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Regenerative Medicine for the Treatment of Teno-desmic Injuries of the Equine. A Series of 150 Horses Treated with Platelet-derived Growth Factors

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Abstract. *Background/Aim: The aim of the present study was to evaluate the safety and the clinical outcome of platelet-rich plasma for the treatment of teno-desmic injures in competition horses. Patients and Methods: From January 2009 to December 2011, 150 sport horses suffering from teno-desmic injuries were treated with no-gelled platelet-concentrate. Results: No horse showed any major adverse reaction as a result of the procedure. Full healing was obtained for 81% of the horses. Twelve percent had clinical improvement and only 7% a failure. Eight percent of cases of relapse were observed. No statistically significant correlation existed between clinical outcome and the area of the lesion. A statistically significant correlation existed between the clinical outcome and the age of the horse. Conclusion: Treatment with platelet-derived growth factors leads to the formation of a tendon with normal morphology and functionality, which translate in the resumption of the agonistic activity for the horses we treated.*

In veterinary as well as in human medicine tenodesmic lesions are of great importance due to their high incidence, difficult wound healing and therefore incomplete full functional recovery, with long periods of inactivity. The specific pathogenesis of these diseases includes continuous microtrauma, forced exercise, high speed, muscle fatigue; they may also be manifestations of a degenerative process in old horses (1).

The connective tissue lesions are characterized by destruction and disorganization of collagen fibers; this process results in the formation of inelastic scar tissue, unable to adapt to the continuous tension changing of the structure (2).

In sport horses, tendon and ligament injuries are a frequent cause of lameness and entail long periods of rest. Conventional therapies, such as the thermo-cautery, extracorporeal shock waves, hyaluronic acid and surgical techniques (radial bridle desmotomy, tendon splitting, carbon fibers implant) do not act on the pathogenesis, lead to an often delayed healing that does not permit resumption of normal agonistic activity and, in some cases, severe recurrences happen.

Recently, regenerative medicine and tissue engineering have focused on the use of growth factors (GFs) and cell-based therapy to improve on the quality and speed of healing in tendons and ligaments (3).

Regenerative medicine aims to restore the normal structure and the biomechanical properties of the injured tissue and is based on the employment of either stem cells with multipotent differentiating potential and/or biological products (Platelet-Rich Plasma, PRP, or its gel formulation Platelet Gel, PG) that have the ability to induce the recruitment, proliferation and differentiation of cells involved in the tissue regeneration.

Tissue repair is a complex biological process facilitated by growth factors (GF), molecules of crucial importance that interplay and exchange biochemical information. GFs are produced by the cells involved in the regenerative process and when they reach a proper concentration they trigger the reparation process. (4)

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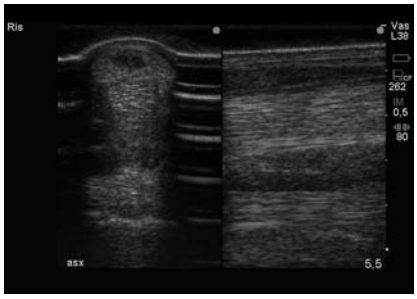


Figure 1. *US-scan shows a superficial digital flexor tendon lesion.*

During soft or hard tissue healing, blood platelets are the main source of released GF necessary for the process. In addition to their functions in hemostasis, platelet α-granules release growth factors (PDGF, TGF, EGF, IGF, FGF, VEGF) which promote tissue regeneration (5). These proteins regulate various processes involved in wound healing and tissue regeneration by regulating cell proliferation and differentiation, angiogenesis, matrix deposition and tissue remodeling (6).

Several *in vitro* studies have been performed with platelet-derived growth factors. PRP treatment improved the gene expression of type I and type III collagen and of COMP (cartilage oligomeric matrix protein) when used on SDFT equine tendon explants (7). Also platelet lysate, a PRP derivative, has been shown to have a positive effect on the proliferation of equine mesenchymal stem cells and tenocytes (8). The promising results obtained by *in vitro* studies have encouraged the *in vivo* PRP use as treatment for the management of tendon injury in sport horses (9, 10) or in surgically treated tendon lesions (11-13). The available data about the therapeutic use of PRP in equine tendon and ligament lesions are promising but show some limitations due for example to the lack of a standardized procedure for the PRP preparation, the variability in the number of platelets of the platelet concentrate and the number of PRP treatments.

The aim of the present study was to evaluate the safety of the procedure and the clinical outcome (*i.e.* the rate of horses that could resume their normal agonistic activity) in competition horses affected by teno-desmic injures treated with platelet-rich plasma obtained with a standardized procedure.

Materials and Methods

Study design. From January 2009 to December 2011, 150 sport horses suffering from teno-desmic injuries were treated with not gelled platelet-concentrate. Only 99 horses have an adequate follow-up (at least 12 months), thus our analysis will be limited to these animals. The basin of origin of the animals was Northern Italy, from Brescia to Pisa and all races were well-represented. The horses were treated in four different veterinary facilities.

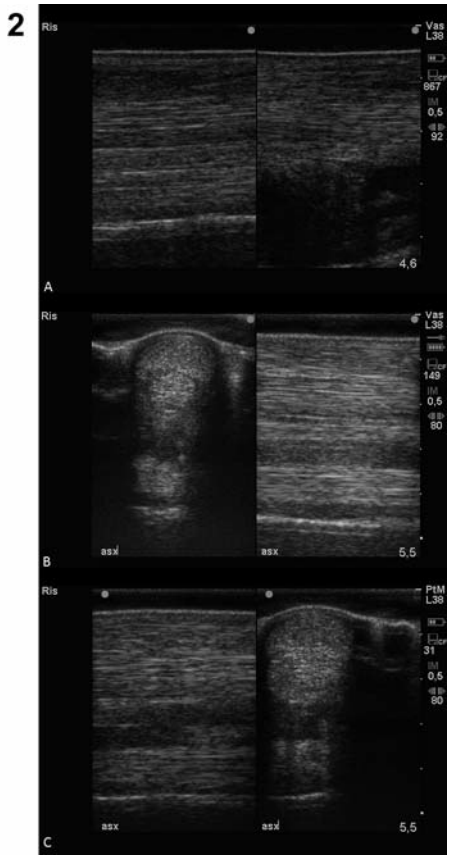
Baseline assessments. All injured horses underwent clinical evaluation to define the lesion by inspection and palpation in order to assess tenderness and heat. Heat was also evaluated by digital camera thermography. The presence of lameness at the walk and trot was evaluated. Lameness grade was > or equal to 2 for all horses. During the physical examination, the involvement of the bone, such as fractures, should be excluded; a radiographic examination was performed if clinically indicated. The location and severity of the damage to the tendon was defined by transverse and longitudinal ultrasound scan. A US-scan was performed at baseline and 3, 6 and 9 weeks after treatment.

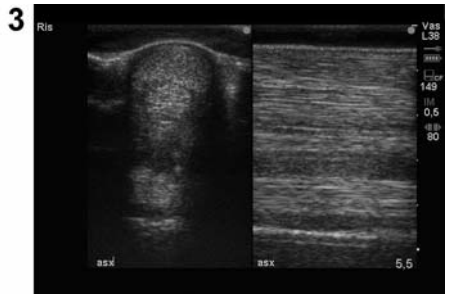
PRP preparation. Two units of 450 mL of blood are collected from the horse through a standard triple-bag system. The first bag (bag 1) contains the CPD anti-coagulant (citrate-phosphate-dextrose). Of the two satellite bags, bag 2 contains SAGM (preservative solution for red blood cells, consisting of saline, adenine, glucose and mannitol), while the other (bag 3) is empty. The method of sampling in the horse is quite simple, due to the easy availability of the sampling site and the size of the animal, that allows easily the removal of 450-900 cc of blood. Sampling was done from the jugular vein after trichotomy and disinfection of the area. The operator always used sterile gloves. Sterility is very important because this is the only time for possible contamination of the sample. The blood was drained by gravity into the first bag (bag 1). Once filled, the infusion tube must be closed and the needle used for sampling is removed. The blood is sent to the laboratory at room temperature (20-24˚C) and platelets should be separated within 6 hours after blood collection. All centrifugation steps were performed in the centrifuge Rotanta 460 R (Hettich Zentrifugen).

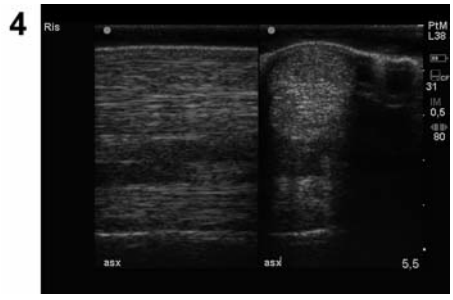
Blood was centrifuged at 1,450 rpm for 10 min at 20˚C, in order to obtain the separation of red blood cells from plasma, which contains platelets and the factors that lead to the formation of a clot. Plasma separation is obtained thanks to a mechanical plasma extractor after locking in a suitable way the bag containing SAGM (bag 2). Plasma is collected into the empty bag (bag 3).

After blocking the entry of liquid into bag 3, containing the plasma, the SAGM solution is made to flow into the bag containing the red blood cells (bag 1), which is then eliminated. Plasma (bag 3) is then centrifuged at 3000 rpm for 20 min at 20˚C, thus obtaining the separation of a platelet pellet and platelet poor plasma (PPP). The PPP is collected into the bag that previously contained the SAGM solution (bag 2).

The bag containing the platelet pellet (bag 3) is weighed. The platelets are then re-suspended in 30-35 mL of PPP in order to have a PRP with a platelet concentration of about 1×10^6 platelets/μL. The bag containing the PRP is placed on a platelet agitator under constant agitation at room temperature in order to obtain a homogeneous platelet suspension; after about 2 hours the bag is transferred under a sterile hood and the platelet concentrate is dispensed into sterile tubes (Monovette, Sarstedt). The PRP product is stored at –20˚C until use. Platelet count is performed on a small aliquot of PRP in order to assess the quality of the product, after having diluted 1:5 the sample with saline solution.







Figures 2-4. *The lesion appears fully recovered at 1, 2 and 3 months, respectively.*

PRP treatment. The laboratory provides the physician with the PRP in the frozen form, contained in sterile tubes; one part of the product is used immediately, other tubes are stored at –20˚C for possible future applications.

The horse is prepared for surgery proceeding to sedation. The degree of sedation depends on the horse and is preferably performed with acepromazine (0.03-0.08 mg/kg) and xylazine (0.003 to 0.005 mg/kg). Disinfection is performed by a massage with alcohol, put in place for about 7 min, and completed with the application of a disinfectant like Betadine iodine solution. The intralesional injection of PRP is performed by ultrasound-driven syringe needle (22 G or 23 G) at the exact point of injury. The amount of injected PRP product varies depending on the size of the lesion. The PRP may also be applied under the skin above and around the injured tendon or ligament. In case of injury to the palmar tenodesmic structures of the metacarpus or plantar tenodesmic structures of the metatarsus, the PRP is injected under the skin *via* a 25 G butterfly needle in the proximal metacarpal/metatarsal region, and it is then scrolled down with massage. In some cases, such as minor injuries to the superficial digital flexor, as it is very difficult to insert the needle into the lesion, it may be sufficient to place the PRP in the subcutaneous tissue. After the procedure, the skin is disinfected with alcohol and iodine product, and dressed with cotton gauze and a Vetrap-type bandage strip. The dressing remains in situ for 48 h.

All animals were treated with not gelled platelet-concentrates. However, the four veterinary facilities applied four slightly different clinical protocols: 1. Intralesional and perilesional injection with the platelet concentrate; 2. Intralesional injection with platelet concentrate, followed by two perilesional injection after 15 and 30 days; 3. Intralesional injection with platelet concentrate, followed by another intralesional injection after 30 days; 4. Intralesional injection with platelet concentrate at Day 1, followed by:
- Day 7: Shock waves therapy. - Days 10, 11 and 12: Tecartherapy.
- Day 14: Shock waves therapy. - Days 17, 18 and 19: Tecartherapy.
- Day 21: Shock waves therapy. - Days 23, 24 and 25: Tecartherapy.

Almost half horses (48%) were treated according to protocol 2; 23% and 20% of them were treated according to protocol 1 and 4, respectively, and only 9% to protocol 3.

Rehabilitation program. After 48 h the horse is sent to the rehabilitative phase. The rehabilitative treatment included:
1st Week: • Rest in stalls; • Robert Jones bandage for 3 days; • In-hand stepping on hard ground for 20 minutes a day.
2nd-3rd Week: • Rest bands in stalls; • In-hand stepping for 10-20 minutes and mounted stepping for 10-20 minutes a day on hard ground.
3rd to 6th Week: • Mounted stepping for 20 minutes a day and trotting for 5-10 minutes a day.
6th to 12th Week: • Trotting for 15 minutes a day.
After 3 months: • 50 minutes a day of work including canter/gallop.

All horses were followed-up by clinical examinations and US-scans (Figures 1-4 show the US-scan follow-up of a superficial digital flexor tendon lesion).

Statistical analysis. All analyses were performed using the survival package of the open source statistical software R. (R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.) Statistical analysis of the data was performed using ANOVA and Chi-square tests. ANOVA evaluates the effects of two or more independent variables simultaneously on a single dependent variable. Chi-square tests are designed to determine that an observed number differs from chance or from what was expected.

Results

99 horses were included in the analysis of clinical activity, while all horses were evaluated regarding the safety of the procedure. Origin of the animals was Northern Italy and all races were well-represented. 9.3 years was the average age of the horses (range=2-23 years).

The lesions included in the analysis were distributed as follows: 45 superficial digital flexor tendon; 8 deep digital flexor tendon; 6 articular ligaments; 5 accessory ligament of the deep digital flexor; 35 fetlock suspensory ligament.

None of the treated animals had any major adverse reactions as a result of the procedure, either locally or systemically. The rehabilitation program was well tolerated.

The grade of therapy success was evaluated as follows: Grade 1 (failure): a complete clinical and ultrasound healing is not obtained; Grade 2 (improvement): a complete clinical and ultrasound healing is obtained but the horse resumes his agonistic activity at an inferior level; Grade 3 (success): a complete clinical and ultrasound healing is obtained and the horse resumes the same agonistic activity he had before the injure within maximum 6 months.

A grade 3 success was obtained for 81% of the horses included in the analysis; 12% had an improvement (grade 2) and only 7% a failure (grade 1). 8 (8%) cases of relapse were observed; three of them, however, obtained a grade 3 success after a second treatment.

No statistically significant correlation existed between the clinical outcome and the area of the lesion (ANOVA, p=0.05) or the kind of protocol applied (Chi-squared, p=0.05). A statistically significant correlation was found between the clinical outcome and the age of the horse (ANOVA, p=0.05).

Discussion

Cell therapy and tissue engineering in equine veterinary arises from the need to find an optimal therapeutic solution to heal difficult injuries of tendons and ligaments in horses. These lesions have a poor regenerative capacity and often relapse, definitely affecting the athletic activity of the horse. Conventional therapies are not optimal because they cause the formation of a tendon scar, an alteration of the elasticity and mechanical properties of the structure, leading to an often delayed healing (1-2 years) that does not permit the resumption of normal agonistic activity (14).

In the scars the collagen is less cross-linked compared to normal tendon collagen and the predominant form is the type III (<1% in normal tendon compared to 20-30% in the scar tissue) (15). Furthermore, the mechanical properties of the scared tendon are worse than the normal tendon due to a deficient structural organization and composition of the extracellular matrix (16). On the contrary, regenerative medicine aims to favor the healing of the tissue recovering its original functional properties (3). On the basis of previous studies, cell therapy and the use of growth factors have proved to succeed in connective tissue regeneration, mainly due to the ability to stimulate the formation of type I collagen in a greater quantity than type III collagen, which is significantly less functional for tendon and ligament biomechanics. In the present study we evaluated the effect of PRP treatment on teno-desmic injuries in competition horses with different clinical protocols.

The success rate of our therapy with platelet concentrate was 93% regardless of the variant of protocol, higher than that observed with traditional treatments, while the relapse rate turned-out to be much lower (17, 18). In addition, cases of relapse were also successfully treated. Regenerative medicine using growth factors is able, by itself, to lead to the healing of the lesions, without the need of any additional treatments. Through the influence on the proliferation of fibroblasts, the promotion of angiogenesis and development of structures vascular mature, in fact, such treatment is not only capable of repairing the lesion with regenerated structure rather than a fibrotic scar, but also to strengthen the entire tendon structure decreasing the risk of recurrence and onset of new lesions. No statistical significance between the four different treatments and clinical outcomes was observed, so some considerations can be made. Protocol 4 is not to be considered the variant-of-choice, because it is more expensive and includes additional treatments which are probably not necessary. Protocol 2 was the most used one, but requires three injections at three different times. Protocol 1 is the most simple with only intra- and perilesional injections in a unique time. Therefore, the protocol of choice may be variant 2, since more data on safety and efficacy are available, or 1, since it is the least expensive and no statistically differences in efficacy were observed with other variants.

There was no statistically significant correlation between the area of the lesion and the response to treatment and this can be explained by the fact that the effectiveness of the treatment extends beyond the difficulty of healing of a particular lesions arising in a "critical" area, such as the core

lesions of the superficial digital flexor tendon. The efficacy of the treatment occurred even in cases of complete tearing of the tendon.

Limitations of our study were the lack of a control animal group and of histological evaluation. Even under certain limitations, the clinical observations derived from our study suggest that PRP treatment may be a promising therapy in treating teno-desmic injures which have a poor healing potential if treated with standard approaches. Full healing was obtained in 81% of the horses included in our analysis, 12% had clinical improvement and only 7% a failure. Future randomised controlled studies are required to confirm our results.

Conflicts of Interest

The Authors declared that there are no conflicts of interest.

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