31).

In friction subjected areas, tendons are enveloped by sheaths; in those tendons, branches from the major vessels pass through the mesotenon and reach the synovial visceral sheath forming a plexus that supplies blood to the superficial part of the tendon (22). Some vessels penetrate the epitenon and enter the endotenon septae forming a connection between the two different vascular networks.

In those tendons where the synovial sheath is not present, vessels enter the paratenon transversely and branch repeatedly forming a vascular network (33): arterial branches penetrate the epitenon and then course in the endotenon septae forming an intratendinous vascular network by many anastomoses (7, 34).

1.3 Innervation

Tendons' innervation is essential for sensing tension, pressure and pain in the tissue.

The innervation of tendons originates from cutaneous, muscular and peritendinous nerves, with both sympathetic and parasympathetic fibers being present (35).

At the MTJ, nerve fibers cross entering the endotenon septa, forming rich plexuses in the paratenon from which some branches penetrate into the epitenon. Most of the fibers though do not enter the main body of the tendon but terminate on its surface as nerve endings.

The nerve endings of myelinated fibers function as mechanoreceptors that can detect changes in tendon's pressure or tension; those receptors are known as Golgi tendon organs.

The Golgi tendon organs consist of a thin layer of connective tissue that surrounds a group of branches of large, myelinated nerve fibers that terminate with a spray of fiber endings between tendon's collagen fibers (36). These organs are more numerous at the MTJ (37).

Unmyelinated nerve endings on the other hand, are responsible for sensing and transmitting pain: they are nociceptors.

1.4 Biomechanics

Tendons transfer tensile loads generated by muscles to the bone, in order to enable joint motion and to stabilize joints; they also act as a buffer that absorbs external forces in order to limit muscle damage (38). Therefore, two types of tendons can be recognized: tendons that function to transmit loads and tendons that mainly transmit motion.

Tendons are viscoelastic tissues with a high mechanical strength, good flexibility and elasticity; they can display a state of stress, relaxation and creep (39, 40). The viscoelastic response is related to the presence of water, proteoglycans and GAGs in the tissue (1, 41-46). Viscoelasticity makes tendons more deformable at low strain rates rather than at high strain rates; therefore, at low strain rates more mechanical energy is absorbed but the tendons are less effective in carrying loads, while at high strain rates, tendons become stiffer and the transmission of large muscular loads to bone is more efficient (46).

Tendon's tensile strength is related to its thickness and collagen content: a tendon with a cross sectional area of $1cm^2$ can bear 500-1.000kg (6, 47); tendon is stronger than muscle per unit area as its tensile strength in approximately equal to that of bone, even though tendons have a much higher flexibility, elasticity and extensibility than bone itself (11, 12).

The mechanical behaviour of tendon's collagen fibers can be better understood with a stress-strain curve; four different regions can be distinguished, as shown in *Figure 1.4*.

1. Toe region: at rest, collagen fibers display a crimped configuration (6, 48); in this region the stretching-out of the crimp-pattern can be seen, when the tendon is lengthened by low tensile loads, up to 2% of its resisting length, after release quickly resumes its initial length. This is the initial concave portion of the curve; with this tensile stress, tendon's fibrils respond with a flattening of the crimp pattern in which the angle and length of the pattern depend on the type of tendon and affects tendon's mechanical properties: fibers with a small crimp angle fail before those with a large one (6, 46, 49, 50).
2. Linear region: beyond the previous point, the tendon deforms due to the intramolecular sliding of collagen triple helices; fibers become more parallel (6, 51). If the strain remains below 4%, when unloaded the tendon returns to its original length (6, 52); from 4% to 8% microscopic failure occurs, a tearing of tendon's fibers can be seen and a pathological irreversible tensile elongation begins to take place (6, 46, 53).
3. Macroscopic failure: beyond 8-10% strain, depending on age, type and organization of the tendon fiber bundle, macroscopic failure with intrafibril damage by molecular slippage (6, 46, 53, 54).

4. Tendon rupture: further stretch causes tendon rupture (6, 53, 54, 55). At this point, fibers recoil into a tangled bud at the ruptured end (6, 38).

   The higher risk of rupture occurs when tension is applied quickly and obliquely. The highest forces can be seen during eccentric muscle contraction as the ones generated by quick eccentric movements where a limb must rapidly be decelerated (6, 11, 56, 57).

Training and mobilization determine changes in tendon's structure: ultimate load and energy absorbed at failure gets higher in exercised animals (46, 58, 59), and running increases strength at the insertion (46, 60); number and size of collagenous fibrils and tendon's cross-sectional area also increases in exercised animals (46, 61, 62).

Biochemical changes are also induced by training. Training stimulates the production of insulin-like growth factor-I (IGF-I) by tenocytes, a stimulus of collagen synthesis and cell proliferation (46, 63, 64). IGF-I can therefore be used as protein marker for tendon's remodelling activities.

Furthermore, exercise increases collagen turnover but decreases its maturation.

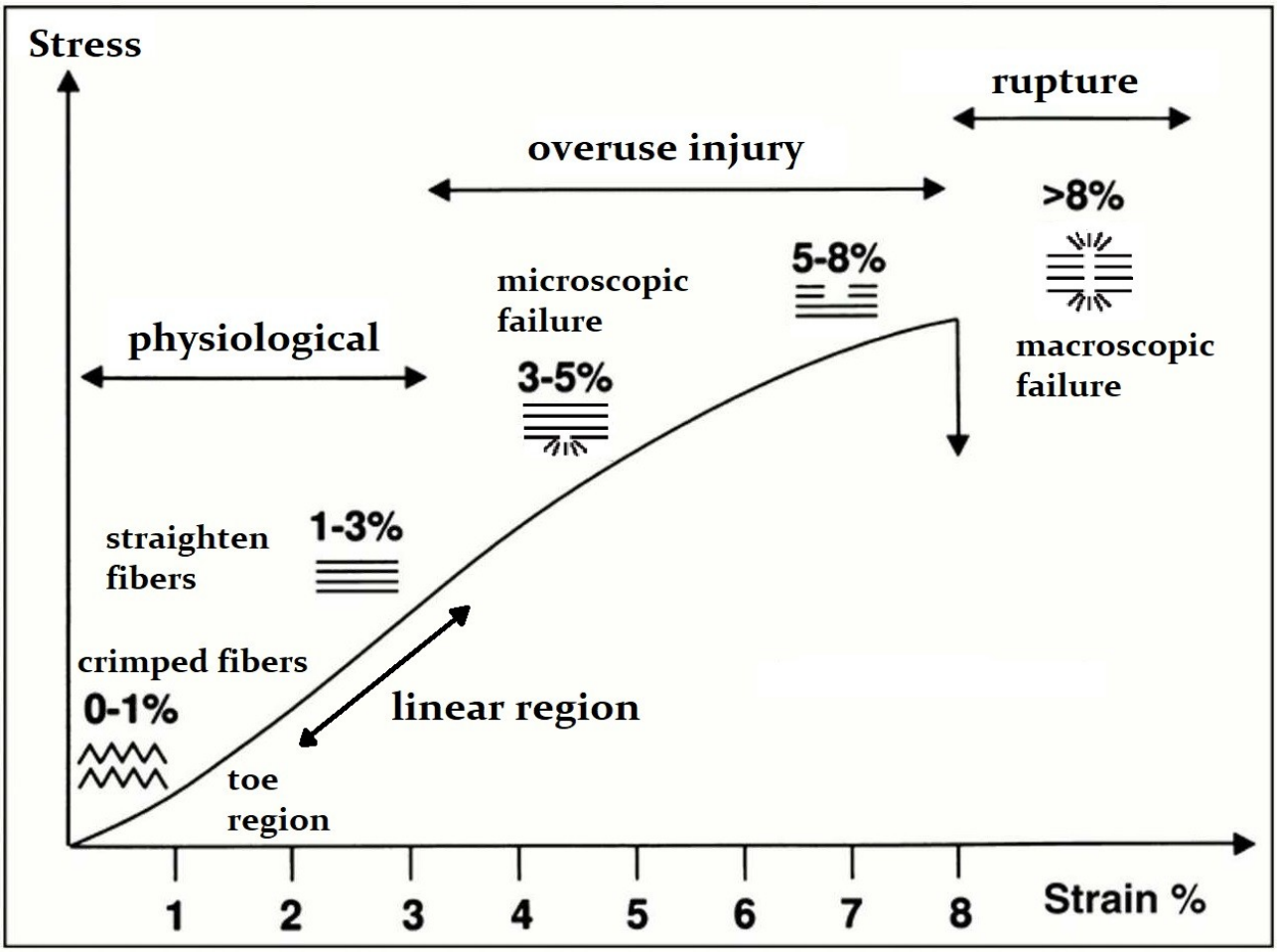

Figure 1.4: Tendon's stress-strain curve showing its viscoelastic properties.
The toe region is associated with elimination of "crimp" while in the linear region the tendon is operating in an elastic way. At the end of the linear region the yield point is reached, and irreversible damage starts to occur before complete tendon rupture.

CHAPTER 2: TENDON INJURY

Tendon injuries can follow acute or chronic events and may be caused by intrinsic or extrinsic factors that can occur either alone or in combination. Generally, extrinsic factors lead to acute traumas whereas, in chronic cases, intrinsic factors are also involved (6).

2.1 Tendinopathy, overuse injury

Although tendinopathies are defined as a form of degeneration (tendinosis) and not inflammation (tendonitis) their aetiology is still unclear (1). Tendinopathies describe the pain induced by the overuse of a tendon which results in impaired function of the associated limb (65, 66).

Many causes have been considered throughout the years: hypoxia and ischaemic damage, oxidative stress, inflammatory mediators, hyperthermia, impaired apoptosis, fluoroquinolones and an imbalance in the matrix metalloproteinase.

As tendinopathies represent a chronic disorder, the interaction between extrinsic and intrinsic factors, as alignment and biomechanical faults, is commonly seen (6, 46, 67, 68, 69).

During training, excessive loading of the tendons frequently occurs and is considered the main pathological stimulus for degeneration (6, 70); furthermore, in presence of intrinsic risk factors, the risk of inducing tendinopathy is higher. Some of the intrinsic factors that have been identified are patient gender, age, body mass and genetic constitution or anatomical variations that may effect the development of tendinopathy (1, 71, 72, 73).

Repetitive overload over the physiological threshold can induce inflammation of tendon's sheaths, degeneration of their body or a combination of both (6, 74); it also leads to the inability of the tendon to endure further tensile stress (46, 75).

Tendon damage might even occur from frequent cumulative microtraumas within physiological limits, as these can cause tendon microinjuries that lead to inflammation and do not allow enough time for tendon repair (6, 11, 46, 76). Cumulative microtraumas can be defined as "fatigue", since they cause a progressive localized structural damage to tendons subjected to cyclic loading (11).

Microtraumas may also result from non-uniform distribution of forces within tendons that leads to abnormal load concentration and frictional forces between the fibrils that result in localised fiber damage (6, 76).

The OTJ is also subjected to tendon overuse injury. In case of enthesopathy, tendons are metabolically active ate the insertion site; collagen bundles loosen, the composition of the extracellular matrix gets altered, lipids accumulate and microcalcifications might occur (46, 77, 78, 79).

Patatenonitis (or peritendinits) can follow either after trauma or excessive loading of the paratenon. In such cases, edema and swelling can be observed; there can be hyperthermia of the tenosynovium, infiltration of lymphocytes and proliferation of blood vessels (46, 77). The inflammation and metabolic activity of the paratenon reflects those of the tendon.

The macroscopical aspect of the affected tendon's portions sees the normal white brilliant appearance turn into a grey-brown amorphous appearance. Tendon's thickening also occurs, and it can be diffuse, fusiform or nodular (6, 80).

Histologically, affected tendons show a scenario characterised by a disordered random healing in absence of inflammatory cells (6, 11). The healing response is poor and non-inflammatory intratendinous collagen degeneration, fiber thinning and disorientation, increased interfibrillar GAGs, hypercellularity and scattered vascular ingrowth can also be observed (6, 22). Instead of continuous crimped well-aligned collagen fibers, the tissue appears fragmented with a disordered collagen matrix often associated with the absence of clear fiber structure within the tendon (65, 81, 82). Tendon deterioration can also show mucoid degeneration, fat infiltration and calcification (1). Tendon's mechanical proprieties can be normal even if its fibril morphology is altered (11).

As it has been suggested, by histological evidence, that cellular changes precede alteration of collagen in the development of tendinopathy (65, 83), tendon's nanostructural level should also be considered. Indeed, in the initial stages of tendon suffering induced by mechanical fatigue, collagen fibril kink bands can be observed whereas this damage could not be visible microscopically; kink banding is a mode of disruption that occurs in anisotropic layered or fibrous material in response to compressive forces (65, 84, 85). With continuous loading, kink bands may extend laterally and grow into microscale fiber ridges, leading to an extension of the wound (65, 83, 86).

Nano kinkbands can be seen in Figure 2.1.

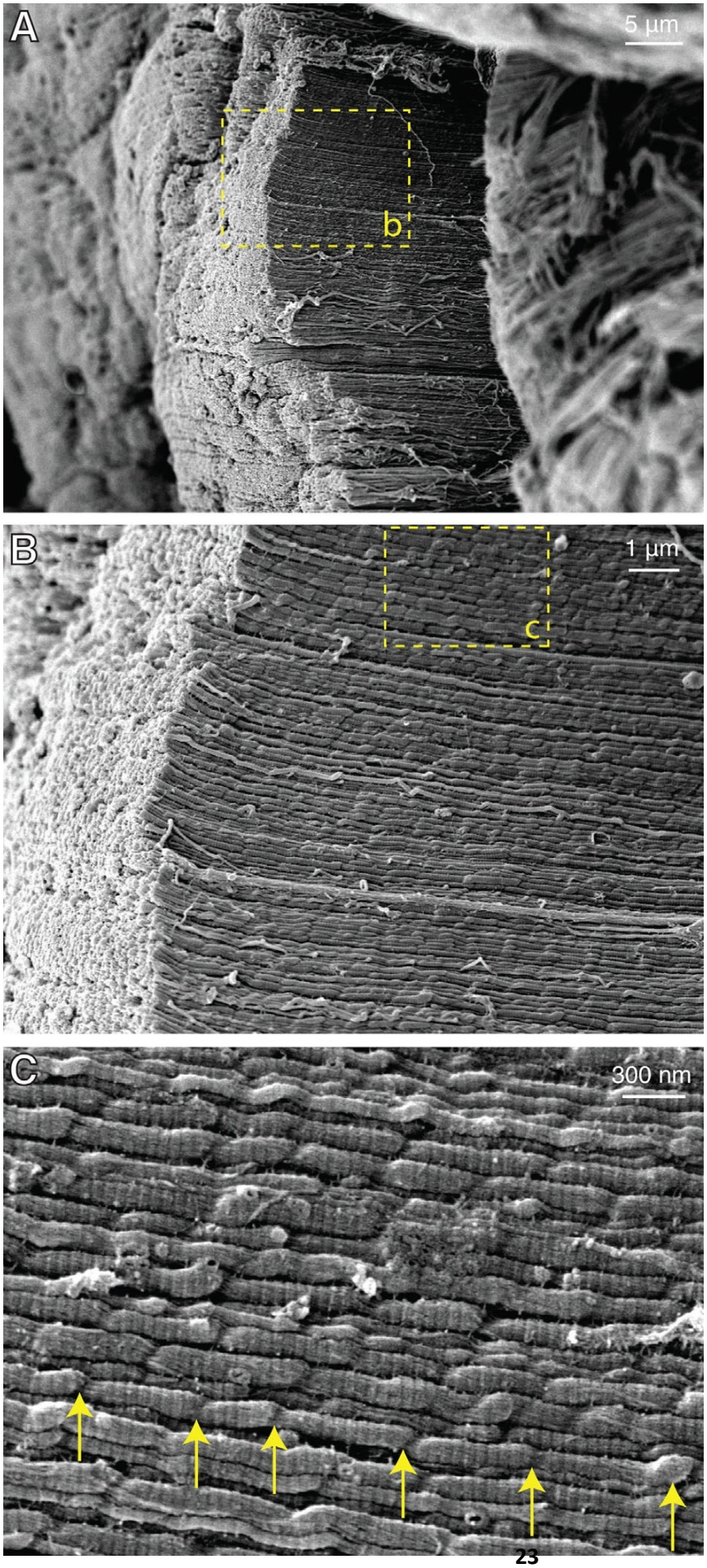

Figure 2.1 (Tyler W., 2017) Normal tendon appearance at the microscale does not indicate the absence of mechanically damaged collagen. (A) 1,800 ×. The collagen fibre in boxed region "b" appears normal at the microscale. (C) At 30,000 ×, it is apparent that the collagen fibrils making up the normal looking fibre in A are actually highly damaged, having undergone discrete plasticity (repeating kinks along each fibril, such as those marked by yellow arrows).52 (B) intermediate image taken at 7,000 ×. The boxed regions b and c show the locations of images (B and C), respectively.

2.2 Disuse and immobilization

The knowledge on the effects of tendon disuse and immobilization are limited, as there their effect is much slower than on muscles and because there only have been few studies on this topic (46, 87).
In general, immobilization causes a decrease in tendon's tensile strength, stiffness and total weight (46, 88, 89, 90). Stress deprivation caused by tendon's disuse is considered to be responsible for the degenerative changes (46, 91). Microscopically, collagen fibers appear irregular and uneven, and dilated veins and capillaries can be found (46, 92).

2.3 Rupture

Tendon rupture is an acute injury which aetiology is still unclear (6, 93).
Even though extrinsic factors predominate in this kind of injury, intrinsic factors are also important; intrinsic abnormalities can increase tendon's injury risk. Degenerative tendinopathy is frequently observed in spontaneous tendon ruptures as it leads to a reduction in tendon's tensile strength and therefore a predisposition to rupture (6). A malfunction in the physiological protective inhibitory pathway of the musculotendinous unit might also cause tendon injury (6, 94).

2.4 Healing process

Acute tendon injury rapidly causes the initiation of the healing process that is generally subdivided into three stages: inflammation, proliferation (or reparation) and remodelling (46, 95-101). Each one of these stages chronologically overlaps with the following one but possesses a different cytokine and cellular profile; generally, in the early stages, pro-inflammatory cytokines predominate, while anti-inflammatory cytokines are mainly expressed in the late phases of the healing process (95, 102).
The first stage of the healing process is the inflammatory stage, which begins immediately after tendon injury. A clot is formed in the damaged vessels, and the activation of inflammatory cells leads to fibroblast recruitment (95, 96); in this phase, the clot acts as a scaffold for the migration and adhesion of the recruited inflammatory cells (95, 103).
In this stage, the main soluble factors, released by platelets, cells in the clot, and the cells surrounding the wound area, are: transforming growth factor-β1 (TGF-β1), IGF-I, and platelet-derived growth factor (PDGF) (1, 95, 97-102, 104, 105, 106). The elaboration of these growth factors causes the recruit of neutrophils, that then leads to the activation of macrophages; these cells clean the wound

site of necrotic tissue and bacteria by phagocytosis (46, 95, 98, 103); PDGF also stimulates mitogenic responses in the tendons in a dose-dependent manner (46, 107, 108).

The proliferative stage begins approximately 48 hours after the injury, when the cytokines released by intrinsic cells of endotenon and epitenon, and by macrophages, recruit fibroblasts (95, 97, 98, 109); it is characterized by the expansion of the ECM, increased cellularity, deposition of fibrovascular scar (95, 96, 103) and other components as proteoglycans (46). In fact, PDGF causes the expression of other growth factors by stimulating DNA and protein synthesis (95, 97), while IGF-I and TGF-β1 expression remains high throughout the second phase of the healing process, causing the continue recruitment of fibroblast at the lesion site and subsequently the increase in ECM production (46, 95, 108, 110-113). An over-expression of TGF- β1 though, causes tissue fibrosis (46, 114). The expression of TGF-β1 receptors is upregulated after the injury, reaching their peak at 14 days post-injury, and gradually decreasing after day 56 post-injury; the highest receptor expression is located at the tendon sheath and epitenon of injured tendons (46, 115).

During the repairing stage, water and glycosaminoglycan levels remains high (46).

Other growth factors are also expressed during this phase. Basic fibroblast growth factor (bFGF) is expressed by tenocytes, fibroblasts and inflammatory cells and promotes angiogenesis, regulating cellular migration and proliferation (1, 46, 95, 97, 98, 116, 117, 118), as well as the vascular endothelial growth factor (VEGF) which also promotes angiogenesis and increases capillary permeability (1, 95, 97, 119, 120, 121); VEGF peak expression is reached 10 days post-injury (46). As collagen synthesis is a high oxygen consuming process, these growth factors are very important in this time of healing (95, 96, 122).

During tendon healing, nitric oxide synthases (NOS) are also expressed, as they mediate VEGF-induced vasodilation in endothelial cells (46, 123); their inhibition causes a decrease in ultimate tendon's cross-sectional area and tensile strength (46, 124).

The remodelling phase overlaps with the proliferative one and begins about 14 days – 6 weeks post-injury (95, 46). During this stage, the newly deposited collagen fibers are reorganized, leading to the alignment of collagen fibers and tenocytes in the direction of stress. This process causes an increase of fibrous matrix, a gradual decrease in cellularity, type III collagen, vascularity, cellularity, and water content (95, 97, 103); also, tenocytes' metabolism and tendon's vascularity declines (46). Type III collagen fibers are replaced with type I fibers that have a higher tensile strength (95, 96, 125). Within the remodelling stage, two different phases can be recognized: consolidation and maturation (6, 126).

The consolidation phase begins approximately 6 weeks post-injury and continues until week 10; during this period tenocyte metabolism is still high and the repair tissue changes into fibrous tissue, with collagen fibers and tenocytes aligning in the direction of stress (6, 46, 127). After this changes,

the maturation phase occurs, with the fibrous tissue gradually changing into scar-like tendon tissue (6, 46, 127). This change can take up to one year and, during the latter half of this period, a decline in tenocyte metabolism and tendon vascularity occurs (6, 128).
Even though the remodelling process continues for years after the injury, the newly formed tissue never reaches the native biochemical, ultrastructural and biomechanical properties of a normal physiological tendon (46, 95, 99, 129, 130), and healing generally results in scar tissue formation (46). During the healing process, fibroblasts generate a force on the ECM (referred to as fibroblast contraction) in order to close the wound (46, 131); when excessive, the contraction may cause wound scarring, whereas its inhibition leads to impaired wound healing (46, 132, 133).

When present, lesions to the OTJ should also be considered, as they cause bone loss and impaired function at first (46, 134), and then require a long time to heal, resulting anyway in a tissue with inferior biomechanical proprieties (46, 78, 135, 136).

Tendon's mechanical loading should be strictly controlled during wound healing, as it effects the quality of the newformed tissue. During the inflammatory phase, tendon's stretching should be avoided to minimize interferences with the healing process. About 1-week post-injury, controlled tendon mobilization should be introduced, as it enhances the quality of the healing process: tendon's ultimate tensile strength is enhanced, gliding surfaces are restored with a reduction of tissue adhesion, and the excursion proprieties are improved (46, 75, 88, 137, 138); scar formation is also reduced (1, 139). When applied on a chronic tendinopathy, mechanical loading seems to relieve symptoms (46, 140).
These beneficial effects of mechanical loading are due to its stimulation of fibroblast proliferation and activity, and of collagen realignment (46, 141, 142).

CHAPTER 3: TREATMENTS FOR TENDINOPHATIES

Lameness due to musculoskeletal disease is the most common diagnosis in equine veterinary medicine, with ligament and tendon injuries accounting for 50% of them and resulting though as the most common lesions (95, 143). These injuries can affect both athletic and sedentary horses and have a significant impact on their athletic performance but also on their quality of life (95, 144).
Even though both tendons and ligaments have the ability to repair themselves, the repaired tissue never reaches the native tissue biomechanical proprieties: tendons and ligaments are poorly vascularized tissues consisting of few cells lying in abundant extracellular matrix, so the healing process is slow and leads to the formation of scar tissue, that does not have the same biomechanical characteristics of the native tissue. For these reasons, there has been a great interest in treatments for these injuries. Moreover, the economic cost of the treatments required is very high and reinjury rates can reach 80% (144, 145, 146), therefore regenerative medicine by the application of innovative treatment has now been taken into consideration.

3.1 Conventional treatments

Acute tendon and ligament injuries require prompt treatment to reduce inflammation rapidly, as these injuries can be considered clinical emergencies and persistent inflammation can cause further damage.

3.1.1 Physical therapies

Physical therapy is used mainly in the early stages of tendonitis to reduce inflammation and, therefore, the degeneration of the ECM caused by the action of proteolytic enzymes released during inflammation, such as collagenases and matrix metalloproteinases (MMPs), especially MMP-1 (146).

- Cold therapy

  Cold therapy can be particularly useful after an acute injury, during the inflammatory stage, as it has both an anti-inflammatory and analgesic action. Indeed, the cold causes an increase in blood vessels constriction, decreases enzymatic activity, thus reducing the generation of inflammatory mediators, and slows down the nerve conduction (147, 148). The vasoconstriction also leads to a decrease of tissue swelling.

The optimal duration and frequency of cold treatments has not been defined yet. The application of a 20 minutes treatment up to three times a day is the most commonly used protocol for acute tendon injuries. It is recommended not to apply cold therapy for periods longer than 30 minutes as it could cause tissue damage: excessive cold causes vasoconstriction that leads to a decrease in the blood supply resulting in tissue necrosis.

There are many available possibilities to provide cold therapy; the most common ones are ice packages, cold hydrotherapy, spas, and underwater treadmills.

Hydrotherapy is superior to ice packages as it increases the contact surface and evaporation, and is less likely to cause adverse reactions, as superficial tissue damage or cold induced nerve palsy (147, 149).

Spas are even more efficient in providing cold hydrotherapy as, using hypertonic saline, they provide both cold and compression (147, 150).

During the rehabilitation program, also underwater treadmills can be used, as they allow the horse to regain its musculoskeletal normal condition without bearing to much weight on the affected limb.

Cold therapy can be used both after injury and surgery, being more effective in the first 24-48h in the latter case.

- Heat therapy

  Heat causes vasodilation, increasing therefore local circulation; it also induces muscle relaxation and, consequently, increases extensibility along with reducing muscle spasms and associated pain (151).

  The increased blood flow increases tissue oxygenation and two or three times the cellular metabolic rate (for a tissue temperature increase of 10°C) (151).

  Heat should be applied after the inflammatory stage in order to enhance wound healing.

- Compression and coaptation

  Following injury, during the acute phase, compression applied to the affected limb can reduce the swelling (edema) as it increases the interstitial hydrostatic pressure. In most cases, a suitable pressure level can be achieved through a modified Robert Jones bandage.

  In particular cases, a splint or a cast may be necessary, especially in case of severe injuries where there is hyperextension of the metacarpophalangeal joint (MCP joint). Specially designed support boots can also be used.

When collateral ligament injury is associated, the joint becomes instable and external coaptation is necessary. If the joint is not significantly destabilized, corrective farriery might be sufficient: a shoe with increased width on the side of the affected collateral ligament, can reduce the strain on the injured ligament by impending downward vertical movement while the horse is exercised on a soft ground (147).

- Controlled exercise

Controlled exercise is a very important part of tendon and ligament injury rehabilitation; it should be strictly controlled as, if excessive, it can cause further damage.

Controlled tendon mobilization helps to resolve residual inflammation, enhances tendon's ultimate tensile strength, restores gliding surfaces with a reduction of tissue adhesion, and improves the excursion proprieties (46, 75, 88, 137, 138, 147); scar formation is also reduced (1, 139).

Mechanical loading also promotes optimal collagen remodelling: it stimulates fibroblast proliferation and activity along with collagen fibers realignment (46, 141, 142, 147, 152).

The rehabilitation program should provide a controlled and ascending exercise program in order to optimize the newly formed tissue without causing further damage; therefore, the program should be studied from the ultrasonographic appearance of the lesion and adapted over time on the basis of serial ultrasonographic monitoring and clinical signs (such as lameness, swelling, heat, and pain). There is an individual variability between different subjects, and the rehabilitation protocol should take it into consideration.

Most tendon injuries require at least 8 months of rehabilitation before complete resumption of function, reaching up to 18 months in severe cases.

- Counter-irritation

Counter-irritation is a method that has been used for several years for the treatment of tendon and ligament injuries, but recent studies have concluded that it is not an effective treatment, and it might also cause further damage.

Chemical or thermal cauterization, also known as "firing", involves the use of topical iodine or mercury-based products, or of heated bars or pins that are applied on the skin over the injured tendon; sometimes heated pins are also used to penetrate the tendon. These techniques are performed under general or local anesthesia, as they can be very painful for the horse.

No histological differences have been found between the collagen arrangement in cases of tendinopathies treated with firing over control cases, whereas it has been demonstrated that this

practice causes the skin of the region that has been cauterized to become thinner and weaker (147, 153).

It has been postulated that any benefit resulted from counter-irritation techniques is the result of the enforced rest and protective bandage that this practice requires. Therefore, this treatment method should no longer be used.

3.1.2 Pharmacologic management

Physical therapy can improve and accelerate the healing process, but in the first stage of the healing process, pharmacotherapy may help to contain the pathological process. The first 24-48 hours post-injury can be considered the critical period on which drugs can help the most.

The first stage of the healing process is inflammation: inflammation leads to the recruitment of inflammatory cells, in particular neutrophils and macrophages, that clean the wound site from necrotic tissue, stimulates the recruitment and activation of fibroblasts, and promotes the release of inflammatory cytokines that provide further cell recruitment but also causes pain, limiting therefore the animal range of motion (ROM) preventing further damage (1,46, 95-106).

On the other hand, excessive inflammation can lead to further tissue damage; for this reason, anti-inflammatory drugs are generally used in case of tendon or ligament injuries.

Both systemic corticosteroids and nonsteroidal anti-inflammatory drugs (NSAIDs) can be used for inflammation management during the acute stage.

The main drugs that are commonly used are:

- Phenylbutazone: NSAID administered at a dose of 2.2mg/kg twice daily (BID); it seems to have more an analgesic than anti-inflammatory effect (147, 154).

  The main side effect that can follow the use of NSAIDs in the horse is the formation of gastrointestinal ulcers.
- Dexamethasone: systemic corticosteroid administered at a dose of 0.1mg/kg at a single dose (SID); as systemic corticosteroids inhibit fibroplasia, hence tendon regeneration, they should be used only within the first 24-48 hours. The risk of laminitis induction should also be taken into consideration when using these drugs; another side effect of corticosteroids is the immunodepression induction.
- Topical or intravenous dimethyl sulfoxide (DMSO) can also be used to reduce inflammation, but recent studies has shown that high concentrations of topical DMSO may weaken the normal tendon tissue (147, 155).

3.1.3 Surgical therapies

When tendon or ligament injuries cannot be treated with drugs or physical therapy, a surgical approach can be useful. Different techniques have been described over the years.

- Tendon splitting

  Tendon splitting technique was introduced in the 1940s to treat chronic tendinopathies, as it was thought that it could increase the blood flow to the damaged tendon. Subsequent research demonstrated that tendon splitting in chronic lesions causes a massive granulation tissue formation and induces further damage to the tendon itself, resulting in persistent lameness after treatment (147, 156, 157). Tendon splitting is therefore no longer suggested as treatment for chronic tendinopathies.

  Nowadays, this surgical treatment is used in acute lesions where an anechoic core lesion is seen on ultrasonographic examination; this image indicates the presence of a seroma or hematoma within the tendon fibers. It is thought that a core lesion of this nature within the tendon causes a compartment syndrome, with consequent decreased tendon perfusion and ischemia of the injured region (147, 158); the presence of fluid also induces a proximo-distal propagation of the lesion. For this reason, tendon splitting in acute cases is used to decompress the core lesion by evacuating the fluid and facilitating vascular ingrowth (147, 158).

  Tendon splitting can be performed under general anaesthesia or standing sedation with the use of a 11-scalpel blade or a 23G needle: the knife is inserted into the tendon and then fanned proximally and distally, while the needle is inserted multiple times, probably resulting in lower damage of the remaining intact tissue. Needle splitting can also be associated to intralesional injections.

  The procedure can be done blindly or with ultrasonographical guidance; in the first case, the blade or needle is inserted in the point of lower resistance, while with ultrasound guidance the insertion can be made in the point where the lesion is closest to the skin, in order to minimize damage to normal tendon tissue.

  After the surgery has been performed, a modified Robert Jones bandage should be applied, and the horse should be put in stall rest for at least 10-14 days; after this period, a controlled exercise program should be initiated (147).

  The procedure is shown in *Figure 3.1*.

- Desmotomy of the accessory ligament of the SDFT

  The desmotomy of the accessory ligament of the SDFT (DALSDFT) was designed as treatment for SDFT tendinopathy; the production of a functionally longer musculotendinous unit should reduce the strain on the SDFT (147, 159). However, studies in horse cadaver models have shown that this surgery actually increases the strain on the SDFT during loading as it increases the extension of the MCP joint (147, 160); *in vivo* studies have also demonstrated that a higher risk of injury of the suspensory ligament is associated to this procedure (147, 161).

  This procedure can be performed by making a 10cm skin incision in the medial aspect of the limb, between the cephalic vein and the caudal radius, with the horse in lateral or dorsal recumbency (147, 162). Recently, the DALSDFT has also been performed tenoscopically, through the carpal sheath (147, 163).

  After surgery, the horse should be put to stall rest for 14 days, after that a controlled exercise program should be started.

- Anular ligament desmotomy

  The anular ligament desmotomy is a surgical technique indicated in serious inflammation, tendinopathies, or diffuse adherences of the SDFT and DDFT in the region of the MCP and metatarsophalangeal (MTP) joint; the procedure is therefore indicated when the normal gliding function of the flexor tendons is impeded.

  This surgery is performed under general anaesthesia; the tenoscopic approach is preferred to other closed or open techniques as it ensures an accurate transection of only the palmar/plantar anular ligament and allows tendons evaluation in order to identify possible primary causes. This approach is also less traumatic as it requires only small entry wounds with less trauma to the surrounding tissues and subsequently a better wound healing with less risk of dehiscence resulting in an earlier postoperative exercise (147).

- Fasciotomy and neurectomy of the deep branch of the lateral plantar nerve

  In cases of chronical proximal suspensory ligament desmopathy (PSLD) of the hindlimb that are not responsive to conservative treatment, tibial neurectomy has been reported to be a successful treatment (147, 164). It has later been described a more specific neurectomy that sees the transection of the deep branch of the palmar nerve only (147, 165).

  When this surgery is needed, it is associated with the fasciotomy of the connective tissue adjacent to the lateral splint bone that covers the suspensory ligament, as hindlimb PSLD is thought to be related to a compressive compartment syndrome that involves the plantar metatarsal nerves.

The surgery is done under general anaesthesia. The horse is the put to stall rest for 14 days, to allow tissues healing; then a controlled gradual exercise program can be started.

This surgery is reported to allow horses to return to full work in high-level competitions with minimal risk of exacerbating the desmopathy (147, 164).

- Desmotomy or desmectomy of the accessory ligament of the DDFT

  In those rare cases where desmopathy of the accessory ligament of the DDFT (ALDDFT) recurs after conservative treatment or causes adhesions between the ALDDFT and the SDFT or a flexural deformity, desmotomy of the ALDDFT can be a valid approach.

  For this surgery general anaesthesia is required, and after the procedure, a modified Robert Johnson bandage should be applied to the distal limb for 3-4 days.

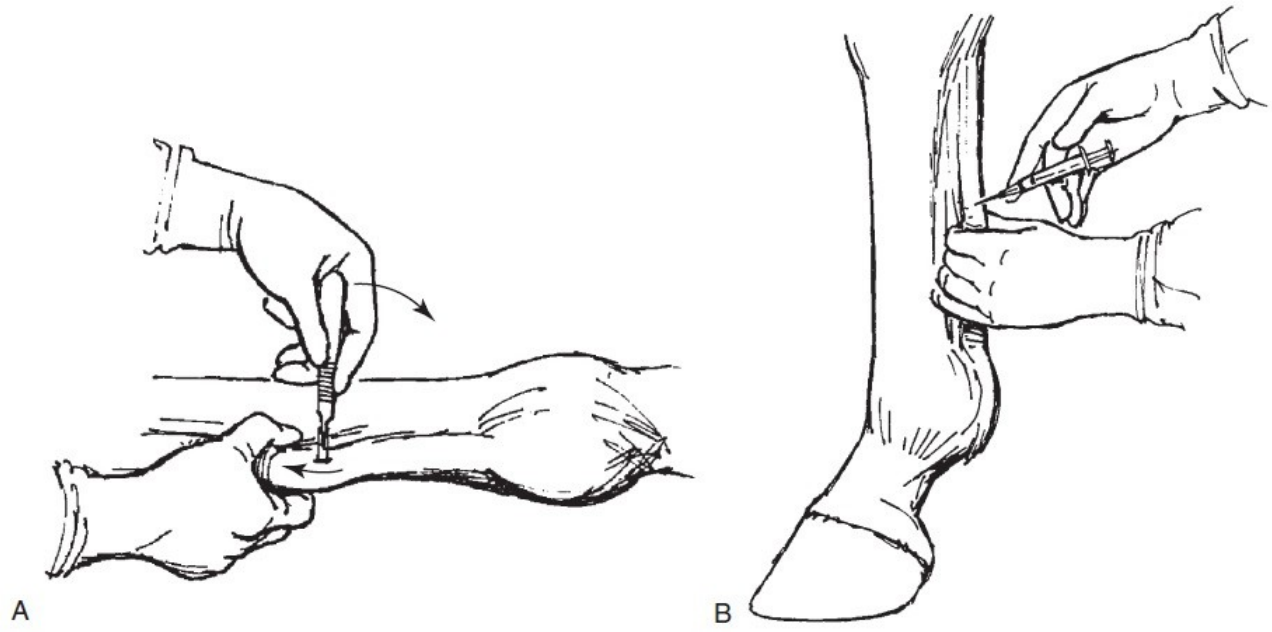

Figure 3.1: Tendon splitting performed with a blade (A) or a needle (B). (Equine surgery, Auer & Stick, 2012)

3.2 Regenerative medicine

The aim of regenerative medicine is not only to provide wound healing, but to regrow, repair or replace damaged cells, tissues or even organs, leading to restore the normal function of the injured tissue.

In the case of tendon and ligament lesions, regenerative medicine aims to restore both the structure of collagen fibers and their biomechanical proprieties, so that after the lesion as healed, tendon's structure and function is the most possible similar to the one of the original tissue; this allows the horse to get back to the same activities he was used to and, moreover, to the same performance level with minimum risk of reinjury.

3.2.1 Physical therapies

With the introduction of new technologies and a higher comprehension of the mechanisms that lead to tissue damage, new physical therapies have been introduced in the equine practice.

- Extracorporeal shock wave therapy

  Extracorporeal shock wave therapy (ECSWT) consists of shock or pressure waves transmitted into the tissues where a specific probe is applied; this kind of therapy is primarily used in the horse for chronic desmopathies.

  The exact mechanism of action of ECSWT is still unclear, but it is likely related to an effect on sensory nerves that induces analgesia (147, 166). Even though the use of ECSWT on normal tendons or ligaments can induce injuries, resulting in ECM disorganization, it was acknowledged that this initial disorganization functions as a stimulus for tissue repair in case of chronic injuries (147, 167).

- Therapeutic ultrasound, laser, and magnetic fields

  Even though there still is a paucity of scientific studies that prove their efficiency, and their effect on tissues is poorly understood, in the last decade these techniques have gained relevance in equine practice.

  Therapeutic ultrasound is thought to influence tissue regeneration due to the conversion of ultrasound energy into thermal energy, resulting in increased vascularization and fibroblastic proliferation (147, 168).

Therapeutic ultrasound works by alternating compression and rarefaction of sound waves with a frequency of 0.7 to 3.3 MHz As the intensity decreases as the sound waves penetrate deeper, the soft tissue maximum energy absorption occurs from 2cm to 5cm.

Low-level laser works through light amplification by stimulated emission of radiation; low-pawer lasers are applied to the patient's skin. This technology stimulates cellular metabolism, fibroblast proliferation and collagen synthesis (147, 169).

Although magnetic therapy is widely used by horse owners, its efficacy on enhancing tissue regeneration has not been proved. It involves a weak static magnetic field generated by a permanent magnet (147).

3.2.2 Intralesional medication

Many intralesional medications have been advocated for tendinopathy treatment.

These medications can be performed under general anaesthesia or standing sedation; it seems that weight-bearing medication is a better option as it keeps the tendon tense, potentially assisting injection.

These medications are mainly executed blindly, simply injecting the medication when lower tendon resistance is detected with the needle. The use of an ultrasound machine can provide a more accurate medication, allowing to reach the core of the lesion, and minimizes tissue damage as the needle can be inserted from the point of lower skin-lesion distance.

It is very important to wait at least 3 days post-injury before performing any intralesional injection, as in the first days (in the inflammatory stage) there is a higher haemorrhage risk.

The volume of the injection should vary between different cases, as the lesion extension must be considered; indeed, large volumes can damage the healing tissue (147, 170).

- PSGAGs

  Polysulfated glycosaminoglycans (PSGAGs) are used as soft tissue anti-inflammatory agents in the treatment of tendinopathies and desmopathies, either intralesionally or intramuscularly (147).

  PSGAGs are used for their anti-inflammatory effect as they have no effect on fibroblasts but inhibit collagenases, metalloproteinases and also macrophage activation, leading to a decrease in the inflammatory response (147, 171, 172).

  While the influence of this treatment on tissue healing is still debated, some studies have shown an improvement in tendon echogenicity after injury, with faster resolution of the core lesion and a higher rate of animals returning to their previous activities (147, 173, 174).

❖ Hyaluronan

Hyaluronan (HA) is a component of the ECM that consists of repeated units of D-glucuronic acid and N-acetyl-D-glucosamine. Over the years HA has been administrated intralesionally, peritendinously, intratechally, and systemically with the attempt to treat tendinopathies.

Many studies have been conducted to understand the potentiality of HA, and it seems that it does not decrease reinjury rate, and has no effect on ultrasonographic or histologic appearance, and biomechanical properties (147, 175); on the other hand, intrathecally administered HA have been shown to decrease the extent of adhesion within the digital sheath (147, 176). Moreover, horses treated with intrathecal HA showed less intratendinous haemorrhage and less infiltrated inflammatory cells (147, 176).

3.2.3 Novel therapies

The research of new therapies that allow a better healing process, resulting in regeneration rather than reparation, is still ongoing. Some of the most promising therapies will be briefly described in this paragraph.

❖ Growth factors

Growth factors are natural substances, generally secreted proteins or steroid hormones that act as signalling molecules intervening in the regulation of cellular processes, such as cellular proliferation, wound healing and, sometimes, cellular differentiation. Each growth factor has its own target and action.

- IGF-I is a potent mitogen that stimulates the tendon EMC synthesis (147, 177) as well as inducing tenocytes proliferation (178). Studies both in vitro and in collagenase-induced lesions have been done, but long-term follow-up data are still missing.
- TGF-β1 is a cytokine produced by white blood cells lineages; it is considered to be a potential beneficial growth factor, but clinical experience is still limited. After binding to its receptor, TGF-β1 activates a signalling cascade, ultimately leading to the induction of the transcription of genes that function on differentiation, proliferation, chemotaxis, and activation of many immune cells. After treatment with TGF-β1, horses showed tendon enlargement; even though the reinjury rates were similar to the ones of conservatively managed horses, the reinjuries were all on the contralateral untreated limb (147, 179).

- Platelet-rich plasma

  Platelet-rich plasma (PRP), is defined as plasma containing at least twice the concentration of normal platelets; it is derived from whole blood centrifuged to remove red blood cells, or by gravity filtration.

  PRP is a reach source of growth factors, in particular: PDGF, TGF-β1, and VEGF. These growth factors enhance tissue healing, stimulating cellular proliferation and matrix synthesis.

  PRP has also been proved to reduce pain and improve function in cases of chronic tendonitis.

- Bone marrow

  Bone marrow has a high growth factor concentration; for this reason, it can be used for the promotion of tissue healing. Bone marrow is primally used in tendinous and ligamentous lesions to promote tissue regeneration rather than scar tissue formation (147, 180, 181).

  Bone marrow also contains mesenchymal stem cells, but their concentration is very low (1 in 10^4 nucleated cells), so treatment whit bone marrow resembles more a growth factor treatment rather than a stem cell therapy.

- Mesenchymal-stem cells

  Intralesional injection of MSCs is used to achieve regeneration rather than repair.

  MSCs are multipotent cells that have therefore the ability to differentiate into tenocytes and to generate tendon ECM, creating a healing tissue with biomechanical proprieties that are far superior to the ones of the normal scar tissue. It is also reported that the reinjury rate in horses treated with MSCs for tendinous or ligamentous injuries is lower than in horses that have followed a conventional therapeutic protocol (147, 182, 183, 184).

  Stem cells' biology and therapeutical proprieties will be furtherly described in the following chapter.

CHAPTER 4: MESENCHYMAL STEM CELLS

The ability of stem cells to replicate and differentiate into different cellular types has gained great interest in regenerative medicine. Clinicians, researchers, and industry members are therefore studying the possibility to harvest stem cells form horses and use them for regenerative purposes.
Moreover, their use is particularly of interest because of their low immunogenic characteristics, which allow the use of allogeneic MSCs without risks of immunoreaction.

4.1 Mesenchymal stem cells' biology

Stem cells therapy is widely spread in veterinary medicine, but the comprehension of the biology underlying their therapeutic effect is still to be investigated.
Stem cells are able to replicate through a process of self-renewal and have the ability to differentiate into different cellular types of the body. These peculiar characteristics are particularly important during embryonic development, as they intervene in organogenesis, but also in adult's life, for the maintenance of tissue physiological turnover, and in tissue regeneration after injury (147, 157).
MSCs are also capable of a peculiar replication mechanism called asymmetric cell division; this process sees some daughter cells develop into a somatic cellular type, while the others retain their stem cell identity within the niche tissue (147, 185).
Different cells have a different ability to differentiate into other cellular types, which is defined as cell potency: the more cellular types a stem cell can differentiate into, the greater its potency is. The cells with the highest differentiation potential are defined totipotent; following a decreasing order in the number of cellular types that a cell can turn into, there are pluripotent, multipotent, oligopotent, and finally unipotent cells. For this reason, stem cells are usually divided into embryonic and adult stem cells.
Embryonic stem cells derive from the inner cell mass of the blastocyst mammalian embryo stage and can differentiate into cells belonging to all three germinal tissues (ectoderm, mesoderm, and endoderm); they are therefore defined pluripotent cells (147, 186, 187). Embryonic stem cells are capable of unlimited, undifferentiated proliferation *in vitro*, but there is still lack of evidence of the presence of these characteristics *in vivo* (147, 188, 189).
After birth, adult stem cells, can be found in many tissues in particular microenvironments called niches; their characteristics vary on the base of the tissue where they are located (147, 190, 191). The stem cells that are involved in the musculo-skeletal regeneration process are mesenchymal cells; even though neither mesenchymal nor stromal are terms that appropriately describe the characteristics of

these adult stem cells, these terms have been used interchangeably, and the term MSCs will therefore be used from this point forward.

Many biological characteristics of adult stem cells are still to be understood: while injuries can activate quiescent MSCs stimulating self-renewal, aging and diseases cannot (147, 191, 192); the signalling pathways that preserve MSCs within the niches in an undifferentiated state and those regulating their activation are also still to be understood (147, 193, 194, 195).

MSCs are described as adherent, clonogenic, non-phagocytic, fibroblastic cells that are capable to differentiate, both *in vitro* and *in vivo*, into a variety of somatic mesenchymal phenotypes (196): bone (147, 197, 198), cartilage (147, 199), tendon (147, 200, 201), muscle (147, 202), and adipose tissue (147, 202). It has also been described the ability of MSCs to differentiate into other mature somatic cells, by a process called transdifferentiation (or lineage reprogramming); this process allows MSCs to tourn also into hematopoietic-supporting stroma (147, 203, 204), cardiomyocytes (147, 205), pneumocytes (147, 206) and neural cells (147, 207), even though the functional ability of MSCs-derived neural cells is still debated.

4.2 MSCs' role in inflammation and immune response modulation

Regenerative medicine is particularly focusing on MSCs as, in addition to their ability to differentiate into different tissues enhancing tissue regeneration rather than repair, they also have an immunomodulatory effect. In fact, MSCs inhibit B-cells function, T-cells activation, and dendritic cell maturation, and have a high protective effect against allograft rejection and experimentally induced autoimmunity (147, 208-211).

As cellular culture requires some time to be performed in order to have a sufficient amount of stem cells for a treatment, studies have been conducted on the use of allogenic cells; this would allow to store cells so that, when a treatment is necessary, there is availability of ready-to-use cells. It has been shown that allogenic cells are as efficient as autologous cells, suggesting that the immunosuppressive action of MSCs is not restricted by class I major histocompatibility complex (MHC) (147, 212-215). Some studies also focused on the detection of histological lesions following treatment with allogenic MSCs, concluding that, as lesions could not be found after 8 weeks, these treatments potentially do not trigger an immunologic response, allowing the use of stocked cells for equine treatments (147, 216, 217). These low immunogenic reactions of the host are probably due to the absence of the MHC-II, normally involved in the immune system antigen recognition process (218).

New studies are now focusing on the potential anti-inflammatory effect of co-culture of MSCs and lymphocytes; it has been shown that these MSCs secrete IL-10, a growth factor that mediates T-cells

response and can antagonize the effects of IL-12 during inflammation (147, 219, 220). They also secrete TGF-β1, a T-cell suppressor that can be used in acellular treatments to modulate inflammation (147, 221, 222).

4.3 MSCs in the treatment of tendonitis

Tendon healed tissue generally lacks normal tendons' biomechanical characteristics: fibrous scar tissue takes the place of normal tenocytes, collagen fibers are not as well-aligned as they used to be, therefore tendon elasticity decreases and the risk of reinjury gets higher (147, 223).

MSCs have been considered in the treatment of tendon injuries as they provide the area with growth factors that may improve the healing response and can differentiate into tenocytes following the in vivo transfer (147, 224-229).

Many studies have been conducted on the efficacy of MSCs treatments for tendinopathies; it can be concluded that treatment with stem cells improves tendon repair by improving fiber organization and alignment, allowing horses to return to precious levels of work with a lower reinjury rate (147, 225, 230-233).

CHAPTER 5: AIMS OF THE STUDY

In equine practice, lameness due to ligament and tendon injury is the most common diagnosis, affecting both athletic and sedentary horses with a severe impact on their quality of life (95, 144).
As tendon and ligaments are poorly vascularized and have a poor cellularity, natural healing occurs very slowly and the replaced tissue does not have the same biomechanical characteristics; moreover, reinjury rate reach the 80% (144, 145, 146).
Regenerative medicine allows though the repairment of the tissue, with the restoration of the normal function allowing the return to the same performance level with a minimum risk of reinjury.

The aim of this thesis was to evaluate the clinical response to a repeated intralesional tendon injection of autologous adipose-derived mesenchymal stem cells (AD-MSCs) combined with autologous platelet rich plasma in two horses chronically affected by tendonitis.
The follow-up period after treatment included clinical evaluation of lameness and pain, along with ultrasound examinations: size of the lesion, fiber pattern and alignment plus new tissue echogenicity were evaluated.
Different plasma molecules values were also estimated, in order to assess the inflammatory state of the animals: total protein level (PT), advanced oxidises protein products (AOPP), total carbonyl groups (CT), thiols, IL-1β, IL-10, PDGF, TGF-β1 and IGF-1.

CHAPTER 6: MATERIAL AND METHODS

This thesis was conducted on two sport horses affected by tendinopathy of the forelimb; they were treated with autologous AD-MSCs and PRP administered by ultrasound guided intralesional injection.
AD-MSCs were isolated and cultivated in our laboratory and cryopreserved for further injections.
Several clinical evaluations followed the treatment to evaluate lameness, pain, ultrasound appearance of the lesion site and different inflammatory plasma molecules.

6.1 Animals

Subject n° 1 was a 10-year-old Sella Italiano gelding, competing in show jumping, presented with a lesion in the of the SDFT of the left forelimb in the middle third of the metacarpal region. The lesion was a reoccurrence, which had developed from a previous healed injury in the same area of the SDFT.
At diagnosis, the horse showed a lameness grade 2.5/5 based on the American Association of Equine Practioners (AAEP) scale, briefly described in Table 6.1. Pain and local heat were noted at palpation along with severe swelling. An ultrasound evaluation of the lesion was also done, showing complete loss of tendon's structure in the lesion site.
When the lesion was first diagnosed six months before this study, the horse stopped competing and was treated with NSAIDs. A controlled rehabilitation exercise program was then started after two weeks of stall rest. However, the horse did not show any improvement at the clinical or ultrasonographic level.
The followed rehabilitation protocol is shown in *Table 6.2*.

Subject n° 2 was a 7-year-old trotter, racing at high level events that was presented with a lesion of the SDFT of the right forelimb in the distal third of the metacarpal region. The lesion was acute, as it occurred during racing activity on the day before the clinical evaluation.
At diagnosis, the horse showed a lameness grade 4/5 based on the AAEP scale. Pain and local heat were noted at palpation along with severe swelling of the whole metacarpal region and MCP joint. An ultrasound evaluation of the lesion was also done, showing complete loss of tendon's structure in the lesion site that was particularly extended on the proximo-distal axis.
When the lesion was diagnosed, the horse was put at stall rest and treated with NSAIDs for seven days.

Score	AAEP degree of lameness
0	Lameness not perceptible under any circumstances
1	Lameness is difficult to observe and is not consistently apparent, regardless of circumstances
2	Lameness is difficult to observe at a walk or when trotting in a straight line but consistently apparent under certain circumstances
3	Lameness is consistently observable at a trot under all circumstances
4	Lameness is obvious at a walk
5	Lameness produces minimal weight bearing in motion and/or at rest or a complete inability to move

Table 6.1 Clinical AAEP scores to assess lameness.

Typical Exercise Program after Tendon Injury

Exercise Level	Weeks	Duration and Nature of Exercise
0	0-2	Stall rest
1	3	10 minutes walking
1	4	15 minutes walking
1	5	20 minutes walking
1	6	25 minutes walking
1	7	30 minutes walking
1	8	35 minutes walking
1	9	40 minutes walking
1	10-12	45 minutes walking
WEEK 12: REPEAT ULTRASOUND EXAMINATION		
2	13-16	40 minutes walking and 5 minutes trotting daily
2	17-20	35 minutes walking and 10 minutes trotting daily
2	21-24	30 minutes walking and 15 minutes trotting daily
WEEK 24: REPEAT ULTRASOUND EXAMINATION		
2	25-28	25 minutes walking and 20 minutes trotting daily
2	29-32	20 minutes walking and 25 minutes trotting daily
WEEK 32: REPEAT ULTRASOUND EXAMINATION		
3	33-40	45 minutes exercise daily with slow canter, gradually increasing in amount
3	41-48	45 minutes exercise daily with fast work three times a week
WEEK 48: REPEAT ULTRASOUND EXAMINATION		
4	48+	Return to full competition/race training

Table 6.2 Typical rehabilitation protocol. (Equine surgery, Auer & Stick, 2012)

The program can be shortened or lengthened depending on the severity of the lesion and the progress of the patient.

6.2 AD-MSCs

The treatment was performed with autologous stem cell s after expanding them in vitro, in order to obtain a proper number to perform the treatment.

6.2.1 Isolation

The adipose tissue was collected from the region above the dorsal gluteal muscle, at the base of the tail, because of the ease of access and absence of large veins.
The horse was intravenously sedated with 0,01mg/kg detomidine (Domodesan®, Orion Pharma, Italy); then the area was shaved, aseptically prepared, and locally anesthetized with 2% lidocaine (Lidor®, Richter Pharma AG, Italy).
An incision of approximately 5-6 cm in length was made parallel 15 cm lateral to the spinal column, in order to allow visualization of adipose tissue between the skin and the musculature. Afterwards, approximately 4 g of subcutaneous adipose tissue was collected and stored in proper medium for transport, consisting of phosphate buffer saline (PBS) supplemented with penicillin-streptomycin (10%).
The incision site was then sutured with absorbable stitches on two different levels: subcutaneously and on the skin.
Pictures of the tissue collection can be seen in *Figure 6.1*.
The adipose sample was stored in a controlled temperature box, at a temperature of about 4°C, for the whole transport to the laboratory.
Upon the arrival to the laboratory, the sample was immediately processed under a laminar flow hood in order to guarantee sterility.
The sample was washed three times with PBS: the sample was submerged consecutively in three Falcon tubes, each containing 20 ml of PBS, util the fluid in the last tube remained completely encolour.
The adipose sample was then placed in a Petri dish containing 10 ml of PBS and then cleaned from blood vessels and connective tissue, in order not to contaminate the cellular culture with fibroblasts, that have a microscopical aspect similar to the one of AD-MSC and are for this reason difficult to distinguish.
After the cleaning process, the adipose tissue was moved in a clean Petri dish containing 5ml of PBS and cut into small pieces, that were put in a 50 ml Falcon tube with a PBS solution containing 0.01% collagenase type IA (Sigma-Aldrich, Italy) solution and kept for 1 hour at 37°C with continuous

shaking in a thermostat agitator. The small diameter of the adipose tissue pieces enhances the surface that can be digested by the collagenase, with a better result; collagenases are enzymes that can break collagen's peptide bonds, so that the cells contained in the ECM (stem cells included) are set free.
After digestion, the solution was filtered using a 100 μm cell strainer, put in a 20 ml Falcon tube and centrifuged at 800g for 10 minutes, in order to allow the sedimentation of the pellet consisting in the stromal vascular fraction (SVF); the triglycerides remain on the top of the tube, in the supernatant that was removed leaving only 5 ml on top of the pellet. 5 ml of PBS were then added, the pellet suspended, and then the tube was centrifuged again at 300g for 5 minutes.
After removing the supernatant, the pellet was suspended in the culture medium, that is composed of: 89% Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Italy), 10% bovine foetal serum (FBS; Sigma-Aldrich, Italy), 1% antibiotics (penicillin/streptomycin; Aurogene, Italy).
The cells (and the culture medium) were seeded in a cell culture flask, spreading them as well as possible to have a uniform distribution that covers the whole surface of the flask. The flask was then put in culture in an incubator that was set at 37°C with 5% CO_2 levels.

6.2.2 Maintenance

AD-MSCs when seeded in culture into flasks, adhere to the bottom of the flask; after adhesion, that usually occurs in 48 hours from the isolation, the cells start the replication process.
It is important to check the cellular culture every day in order to evaluate the confluency. Cell confluence is the percentage of the surface area (in a two-dimensional culture) that is covered with cells; confluence assessment is used to determine when cells need to be passaged, as proper timing is essential to maintaining the cell phenotype and a high culture quality. In fact, the growth of some cells, including MSCs, is limited by contact inhibition: when cells contact each other, their growth eventually stops, in a cell-density dependent manner, regulating *in vivo* tissue growth and development. Cell contact might also induce the differentiation of MSCs into fibroblasts, so it should be avoided when expanding stem cells population.
Stem cells are usually detached from the bottom of the flask and split into more flasks, in order to have a lower cellularity, before maximum confluence is reached.
The detachment process requires the use of an enzyme named trypsin, a proteolytic enzyme; the process is therefore named trypsinization.
The trypsinization of a cellular culture requires a series of different steps. First of all, as the FBS contains protease inhibitors enzymes, such as α1-antitrypsin, the culture medium has to be removed from the flask, paying attention not to touch the bottom of the flask where the cells are attached. The

flask is then gently washed with PBS from one to three times, in order to remove all of the culture medium and the dead cells that could be present. Trypsin can then be added as trypsin-EDTA 0,0025%, then the flask is put in the incubator at 37°C for 4 minutes, and cells are detached from the bottom of the flask. To be sure that the detachment process is ultimate, the flask is observed with a light microscope: detached cells are round shaped, while adherent cells are spindle shaped. Culture medium is then added to inhibit the trypsin, blocking the process so that the cells do not get damaged; PBS is also added to reach a higher volume.

The whole content of the flask is subsequently put in a Falcon tube and centrifuged at 300g for 5 minutes. The supernatant was removed, and the pellet diluted in culture medium; the content of the tube is then spread into different flasks to achieve the desired cellularity in each flask.

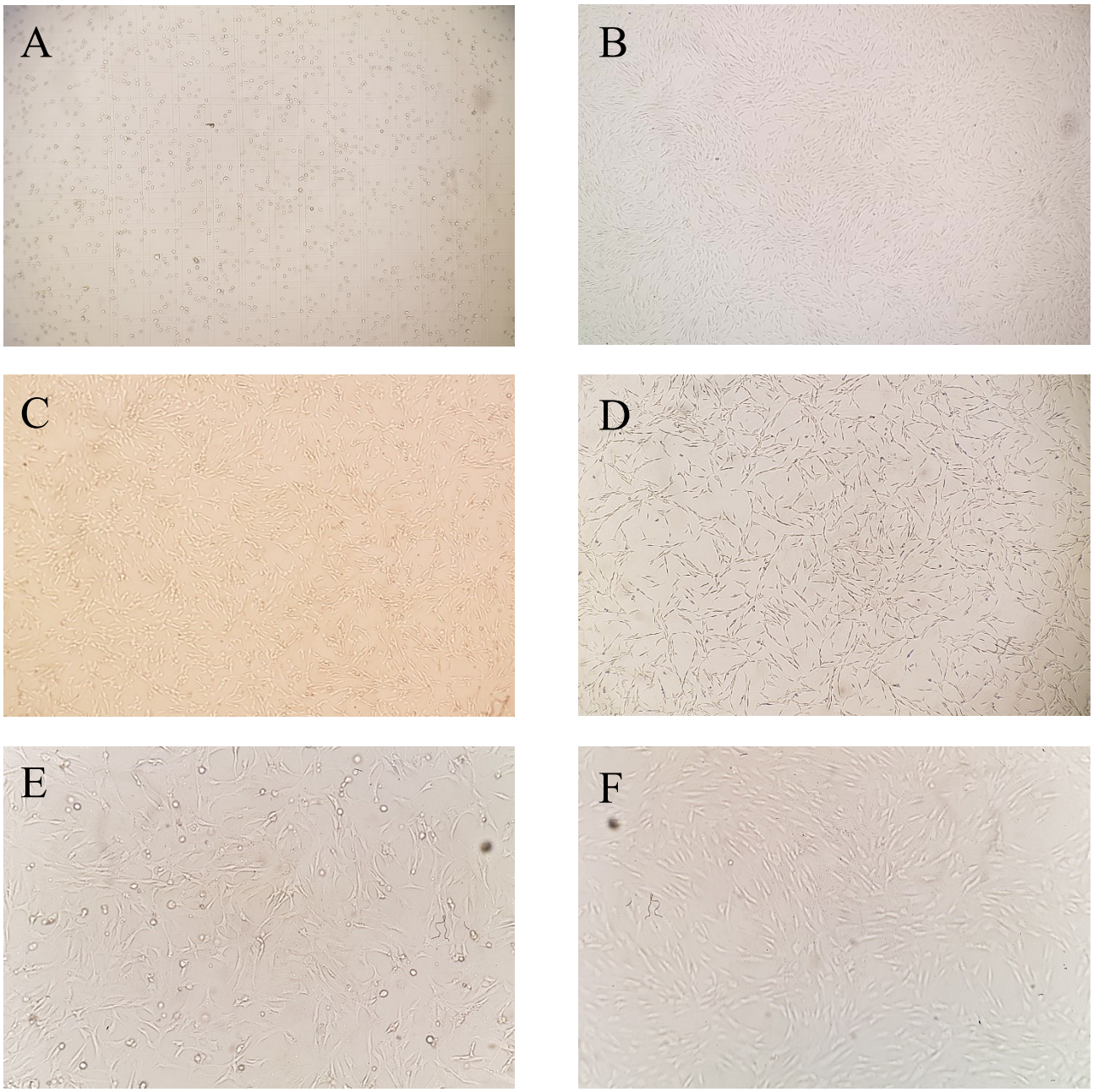

Figure 6.1: AD-MSCs in culture

(A) trypsinized cells that can be recognized by their round appearance

(B), (C) attached cells at confluence

(D) attached cells

(E), (F) attached cells at higher magnification

(G) cellular pellet at the bottom of a Falcon tube

6.2.3 Cryopreservation

MSCs can be easily stocked in a -80°C refrigerator or in liquid nitrogen if suspended in an appropriate medium. Cellular cryopreservation is very useful as it allows to keep a wanted number of flasks in culture, but mainly as it allows to store ready-to-use amounts of cells, that only need to be unfrozen before usage.

The process that allows the cryopreservation of cells is similar to the one previously described to expand the cell culture: cells need to be trypsinized, centrifuged and resuspended in an appropriate frosting medium that is made of 10% FBS and 90% dimethyl sulfoxide (DMSO). Generally, the cells are suspended in 1 ml of frosting medium and put in an Eppendorf tube; the presence of air bubbles should be avoided as they do not allow the direct contact between cells and frosting medium so the cells that lay next to the bubbles do not survive the frosting process.

Once the Eppendorf tube is properly closed, it can be stocked in a -80°C refrigerator or in liquid nitrogen. When stocking the cells at -80°C, the Eppendorf tube is put in a specific box, named Mr Frosty, that contains isopropanol (isopropyl alcohol) which allows a gradual decrease of the Eppendorf tube temperature and keeps it at a more uniform temperature. If stocking in liquid nitrogen is necessary, the Eppendorf tube is kept at -20°C for two to three hours, then moved overnight in the -80°C refrigerator and finally stored in liquid nitrogen.

6.3 PRP preparation

The treatment was performed with AD-MSCs suspended in autologous PRP.

PRP was obtained by following a double centrifugation tube method in sterile conditions as described by (234).

16 ml of whole blood were taken from each subject from the jugular vein and collected into two citrate tubes that were centrifuged for 20 minutes at 2800 rpm. In this way the blood was divided into three layers: red blood cells, PRP in the form of a gel-like plug, and platelet poor plasma (PPP) as supernatant. 80% of the PPP (approximately 4-5 ml from each tube) was discarded, and the buffy coat of each tube (containing platelets and mononuclear cells) was suspended in the remaining PPP. The final solution was centrifuged for 15 minutes at 1300 rpm, determining the sedimentation of the platelets on the bottom of the Falcon tube.

The final PRP is obtained after removing the excessive PPP on the top and resuspending the pellet with a vortex mixer in the remaining volume of plasma.

6.4 Treatment

The treatment of both horses was performed with ultrasound-guided injections of AD-MSCs suspended in autologous PRP, that were isolated and prepared for the treatment as previously described.

The cells used for the treatment were at passage 3 and 5 and were characterized by flow cytometry and *in vitro* tri*lineage* differentiation.

On the day of treatment, about $1x10^6$ AD-MSCs were diluted in 4 mL of autologous PRP. Cell viability was assessed by trypan blue staining and more than 90% of cells were viable. Before injection, the horse was sedated with 0,01 mg/Kg detomidine (Domodesan®, Orion Pharma, Italy) and 0,1 mg/Kg butorphanol (Nargesic®, Acme Srl, Italy), and the injection area was aseptically prepared. The treatment was inoculated in the lesion site using a sterile 14G needle via ultrasonic guidance.

Afterwards, protective sterile bandages were applied to the limb and the horse followed the rehabilitation protocol that was previously described and that is summarized in *Table 6.2*.

Pictures of the treatment procedure can be seen in *Figure 6.2*.

A second injection was performed 4 weeks later in order to continue the stimulation of the healing process. The applied protocol was the same as that used for the first treatment.

Figure 6.2: Clinical illustration of collection of adipose tissue and injection of MSC and PRP in the SDFT.

(A) 10 cm linear incision over the superficial gluteus muscle parallel to spinal column.

(B) adipose tissue retrieval from subcutaneous region superior to superficial gluteus muscle.

(C) Suturing of the surgery site using non absorbable monofilament nylon suture.

(D) Aspiration of AD-MSCs and PRP using a 14 G needle.

(E) swollen and inflamed forelimb due to acute tendonitis

(F). Guiding the injection direction using ultrasonography.

(G) intralesional injection of AD-MSCs and PRP using a 14 G needle in the SDFT.

(H) Securing of the treated forelimb with a two-layer bandage.

6.5 Clinical evaluation and follow up

Before treatment, the horses presented pain at palpation along with local heat and severe swelling in the mid-metacarpal region of the left forelimb in the first subject while in the second one the affected area was the distal metacarpal region of the right forearm, that presented a more severe swelling which included the MCP joint. In addition, on the AAEP scoring system the first horse showed a grade 2.5/5 of lameness while the second 4/5.

Clinical assessments were performed every 2 weeks starting from the day of the first injection. The clinical outcomes are reported on the day of injection (T0), at 4 weeks (T1) and 52 weeks (T2) after injection. The most important parameters that were considered during the follow-up evaluations were: general condition, pain, heat and swelling at the site of the injury, grade of lameness, horse keeper's evaluation.

6.6 Ultrasound examination and follow up

Ultrasonographic evaluations of the metacarpal region of both forelimbs were performed using a 7.5-MHz linear transductor probe. For each assessment, a complete examination of the SDFT was conducted by means of longitudinal and transverse scans. The obtained images were evaluated and scored (from 0 to 3) at each examination for two parameters (235, 236, 237): lesion echogenicity and lesion longitudinal fiber alignment (FA). Criteria for scoring are listed in *Table 6.3*. The contralateral healthy limb was used as comparison.

The affected SDFT presented a focal hypo-echogenic area together with an irregular fiber alignment/pattern at the level of the injury site (mid metacarpal region in the first subject and distal metacarpal region in the second subject). At T0, the tendons presented a focal hypoechoic area (Grade 2.5/3) with a low FA (Grade 3/3).

Follow-up ultrasound evaluation were performed one month and one year post treatment.

Table 6.3 Clinical and ultrasonographic scores to assess lameness, echogenicity and fiber alignment.

Score	AAEP degree of lameness	Echogenicity	Fiber alignment (FA)
0	Lameness not perceptible under any circumstances	Normal echogenicity	≥75% parallel fiber bundles in the lesion
1	Lameness is difficult to observe and is not consistently apparent, regardless of circumstances	Mildly hypoechoic	50-74% parallel fiber bundles in the lesion
2	Lameness is difficult to observe at a walk or when trotting in a straight line but consistently apparent under certain circumstances	Moderate hypoechoicity	25-49% parallel fiber bundles in the lesion
3	Lameness is consistently observable at a trot under all circumstances	Severe hypoechoicity	≤25% parallel fiber bundles in the lesion
4	Lameness is obvious at a walk	-	-
5	Lameness produces minimal weight bearing in motion and/or at rest or a complete inability to move	-	-

6.7 Plasma molecules analysis

Horse plasma was analyzed in both horses at baseline, one week and two weeks post-injection, and in subject n°2 also one week prior to treatment and one month after treatment, in order to assess different oxidative stress molecules' levels: total protein, advanced oxidation protein products, carbonyl group and malondialdehyde. Two interleukins important in the inflammatory process were also estimated: IL-1 and IL-10. PDGF, IGF-1, TGF-β1 values were also determined.

6.7.1 Oxidative plasma products

The plasma concentration of AOPP was estimated according to (238). Briefly, AOPP were measured by spectrophotometry on a microplate reader (2023 Multilabel Reader VictorX4, Perkin Elmer, Waltham, MA, USA) at 340nm. 200µL of plasma diluted 1:5 in PBS (v/v, 5mM, pH 7.2) was placed into a microtiter plate with 96 flat-bottom wells (Perkin Elmer) and 20µL glacial acetic acid (Fluka, St. Louis, MO, USA) was added. A chloramine-T solution (Sigma-Aldrich Co., St. Louis, MO, USA) was used to perform a standard curve ranging from 0-200µmol/L. In standard wells, 10µL of 1.16M potassium iodide (Reagent Plus; Sigma-Aldrich St. Louis, MO, USA) was added to200 µL of the chloramine-T solution followed by 20µL of acetic acid. The absorbance of the reaction mixture was read after 5 minutes against a blank containing 200µL PBS, 10µL potassium iodide and 20µL acetic acid. AOPP concentrations were expressed as micromoles per liter of chloramine-T equivalents.
Carbonyl residues were measured as previously described by (239) using dinitrophenylhydrazine (Sigma-Aldrich St. Louis, MO, USA). Briefly, samples were submitted to 10mM dinitrophenylhydrazine in 2.5M HCl for 1h, followed by deproteinization with 20% TCA. The proteins were washed three times in ethanol/ethyl acetate and solubilized in potassium phosphate 20mM pH 2.3 (Sigma-Aldrich St. Louis, MO, USA). The carbonyl concentration was measured by spectrophotometry at an OD of 370nm with $\varepsilon_{370} = 22 mM^{-1}cm^{-1}$ and expressed as nmol/mg total protein.

The lipid peroxidase assay described by (240, 241) was followed to estimate the plasma concentration of MDA. In summary, the reaction was carried out by mixing 25µL sodium dodecyl sulfate solution (8.1% w/v; Sigma-Aldrich, Milan, Italy), 187.5µL acetic acid buffer (20% v/v, pH 3.5; Sigma-Aldrich, Milan, Italy), 187.5µL thiobarbituric acid (1% v/v; Sigma-Aldrich, Milan, Italy), 100µL plasma. The reaction mixture was incubated at 100°C for 10min and then cooled in ice. 100µL of water and 625µL of N-butyl alcohol and pyridine (Fluka, Milan, Italy) were added (15:1 v/v). The

mixture was centrifuged (2000g, 4°C, 10min), and the supernatant was measured spectrophotometrically on a microplate reader at 535nm (2023 Multilabel Reader VictorX4, Perkin Elmer, Waltham, MA, USA). MDA (tetramethoxypropane; Sigma-Aldrich, Milan, Italy) was used to perform a standard curve ranging from 0.154 -5mM. The MDA value was calculated from the MDA standard graph and expressed as nmol/mg total protein.

6.7.2 Antioxidant product assessment

Following the evaluation of the oxidative product, thiol group's level was measured according to thiol/disulfide reaction of thiol and Ellman's reagent (5.5'-dithiobisnitrobenzoic acid) (242). Succinctly, twenty µl microliters of plasma were mixed with 180µl PBS 0.1M, EDTA 1mM pH 8 and 3.5µl DTNB (Sigma-Aldrich, Milan, Italy). Sulfhydryl groups are estimated in a sample by comparison to a standard curve composed of known concentrations of a sulfhydryl-containing compound such as cysteine (0.25-1.5mM). The THIOL concentration was measured by spectrophotometry at an OD of 412nm (2023 Multilabel Reader VictorX4, Perkin Elmer, Waltham, MA, USA) and expressed as nmol/mg total protein.

6.7.3 Inflammatory markers and growth factors

Inflammatory mediators, such as IL-1β, IL-10, PDGF, IGF-1 and TGF-1β, were estimated using commercial kits specific for the equine species (MyBiosource, California, USA) at the same time intervals of the previously mentioned factors. All of the previous analysis were done following the manufactured product protocol.

CHAPTER 7: RESULTS

7.1 Clinical evaluation

Before treatment, the horses presented pain at palpation along with local heat and severe swelling; moreover, on the AAEP scoring system the first horse showed a grade 2.5/5 of lameness while the second 4/5.

At T1, clinical assessment revealed a decrease in the inflammatory signs and symptoms: swelling and pain of the injured region were decreased, and a reduction of lameness (Grade 1.5/5 in subject n°1 and 2.5/5 in subject n°2) was observed; a partial restoration of function was observed as both horses were able to load more weight on the affected limb. A second injection of the combined treatment was performed on the same day.

After one year (T2), no signs of swelling, pain at palpation and lameness of the affected limb were observed. Subject n°1 presented with a Grade 0/5 lameness as it was able to trot fine under all tested circumstances and showed a complete restoration of function with the return to sport activity; currently, the horse is still competing. Subject n°2 presented with a Grade 0.5/5 lameness as it was able to trot fine under almost all tested circumstances, with a very light lameness under circumstances after a particularly stressful training. The horse was able to return to sport activity and is currently still competing.

7.2 Ultrasound evaluation

Follow-up ultrasound evaluation was performed one month post treatment, at the moment of the second injection, and one year post treatment.

Progressive reduction of the defect area in the SDFT was recorded ultrasonographically; tendon echogenicity showed an increase across time, whereas the overall tendon injury and LFP gradually decreased across time, reaching the lowest values at one-year post-treatment. An improvement of tendon's fibre alignment could also be noted.

At T0, the tendons presented a focal hypoechoic area (Grade 2.5/3) with a low FA (Grade 3/3). At T1, a slight increase in FA (Grade 2/3) was observed along with a small increase of echogenicity in the wounded area (Grade 2/3). Progressive reduction of the defect area in the SDFT was also recorded.

At T2, one year after the first injection, echogenicity and fibres alignment were similar to the contralateral sound tendon (Grade 0/3). All these markers suggest restoration of tendon's structure, function and natural features.

Ultrasound images of subject n°1 and n°2 can be seen respectively in *Figure 7.1* and *Figure 7.2*

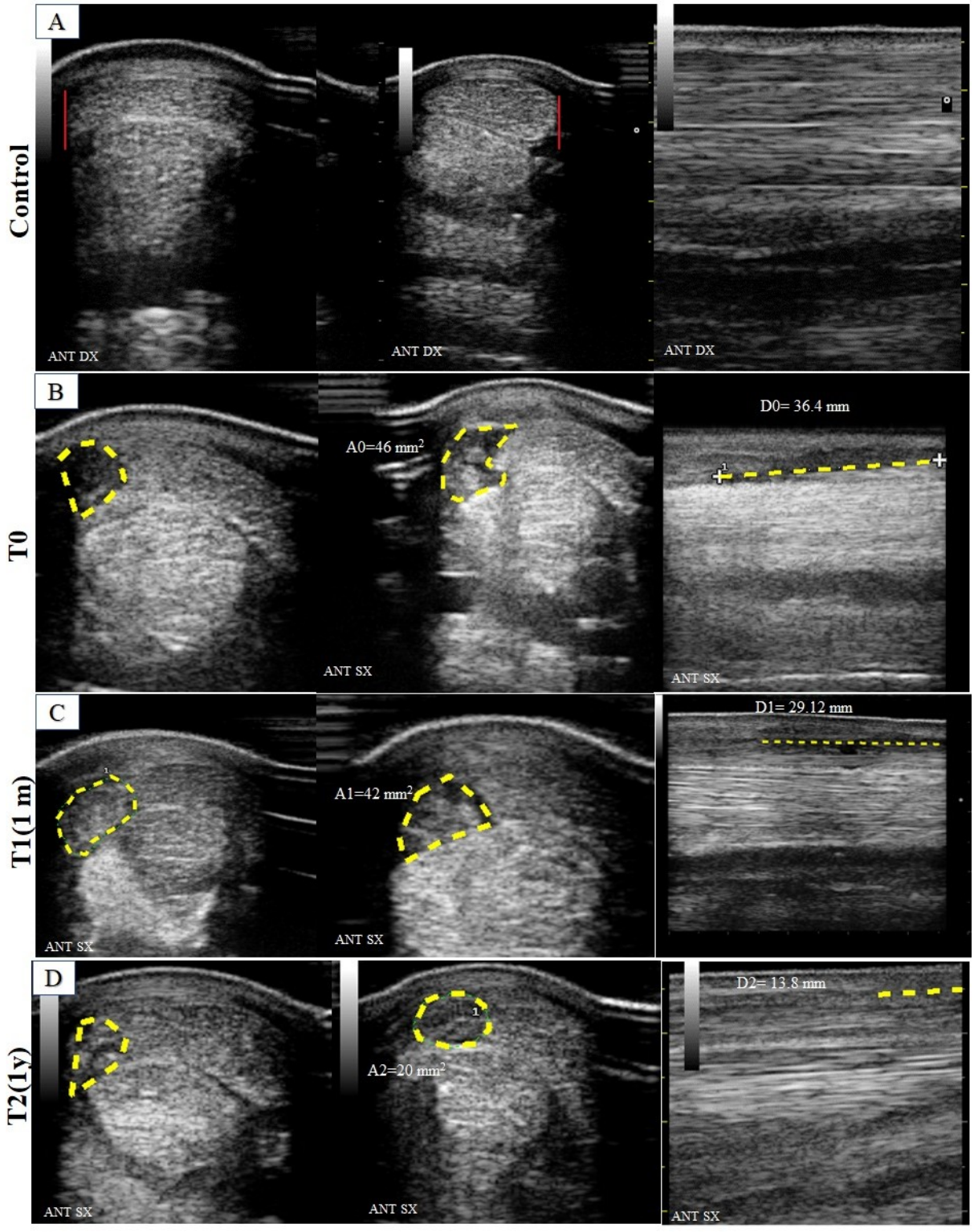
A
Control
ANT DX
ANT DX
ANT DX
B
T0
A0=46 mm²
D0= 36.4 mm
ANT SX
ANT SX
C
T1(1 m)
A1=42 mm²
D1= 29.12 mm
ANT SX
ANT SX
D
T2(1y)
A2=20 mm²
D2= 13.8 mm
ANT SX
ANT SX
ANT SX

Figure 7.1

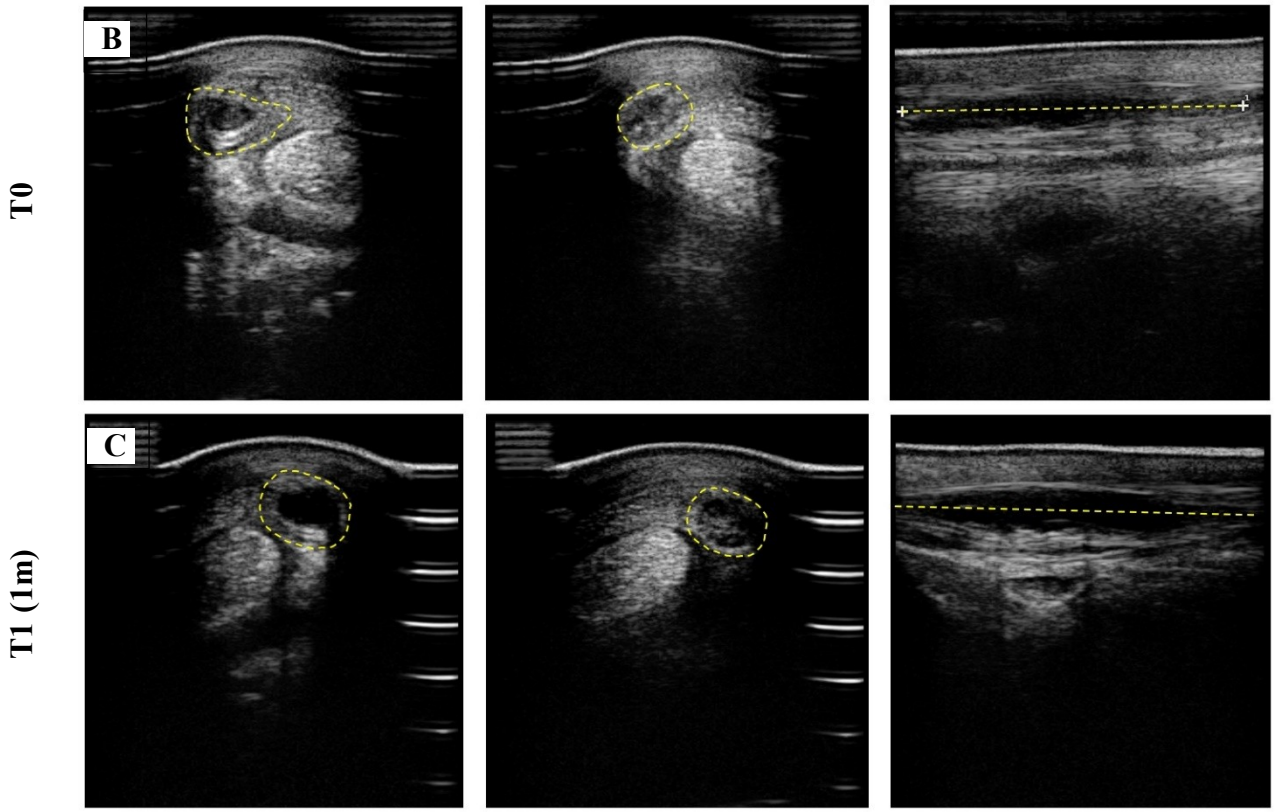

Figure 7.2

Figure 7.1, 7.2: Transverse and longitudinal ultrasonographic images of the SDFT of right and left forelimb of the horse. (A) Healthy right SDFT subject n°1. (B) Lesioned SDFT at T0 (before injection) showing focal hyperechoic defect subject n°1 and n°2. (C), (D) Tendon injected with PRP + (AD-MSC) at different time points (1month, subject n°1 and n°2, and 1year subject n°1, respectively) showing gradual decrease in the lesion size and restoration of the tendon echogenicity.

7.3 Plasma molecules evaluation

The concentration level of inflammatory molecules and growth factors present in blood plasma was assessed on the day of injection, and after 1 and 2 weeks to evaluate markers involved in the tendon healing process in subject n°1, while the same parameters were evaluated also 1 week prior to treatment and one month after treatment in subject n°2 (*Table 6.4*).

Plasma oxidative stress products, as AOPP and T-BARS , decreased over time, reaching the lowest value 14 days post-treatment; CT and thiol group showed a little fluctuation and then reached their lowest values at 14 days, as the other oxidative stress products.

Growth factors, such as PDGF and IGF-1, documented an increase in their concentration at 7days post-treatment compared to the baseline values, but again a decline of their values after 14 days, with the PDGF still recorded at higher concentration than the baseline value. In contrast, TGF-β1 showed steady concentration across the whole-time intervals. Inflammatory markers as the pro-inflammatory cytokine IL-1β accounted for a detectable decrease at 14 days while IL-10, an anti-inflammatory interleukin, recorded an increase in its concentration at the previously mentioned time interval.

All of the levels are listed in *Table 7.1*.

subject n°	days from treatment	PT mg/ml	AOPP nmol/ml	AOPP nmol/mg	CT nmol/ml	PT_CT mg/mg	CT nmol/mg	thiol nmol/ml	thiol nmol/mg	T-BARs nmol/ml	T-BARs nmol/mg	IL-1b pg/ml	IL-10 ng/ml	PDGF ng/ml	TGF-β1 pg/ml	IGF-1 ng/ml
1	0	82,90	64,11	0,77	0,67	4,15	0,16	517,67	6,24	2,73	0,033	83,86	1,23	3,12	781,0	56,24
1	7	74,90	52,64	0,70	0,69	3,75	0,18	521,00	6,96	2,48	0,033	85,05	1,23	14,50	781,0	61,55
1	14	86,87	37,33	0,43	0,37	4,34	0,08	437,84	5,04	2,00	0,023	68,73	1,37	6,43	781,0	51,36
2	-7	68,45	37,30	0,54	0,80	3,42	0,23	415,85	6,08	2,41	0,035					
2	0	75,42	41,04	0,54	0,63	3,77	0,17	479,48	6,36	2,49	0,033	68,40	< 0,2	3,12	781,0	45,94
2	7	59,65	43,19	0,72	0,89	2,98	0,30	468,66	7,86	1,92	0,032					
2	14	55,17	34,10	0,62	0,33	2,76	0,12	405,10	7,34	2,08	0,038			3,12	781,0	42,88
2	30	61,25	24,71	0,40	0,86	3,06	0,28	260,90	4,26	1,36	0,022	48,16	0,84			

Table 7.1: Plasma molecule's assessment

CHAPTER 8: DISCUSSION

Superficial digital flexor tendon (SDFT) injuries are a severe problem that affect a large percentage of athletic and pleasure horses; often they develop recidivisms and, in the worst scenario, have to retire from competition early (235, 243, 244). In a One Health perspective, the equine could play an important role as model for human musculoskeletal disorders because of their similarities (235, 245, 246, 247), especially for investigating the regenerative efficacies of innovative treatments such as PRP, bone marrow aspirate (BMA), or MSCs. However, in human medicine there is a limited knowledge about the combinatory application of these therapies, which is restricted to the treatment of knee osteoarthritis (235, 248, 249) and rotator cuff rupture (235, 250).

In the present study, repeated ultrasound-guided intralesional injections of autologous AD-MSCs combined with autologous PRP were applied for the treatment of SDFT lesions, that had naturally occurred in the left forelimb of a show jumping horse and in the right forelimb of a trotter. The first subject presented with a chronic tendonitis of the SDFT of the left forelimb occurred during sporting activity, which developed from a previous injury six months before the treatment herein described; the horse was unresponsive to conventional treatments such as NSAIDs and a controlled rehabilitation program. The second subject presented with an acute tendonitis of the right forelimb naturally occurred during racing activity.

Therefore, the double application of AD-MSCs with PRP for the treatment of a naturally occurring lesion in the SDFT was the novel approach chosen.

Application of regenerative therapy in a naturally existing tendon lesion gives a more precise idea regarding its effects and is considered one of the privileges of this study. Even though mechanically or enzymatically induced tendon lesions can simulate tendinopathy to a large extend, some differences related to aetiology and physiopathology should be investigated (251).

Results were beneficial for the horses as the repeated injections of AD-MSCs and PRP resulted in a positive development of SDFT tendonitis over a one-year follow-up. In addition, during this period the horses did not suffer any re-injury. The combined treatment might be accountable for the decrease of inflammatory markers (i.e. plasma protein levels) as observed during the first 2 weeks, which might have eventually led to the slight reduction of clinical symptoms observed at T1.

The ultrasonographic evaluation showed restoration in structure, echogenicity, and fibre organization of the affected tendon after one year. This could also suggest that the biomechanical properties of the tendon were restored to an adequate level for allowing the horses to go back to the same performance status as before the injury.

Similar results were reported by (252), where the ultrasonographic evaluation of collagenase induced SDFT defects treated with AD-MSC reported constant CSA% in the first 4 weeks post-treatment and then a gradual decrease, whereas the control group showed an increase in the CSA% at weeks 2, 4 and 6 followed by a decrease.

The addition of PRP to MSCs for the treatment of different disorders as skin wounds (235, 253) or bone defects (235, 254), has demonstrated to boost the regenerative effects of MSCs, both morphologically and functionally. The same effect was also observed for treating degenerative joint disorders in equine specie (235, 255). In all models, the observed results were achieved principally by extracellular matrix remodelling, mainly dictated by the structural action of MSCs, which is a fundamental component in tendon healing. The underlying reason may be ascribed to the soluble molecules (e.g., growth factors) present in the PRP that might stimulate MSCs proliferation and release of bioactive factors (235, 256). Indeed, MSCs themselves are able to release a plethora of soluble bioactive molecules that possess beneficial effects (235, 257).

During the first 2 weeks after treatment, blood plasma analysis showed a reduced concentration of inflammatory markers (AOPP, TBARS, CT, and IL-1β) along with an increase of thiols at day 7 and a reduction at day 14. The substantial increase in the concentration of thiols after 1 week may be related to their function as antioxidants (235, 258), while reducing at day 14 as the inflammatory process started to subside. Concomitantly, the concentration of IL-10, an anti-inflammatory cytokine, increased after 2 weeks. IL-10 is known to have a pro-mitotic effect on tenocytes and tendon-derived stem cells, stimulating cell proliferation and migration (235, 259). In addition, the growth factors IGF-1 and PDGF showed an increase one-week post-treatment. Both growth factors have positive effects on tendon cells, inducing proliferation plus attracting them to the wound area and stimulating ECM deposition (235, 260, 261).

These observations were reflected 4 weeks after treatment (T1) by a reduction of pain and swelling of the affected forelimb area along with a partially reduced lameness. However, at the same time point, ultrasound images still presented with a hypoechoic area in the SDFT, corresponding to the diagnosed lesion. For this reason, the same treatment was applied a second time. The double application of AD-MSCs and PRP in a 30-day interval might have provided a prolonged activation of tendon regeneration due to a more protracted exposure, at the injury site, to both constituents in comparison to a single injection. This is highly related to the mean platelet half-life that in horses ranges from 4 to 6 days (262, 263). Moreover, a recession of topically applied AD-MSCs population in SDFT induced lesions throughout the study period was reported by (264). As a result, the absence of a significant difference between the treated and the control group was reported. In another study

conducted by (265), only about 5 % of BM-MSCs survived more than 10 days and just 0.02% persisted over 90 days following implantation in an equine surgical model of tendinitis.

Moreover, the use of autologous sources for the therapy did not provoke any immune reactions to the horse after application, confirming the safety of MSCs (235, 266).

To conclude, a repeated injection of autologous AD-MSCs coupled with PRP over a 52-week period supported a positive progression of a chronic SDFT lesion, which developed from a previous healed injury in a show jumping horse, allowing the animal to go back to competition. Probably, the measurement of inflammatory and oxidative stress markers in blood plasma can play a pivotal role in monitoring the healing process. However, these outcomes should be confirmed in the future by large placebo-controlled studies with animals affected by SDFT acute and chronic tendonitis.

CHAPTER 9: CONCLUSION

To our knowledge the horse n°1 was the first included in a case report of a successful treatment of a naturally occurring chronic SDFT tendonitis developed from a re-injury in a show jumping horse by a repeated and combined application of AD-MSCs with PRP. The therapy demonstrated to be safe and effective as no adverse reactions were observed; moreover, the horse was able to go back to competition. The same results could also be noted on subject n°2 that was affected by a naturally occurring acute tendonitis. Our results might therefore encourage the combined application of MSCs and PRP for the treatment of tendon injuries in equine clinical practice.

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Case Report: Repeated Intralesional Injections of Autologous Mesenchymal Stem Cells Combined With Platelet-Rich Plasma for Superficial Digital Flexor Tendon Healing in a Show Jumping Horse

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Case Report: Repeated Intralesional Injections of Autologous Mesenchymal Stem Cells Combined With Platelet-Rich Plasma for Superficial Digital Flexor Tendon Healing in a Show Jumping Horse

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In the present case report a show jumping 10-year-old Sella Italiano gelding, presented with severe lameness, swelling and pain at palpation of the mid-metacarpal region of the left forelimb. Clinical and ultrasound examination diagnosed a chronic tendonitis of the central region of the superficial digital flexor tendon (SDFT). The lesion was a reoccurrence since it developed from a previously healed injury. The horse had to stop competing and was unresponsive to gold-standard treatments as Non-steroidal anti-inflammatory drugs (NSAIDs) and conservative management after 6 months of therapy. The animal was subjected to repeated intralesional injections of autologous adipose-derived mesenchymal stem cells (AD-MSCs) combined with autologous platelet-rich plasma (PRP). The combined treatment was administered twice in a 1-month interval. The healing process was assessed through clinical examination, ultrasound imaging and quantification of oxidative stress products and inflammatory mediators in blood plasma. After 2 weeks from first injection, a reduction of concentration of oxidative-derived products was observed, together with an increase of anti-inflammatory cytokines and pro-mitotic growth factors. These results were reflected clinically as the horse showed a reduction of lameness along with swelling and pain after 4 weeks. At the 1-year follow-up, the horse showed no signs of lameness and swelling. The ultrasonographic examination highlighted a compact fiber alignment with a normal echogenic tendon as observed in the sound contralateral limb. Moreover, the horse went back to the previous level of competition. Our results suggest the positive effects of a repeated intralesional injection of AD-MSCs and PRP for the treatment of a chronic tendonitis with long-term effects and an improvement for both equine quality of life and athletic performance.

Keywords: SDFT, platelet-rich plasma, mesenchymal stem cells, regenerative medicine, equine orthopedics, tissue regeneration, horse

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INTRODUCTION

Tendinopathies are one of the most common orthopedic disorders in equine and human athletes, leading to lameness and pain (1–5). Tendon injuries are responsible for approximately one third of traumas that occur during the sporting career of horses (6, 7), forcing a significant number of individuals to an early retirement from competition (8, 9). The superficial digital flexor tendon (SDFT) is frequently injured in show jumping discipline due to the repeated and excessive loading forces that the tendon has to sustain after jumping and landing (10, 11). Tendonitis affecting the SDFT have an incidence up to 43%, and most of them occur in the central tendon region (6, 12).

Tendons possess a limited regenerative capacity and usually heal by forming a fibrotic scar, but the repaired tissue possesses inferior biomechanical characteristics compared to its normal physiological counterpart (13–15). Consequently, horses that have previously sustained a tendon injury are more prone to re-injury (up to 80%) or to chronicity (9, 16).

The gold-standard treatments for tendon injuries consist of conservative therapies including administration of Non-steroidal anti-inflammatory drugs (NSAIDs) and rehabilitation aiming to attenuate symptoms and to recover tendon function. Although clinical improvements might be observed (i.e., relief of symptoms), most of these options lack long-term therapeutic success (17, 18). Over the last two decades, regenerative therapies have been gaining interest because of their beneficial effects in supporting and stimulating the healing process, leading to a healed tissue that resembles healthy tendon in structure and function (19, 20).

Mesenchymal stem cells (MSCs) derived from multiple sources, as bone marrow (BM-MSCs), adipose tissue (AD-MSCs), or peripheral blood (PB-MSCs), have proved their efficacy in improving tendon healing in horses thus reducing the reoccurrence rate of injury, mainly because of their paracrine activity (21–24). Different route of administration of MSCs, including intralesional injection, have demonstrated to be a safe and effective practice to treat tendon injury in equine medicine (25, 26). Another product of interest in equine regenerative practice is platelet-rich plasma (PRP), a blood-derived product rich in growth factors and cytokines that can sustain and boost the tissue healing process (20, 27, 28). When combined, these two treatments possess a higher regenerative potential in comparison to their application alone as it has been demonstrated for treating different tissue (e.g., skin, bone, joint) in human and horses (9, 29–33). Nevertheless, only one study describes the repeated application of MSCs and PRP for the treatment of naturally occurring chronic tendonitis, which was not a re-injury, in the equine in a 16-weeks time interval (34).

In terms of risk factors (e.g., age and over-exercise) and etiology, human and horses share a similar pathophysiology of tendinopathies. For this reason, studies to test regenerative therapies in the horse including cell and cell-free treatments, or their combination, for tendon healing might be useful as preclinical data for translation purposes to human medicine (35–38).

TABLE 1 | Clinical and ultrasonographic scores to assess lameness, echogenicity and fiber alignment.

Score	AAEP degree of lameness	Echogenicity	Fiber alignment (FA)
0	Lameness not perceptible under any circumstances	Normal echogenicity	≥75% parallel fiber bundles in the lesion
1	Lameness is difficult to observe is not consistently apparent regardless of circumstances	Mildly hypoechoic	50–74% parallel fiber bundles in the lesion
2	Lameness is difficult to observe at a walk or when trotting in a straight line but consistently apparent under certain circumstances	Moderate hypoechoicity	25–49% parallel fiber bundles in the lesion
3	Lameness is consistently observable at a trot under all circumstances	Severe hypoechoicity	≤25% parallel fiber bundles in the lesion
4	Lameness is obvious at a walk	–	–
5	Lameness produces minimal weight bearing in motion and/or at rest or a complete inability to move	–	–

In this case report, we describe the repeated application of autologous adipose-derived MSCs and autologous PRP for the treatment of a chronic recurrent SDFT tendonitis developed from a previous injury in a show jumping horse.

CASE DESCRIPTION

Clinical History

A 10-year-old Sella Italiano gelding, competing in show jumping, presented with a lesion in the of the SDFT of the left forelimb in the middle third of the metacarpal region. The lesion was a reoccurrence, which had developed from a previous healed injury in the same area of the SDFT. At diagnosis, the horse showed a lameness grade 2.5/5 based on the American Association of Equine Practioners (AAEP) scale (as reported in **Table 1**). Pain and local heat were noted at palpation along with severe swelling.

Six months ago, when diagnosed, the horse stopped competing and was treated with NSAIDs. Furthermore, a controlled rehabilitation exercise program was followed, adapted and based on the type of injury. The program started with a complete stall rest for the first 2 weeks. Then it was followed by a gradual increase of walking and trotting exercises, starting with 5 min walking a day with an increase of 5 min every 2 weeks, up to 40 min. After 20 weeks, the horse began to trot for 2 min each day with a progressive increase of trotting time, alternated with walking, every 2 weeks (up to 20 min of trot with 20 min of walking). However, the animal did not show any improvement at the clinical or ultrasonographic level.

In **Figure 1** it is showed a schematic timeline of the clinical case.

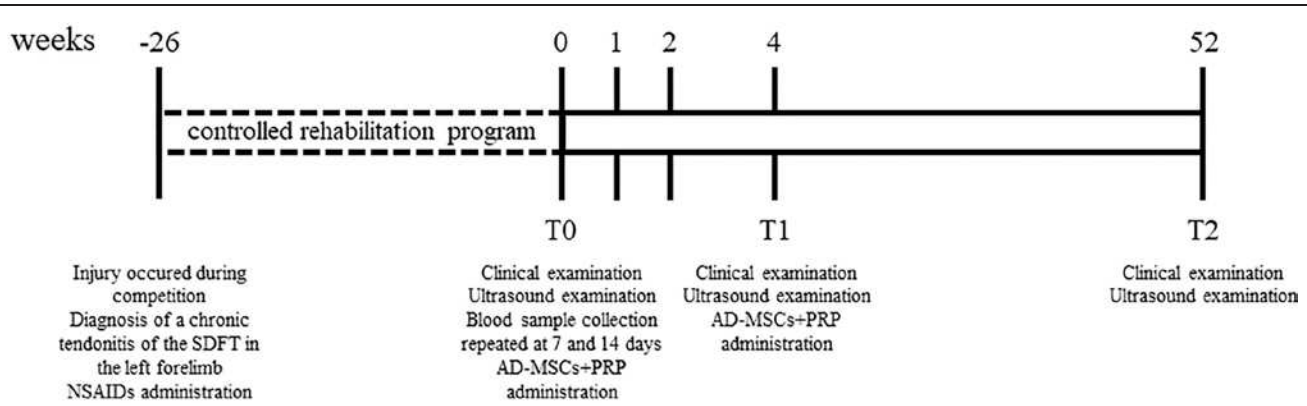

FIGURE 1 | Schematic timeline of the clinical case reporting clinical examinations, ultrasonographic evaluations and therapy protocol.

Diagnostic Imaging: Ultrasound Evaluation

Ultrasonographic evaluations of the central metacarpal region of both forelimbs were performed using a 7.5-MHz linear transductor probe. For each assessment, a complete examination of the SDFT was conducted by means of longitudinal and transverse scans. The obtained images were evaluated and scored (from 0 to 3) at each examination for two parameters as previously described (24, 39): lesion echogenicity and lesion longitudinal fiber alignment (FA). Criteria for scoring are listed in **Table 1**. The contralateral healthy limb was used as comparison.

The SDFT, in the middle third of the metacarpal region, presented with a focal hypo-echogenic area together with an irregular fiber alignment/pattern, which corresponded to ~ 30% of the cross-sectional area of the tendon. The lesion presented with a proximo-distal size of 36.4 mm.

Treatment and Follow-Up

Treatment protocol consisted of ultrasound-guided intralesional injection with autologous adipose-derived MSCs (AD-MSCs) combined with PRP. AD-MSCs and PRP isolated, characterized, and prepared for treatment as previously described (22, 40).

The adipose tissue was collected from the region above the dorsal gluteal muscle, at the base of the tail, because of the ease of access and absence of large veins. The horse was intravenously sedated with 0.01 mg/kg detomidine (Domodesan® Orion Pharma, Italy); then the area was shaved, aseptically prepared, and locally anesthetized with 2% lidocaine (Lidor® Richter Pharma AG, Italy). An incision of ~ 5–6 cm in length was made parallel 15 cm lateral to the spinal column, in order to allow visualization of adipose tissue between the skin and the musculature. Afterwards, ~ 4 g of subcutaneous adipose tissue was collected and stored in proper medium for transport, consisting of phosphate buffer saline (PBS) supplemented with penicillin-streptomycin (10%). Upon arrival to the laboratory, the sample was washed with PBS three times, minced and placed in a 0.01% collagenase type IA (Sigma-Aldrich, Italy) solution for 1 h at 37°C with continuous shaking. After digestion, the solution was filtered using a 100 μm cell strainer and diluted in DMEM high glucose (Sigma-Aldrich, Italy) supplemented with 10% FBS (Sigma-Aldrich, Italy). Afterwards, the solution was centrifuged

ce at 300 xg for 10 min. Isolated cells were seeded in a culture sk in complete cell growth medium consisting of DMEM high cose (Sigma-Aldrich, Italy), FBS 10% (Sigma-Aldrich, Italy), nd 1% antibiotics (penicillin/streptomycin; Aurogene, Italy), intained in culture, and expanded. Cells used for application re at passage 3 and 5. Isolated cells were characterized by w cytometry and *in vitro* tri*lineage* differentiation as previously cribed by (40) and stated by (41).

The PRP was obtained by following a double centrifugation e method in sterile conditions (first centrifuge at 1300 xg for min and then at 300 xg for 15 min) as described by (42).

On the day of treatment, 10^7 AD-MSCs were diluted in 4 mL of autologous PRP. Cell viability was assessed by pan blue staining and more than 90% of cells were viable.

Before injection, the horse was sedated with 0.01 mg/Kg etomidine (Domodesan® Orion Pharma, Italy) and 0.1 mg/Kg utorphanol (Nargesic® Acme Srl, Italy), and the injection area s aseptically prepared. The treatment was inoculated in the ion site using a sterile 14G needle *via* ultrasonic guidance. terwards, protective sterile bandages were applied to the limb l the horse followed a rehabilitation protocol.

A second injection was performed 4 weeks later in order to continue the stimulation of the healing process. The applied tocol was the same as that used for the first treatment.

ood Plasma Analysis

ood plasma was obtained after collection of peripheral blood m the jugular vein of the horse using a lithium-heparin sterile be (BD Vacutainer® BD, Italy) on the day of injection, and r 1 and 2 weeks Post-treatment.

The blood plasma was analyzed for assessing levels of protein ated to inflammation and the relative oxidative stress such advance oxidation protein products (AOPP), carbonyl group (CT), malondialdehyde (lipid peroxidation *via* thiobarbituric acid reactive substance, TBARS), and the presence of thiols iol/disulfide reaction of thiol); all parameters were measured following published protocols (43–46).

In addition, the concentration of proteins involved in the inflammatory process (IL-1β, MBS2020285, MyBioSource, ; IL-10, ab155466, Abcam, UK) or tendon wound healing

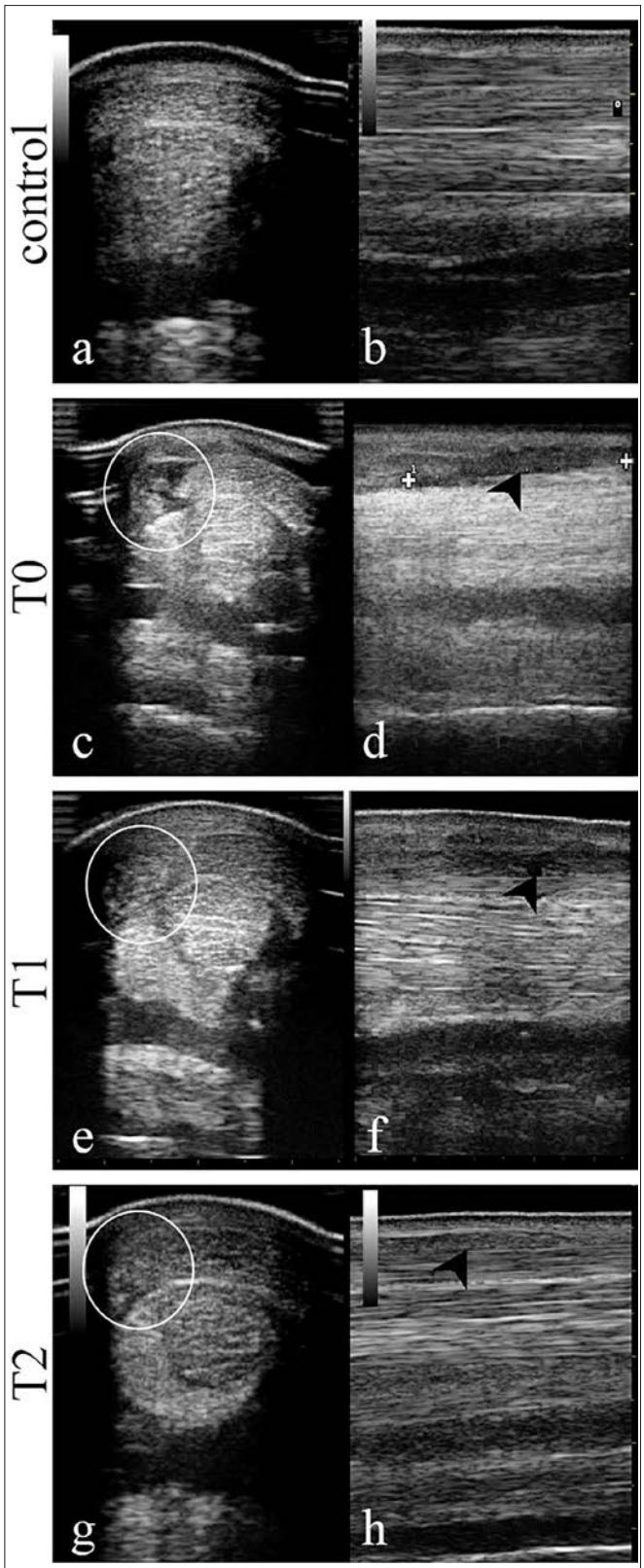

FIGURE 2 | Transversal and longitudinal images of the central area of the superficial digital flexor tendon in the mid-metacarpal region performed at day 0 (T0, day of therapy injection), T1 (4 weeks), and T2 (52 weeks). At T0 the *(Continued)*

FIGURE 2 | SDFT is characterized by a hypoechoic area (c) along with an abnormal fiber alignment (d). After 4 weeks (T1), the same area resulted less hypoechoic (e) and the fiber pattern was more aligned (f) while at T2 the area showed a normal echogenicity (g) and fiber disposition (h) as in the sound contralateral limb (a,b). White circle, corresponding injury area in transverse images; black arrow-head, injury area in longitudinal images.

(PDGF, MBS907132, MyBioSource, US; IGF-1, MBS7606417, MyBioSource, US) was estimated using enzyme-linked immunosorbent assay (ELISA) commercial kits specific for the equine specie. The analyses were performed following the manufacturer's protocol.

All samples were analyzed in triplicate in a 96-well plate and absorbance was obtained by using a VICTOR multilabel plate reader (Perkin Elmer, US).

OUTCOMES

Clinical Evaluation

Clinical assessments were performed every 2 weeks starting from the day of the first injection. In this study we report clinical outcomes on the day of injection (T0), at 4 weeks (T1) and 52 weeks (T2) after injection. Before treatment, the horse presented pain at palpation along with local heat and severe swelling in the mid-metacarpal region of the left forelimb. In addition, on the AAEP scoring system the horse showed a Grade 2.5/5 lameness.

At T1, swelling and pain of the injured region of the left forelimb were decreased; moreover, a reduction of lameness (Grade 1.5/5) was observed. The horse was able to load more weight on the affected limb. A second injection of the combined treatment was performed on the same day. After 1 year (T2), no signs of swelling, pain at palpation and lameness of the affected limb were observed. The horse presented with a Grade 0/5 lameness as it was able to trot fine under all tested circumstances. The horse showed a complete restoration of function and returned to sport activity; currently, the horse is still competing.

Ultrasound Evaluation

Ultrasound examinations were assessed using the scoring system previously described (**Figure 2**). At T0, the tendon presented with a focal hypoechoic area (Grade 2.5/3) with a low FA (Grade 3/3). At T1, a slight increase in FA (Grade 2/3) was observed along with a small increase of echogenicity in the wounded area (Grade 2/3). At T2, 1 year after the first injection, echogenicity and fibers alignment were similar to the contralateral sound tendon (Grade 0/3).

Blood Plasma Analysis

The concentration level of inflammatory and growth factors present in blood plasma was assessed on the day of injection, and after 1 and 2 weeks to evaluate markers involved in the tendon healing process (**Table 2**).

The quantity of plasma oxidative stress products, as AOPP and TBARS, decreased gradually over time, with the lowest value at 14 days after the first treatment; CT and thiol groups showed a little increase at day 7, and then decreased at 14 days.

TABLE 2 | Obtained data from analysis of blood plasma markers related to inflammation and tendon healing process.

Test \ Days	0	7	14	Unit
AOPP	0,77	0,70	0,43	nmol/mg
TBARS (MDA)	0,013	0,011	0,007	nmol/mg
CT	0,16	0,18	0,08	nmol/mg
Thiols	6,24	6,96	5,04	nmol/mg
IL-1β	0,084	0,085	0,069	ng/mL
IL-10	1,23	1,23	1,37	ng/mL
IGF-1	56,24	61,55	51,36	ng/mL
PDGF	3,12	14,50	6,43	ng/mL

The pro-inflammatory cytokine IL-1β showed a decrease at 14 days while IL-10, an anti-inflammatory interleukin, showed an increase in its concentration on the same day.

The plasma concentration of growth factors, PDGF and IGF-1, showed an increase in their concentration at 7 days Post-treatment compared to the baseline values. After 14 days, a decrease of concentration of both factors was observed.

DISCUSSION

Superficial digital flexor tendon (SDFT) injuries are a severe problem that affect a large percentage of athletic and pleasure horses; often they develop recidivisms and, in the worst scenario, have to retire from competition early (9, 47). In a One Health perspective, the equine could play an important role as model for human musculoskeletal disorders because of their similarities (48–50), especially for investigating the regenerative efficacies of innovative treatments such as PRP, bone marrow aspirate (BMA), or MSCs. However, in human medicine there is a limited knowledge about the combinatory application of these therapies, which is restricted to the treatment of knee osteoarthritis (51, 52) and rotator cuff rupture (53).

In the present case report, repeated ultrasound-guided intralesional injections of autologous AD-MSCs combined with autologous PRP were applied for the treatment of a recurrent SDFT lesion, that had naturally occurred in the left forelimb of a show jumping horse. The horse presented with a chronic tendonitis of the SDFT of the left forelimb occurred during sporting activity, which developed from a previous injury 6 months before the treatment herein described. The horse was unresponsive to conventional treatments such as NSAIDs and a controlled rehabilitation program. Therefore, the double application of AD-MSCs with PRP for the treatment of a naturally occurring lesion in the SDFT was the novel approach chosen.

Results were beneficial for the horse as the repeated injections of AD-MSCs and PRP resulted in a positive development of a SDFT chronic tendonitis over a 1-year follow-up. In addition, during this period the horse did not suffer any re-injury. The combined treatment might be accountable for the decrease of inflammatory markers (i.e., plasma protein levels) as observed during the first 2 weeks, which might have eventually led to the slight reduction of clinical symptoms observed at T1. The ultrasonographic evaluation showed restoration in structure, echogenicity, and fiber organization of the affected tendon after 1 year. This could also suggest that the biomechanical properties of the tendon were restored to an adequate level for allowing the horse to go back to the same performance status as before the injury. The addition of PRP to MSCs for the treatment of different disorders as skin wounds (54) or bone defects (55), has demonstrated to boost the regenerative effects of MSCs, both morphologically and functionally. The same effect was also observed for treating degenerative joint disorders in equine specie (56). In all models, the observed results were achieved principally by extracellular matrix remodeling, mainly dictated by the structural action of MSCs, which is a fundamental component in tendon healing. The underlying reason may be ascribed to the soluble molecules (e.g., growth factors) present in the PRP that might stimulate MSCs proliferation and release of bioactive factors (57). Indeed, MSCs themselves are able to release a plethora of soluble bioactive molecules that possess beneficial effects (58).

During the first 2 weeks after treatment, blood plasma analysis showed a reduced concentration of inflammatory markers (AOPP, TBARS, CT, and IL-1β) along with an increase of thiols at day 7 and a reduction at day 14. The substantial increase in the concentration of thiols after 1 week may be related to their function as antioxidants (59), while reducing at day 14 as the inflammatory process started to subside. Concomitantly, the concentration of IL-10, an anti-inflammatory cytokine, increased after 2 weeks. IL-10 is known to have a pro-mitotic effect on tenocytes and tendon-derived stem cells, stimulating cell proliferation and migration (60). In addition, the growth factors IGF-1 and PDGF showed an increase 1 week Post-treatment. Both growth factors have positive effects on tendon cells, inducing proliferation plus attracting them to the wound area and stimulating ECM deposition (61, 62).

These observations were reflected 4 weeks after treatment (T1) by a reduction of pain and swelling of the affected forelimb area along with a partially reduced lameness. However, at the same time point, ultrasound images still presented with a hypoechoic area in the SDFT, corresponding to the diagnosed lesion. For this reason, the same treatment was applied a second time. The double application of AD-MSCs and PRP in a 30-day interval might have provided a prolonged activation of tendon regeneration due to a more protracted exposure, at the injury site, to both constituents. Moreover, the use of autologous sources for the therapy did not provoke any immune reactions to the horse after application, confirming the safety of MSCs (63).

To conclude, a repeated injection of autologous AD-MSCs coupled with PRP over a 52-week period supported a positive progression of a chronic SDFT lesion, which developed from a previous healed injury in a show jumping horse, allowing the animal to go back to competition. Probably, the measurement of inflammatory and oxidative stress markers in blood plasma can play a pivotal role in monitoring the healing process. However, these outcomes should be confirmed in the future by

large placebo-controlled studies with animals affected by SDFT chronic tendonitis.

CONCLUSION

To our knowledge this is the first case report of a successful treatment of a naturally occurring chronic SDFT tendonitis developed from a re-injury in a show jumping horse by a repeated and combined application of AD-MSCs with PRP. The therapy demonstrated to be safe and effective as no adverse reactions were observed; moreover, the horse was able to go back to competition. Our result might encourage the combined application of MSCs and PRP for the treatment of tendon injuries in equine clinical practice.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the owner of the horse signed a written consent, in which all treatment procedures were widely explained. All consent items were discussed in detail during the consultation visit to provide the owner with an overview regarding the benefits of the treatment and the expected results. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

AC, FP, MP, and II followed the clinical case and performed the sample collection. LM, AC, LD, and AP performed cell processing and laboratory analysis. LM, AC, and NE prepared the manuscript. LM and MP contributed to study design and supervised it. GG and MP revised and edited the manuscript. All authors read and approved the final manuscript.

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