Autologous platelet-rich plasma injected intraarticularly diminished synovial effusion and degree of lameness in horses affected with severe joint disease

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Autologous platelet-rich plasma injected intraarticularly diminished synovial effusion and degree of lameness in horses affected with severe joint disease
Summary

The clinical effect of the intraarticular injection of platelet rich plasma (PRP) in 7 horses with severe joint disease (4 with osteoarthritis and 2 with osteochondrosis) was evaluated. The degree of lameness (DL) and joint effusion (JE), and clinical follow-up were recorded. Three injections of PRP were performed at two week intervals. Horses were evaluated before each injection and two months after the last treatment. Clinical follow-up was conducted during 1 year. Count of platelets, leucocytes, and determination of TGF-β₁ levels per ml of PRP were performed, as well as leukocyte count, cytology and protein levels in synovial fluid (SF). PRP produced a statistically significant improvement in both the DL and JE (p<0.05). The most marked improvement was observed 2 months following the last treatment and apparently persisted up until 8 months later. A mean of 250 ± 71.8 x 10⁶ platelets, 8.68 ± 3.78 leucocytes x 10⁶, and 12515 ± 2443 pg of TGF-β₁ per ml of PRP were obtained. No adverse clinical signs resulted from this treatment. Despite the seemingly positive effects of this substance, the clinical use of PRP cannot be recommended until further studies with higher number of cases and longer follow up can be undertaken.

Keywords

Equine joint disease, osteoarthritis, osteochondrosis, platelet-rich plasma, growth factors, TGF-β₁
Introduction

Joint disease frequently produces lameness in horses. This problem could be produced for several causes, like repeated traumatic injury, joint instability, infection and developmental problems of the endochondral ossification (osteochondrosis (OCD)), amongst others. All of these factors can potentially produce severe damage of the articular cartilage. If the inciting cause is not timely diagnosed and treated, an irreversible degenerative process of the cartilage is produced and the final result is known as osteoarthritis (OA) (6).

Biochemically, OA may result from the final imbalance between anabolic and catabolic peptides which stimulate the production and the remodelling of extracellular matrix (ECM) components of the articular cartilage (6, 20). Joint homeostasis depends in many ways on the adequate expression of various growth factors (GFs). Some of these have anabolic effects (6, 20). Transforming growth factor beta-1 (TGF-β₁) (11, 14, 31), insulin-like growth factor-1 (IGF-I) (13, 19, 23), IGF-II (10), and platelet derived growth factor (PDGF) (30) all have been shown to promote both chondrocyte proliferation and ECM synthesis (20). Other peptides such as, interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) produce chemotaxis, leucocytic degranulation, expression of proinflammatory mediators, like prostaglandin E₂ (PGE₂), leucotriene B₄, nitric oxide (NO) and, metalloproteinases (MMPs) which degrade articular cartilage (20, 21, 24, 25).

Numerous studies document the in vitro effects of GFs in equine chondrocytes and cartilage (10, 11, 13, 15, 23). The results of these studies
support the possibility of using purified extracts (20) or of manipulating the genes that encode the production of these peptides (12) in order to manage equine OA. Platelet rich plasma (PRP) is an autologous source of many GFs, like TGF-β₁, platelet-derived growth factor (PDGF), and IGF-I (2, 8, 24, 28, 29) and other molecules that modulate inflammation and tissue repair (2, 8). This substance has been used in maxillo-alveolar reconstruction (5), and plastic surgery (4). There is a case report documenting its use in the successful treatment of a non traumatic cartilage avulsion in a soccer player (22). Furthermore, the use of PRP in a gel form has been reported in an experimental study involving skin healing mechanisms in a horse (8). Excellent results were reported but the investigation was limited to one horse only.

There has been an increase in the popularity of PRP in human (2, 4, 5) and equine (8, 24) medicine but little is known about the biological behaviour of this substance (24, 28, 29). In the present clinical study the therapeutic effects of PRP administered within the joint in a group of horses affected with severe joint disease was investigated. The effects of PRP were evaluated clinically by documentation of its effects on degree of lameness (DL), joint effusion (JE), and some synovial fluid (SF) parameters.
Material and methods

This pilot, clinical, prospective study was approved by the Ethical Committee of our institution. All the owners signed a sheet of consent authorizing treatment and were informed of the possible risks of joint injection or complications derived from PRP injection.

Patients

Seven horses with signs of severe joint disease were included in the study. They were of different breeds and sex and their average age was 6 years (range 1.2-15 years). A total of 10 joints were evaluated, since 3 of these horses had bilateral joint compromise (Table 1). Case selection criteria included detailed musculoskeletal examination and diagnostic procedures including radiography, ultrasound examination, regional anesthesia and arthroscopy in 2 horses.

Clinical study design

Lameness examination. Each patient was scored independently by 2 veterinarians. The lameness exam was scored from 0 to 5 according to the parameters of the AAEP (1). Only trot in a straight line on a hard surface was considered. A one minute long flexion test was performed in all the adult horses (n: 4) and in the fillies (n: 2) only in the last exams (since they had not been handled)
but they were not included in the statistical analysis. Lameness after flexion was graded from 0 to 3, where 0= negative response - 3= markedly positive response.

**Ultrasonic examination.** An ultrasonic evaluation of the affected joints was performed. Joint surface, degree of synovial effusion, thickness and appearance of synovial membrane were evaluated. Degree of JE was graded from 0 to 3, where 0= joint normal in appearance - 3= severe effusion and synovial membrane thickening. Ultrasonic measures were always performed in the same anatomical point in each patient by the same clinician.

**Synovial fluid analysis.** Synovial fluid analysis and cytology was performed in those events when fluid was obtained prior to joint injection.

**Clinical follow-up.** Clinical follow-up of each patient was conducted during 1 year. Four patients were evaluated personally by us; the other 3 were evaluated by the referring veterinarian.

**Preparation and analysis of PRP.** Whole blood was aseptically drawn of the jugular vein of each patient via 23G butterfly catheter (Terumo, Belgium), and deposited in 3.2% (wth/vol) sodium citrate tubes with capacity for 5 ml (BD Vacutainer™ (9NC 0.129M) systems, Uk). Afterwards, they were centrifuged at 120 g during 5 minutes. The first supernatant plasma fraction (50%), adjacent to the buffy coat, was obtained under aseptic conditions in a laminar flow chamber. This fraction was centrifuged at 240 g during 5 minutes and the 25% from the first
fraction was obtained. This last fraction was placed into sterile syringes and activated with calcium chloride (B. Braun Medial SA, Europe, (4.5 mEq/5 ml)), using 50 µl per ml of PRP. A fraction of PRP (2 ml) of each patient was analyzed for platelet and leukocyte count and TGF-β1 levels determination. Cell count was performed by an flow cytometry hematology system (ADVIA 120, Bayer, USA), and TGF-β1 levels were determined by a human commercial ELISA kit (R&D System, USA).

**Schedule of the PRP treatment.** The affected joint(s) of each horse were prepared aseptically for injection. The horses were sedated with an intravenous bolus of detomidine (Domosedan, Pfizer) and butorphanol tartrate (Torbugesic, Fort Dodge Laboratories Inc). Horses were injected 3 times at 2 weeks intervals. A complete clinical examination was performed before each PRP administration and 2 months after the last joint injection. The amount of PRP used in each patient was subjectively determined depending on the type and size of the joint, and weight of each patient (table 1). The horses were generally kept at a lower level of exercise during treatment and for 2 weeks following the last injection.

**Statistical analysis**

Values of DL and JE were expressed as means with their respective ranges. Data was analyzed with a Kruskall Wallis test. It was assumed that in the case statistical significance was found a Wilcoxon paired test of would be performed. The level of
significance for both tests was $p \leq 0.05$. Synovial fluid parameters and PRP values were presented in a descriptive manner.
Results

The 7 horses included in the study suffered severe joint disease. Four horses had OA (1 mare had OA of the left tibiotarsal and proximal intertarsal joints, 1 horse had OA of the left coffin joint, 1 horse had bilateral OA of the anterior fetlocks), 1 horse had OA of the medial femorotibial joint with a medial meniscal lesion, 2 fillies had bilateral OCD of the stifle, an 1 horse had OCD of the left tibiotarsal joint. The horses with OA had been treated with rest for 2 months up to 1 year, corticosteroids and hyaluronic acid. The 2 fillies with stifle OCD had undergone bilateral arthroscopic procedures with debridement of 40% of the lateral trochlear ridges of the femur. The fillies were included in the study because there were fragments remaining in the joint and a second surgery was not within the economic reach of the owners. One of these animals had been operated 3 months before the treatment with PRP and had been turned out in the paddock a month prior to treatment. The other one had had surgery 6 months prior to treatment with PRP. The 2 fillies and the colt with OCD were kept turned out. One mare was kept in training for carriage but at a lower intensity. Two horses were ridden lightly and finally 1 horse in training for endurance was maintained at the same level of work despite our recommendations of reducing work load during the treatment period.

The lameness scores improved after intraarticular administration of PRP and this was statistically significant (p= 0.048) in the horses in the study. The improvement of the lameness score was gradual and was most
significant after the third treatment and 2 months after (Table 2). Flexion tests could not be performed at the beginning on the 2 younger not very handled fillies, but the trend was of generalized improvement in all of the flexion tests performed. There was a statistically significant improvement in the JE (p=0.00043) during the treatment and 2 months after the last injection (Table 1). Joint fluid cytology performed when fluid could be obtained samples revealed a predominance of mononuclear cells (98%). It was not possible to perform statistical evaluation of SF samples (see details in Table 3). A mean of 250 ± 71.8 x 10^6 platelets, 8.68 ± 3.78 leucocytes x 10^6, and 12515 ± 2443 pg of TGF-β1 per ml of PRP were obtained. No adverse clinical signs resulted from this treatment. However, a moderate, transient synovial effusion was observed after the two first PRP injections in a filly (horse No 4) with OCD of the stifle and in patient No 6 with OA of the stifle.

The horses with OA maintained their final lameness score for about 8 months after the last PRP injection and, then showed a gradual increase of the DL. The two fillies and the colt with OCD remain without lameness, but they have not yet been trained at high intensity.
Discussion

This is the first report, to our knowledge, of the use of PRP for the intraarticular treatment of joint disease in the horse. We describe an easy, reliable and inexpensive technique for obtaining equine PRP. A schedule for treatment with PRP is proposed. However, this fact is merely empiric and it is based in a schedule of treatment used for the management of severe inflammatory arthropathies in human beings (R. Soler, unpublished data). We decided to begin this pilot clinical study because there is scientific and anecdotic evidence about the positive effects of this substance on human cartilage and chondrocytes (14, 22).

The improvement in both DL and JE in the horses of this study could be explained by the effect some GFs have on inflammation and tissue repair (27). During joint disease, especially OA, IL-1 and TNF-α promote the expression of nuclear factors like, nuclear factor kappa-beta (NF-κB) (17) which upregulate the genes that encode secondary proinflammatory peptides such as, IL-6, IL-8, IL-12, quimioquines, leucotrienes, PGE$_2$, NO and MMPs (21, 25, 26). From a clinical point of view the sum of all these substances can be manifested as pain and synovial effusion in horses affected with chronic joint disease (6). One could think that the local administration of PRP may have had an analgesic and antiinflammatory effect in the patients of this study by possibly inhibiting the expression of these nuclear factors or by blocking the effects of their metabolic catabolites (2, 27). Nevertheless, this hypothesis remains to be validated experimentally.
Joint effusion is produced by an increase in blood flow and capillary permeability with protein leakage which produce interstitial edema and an overall increase in joint fluid. Moderate synovitis may have beneficial effects on joint nutrition, but severe effusion affects joint function by creating articular surface incongruency, instability and joint pain (3). The increase in joint distension after each injection with PRP could have been produced by the fact that a relatively large volume of PRP was being administered, by the osmotic effect of the proteins injected in the joint space (3, 6), by the biochemical effects of this substance in the joint environment (22) or perhaps by the chemiotactic effects of the leukocytes present in the prepared platelet concentrate (24, 28, 29). However, this phenomenon was transient since the joint fluid effusion reverted to a significant decrease in joint distension before each subsequent treatment and at the end of the study (Table 2).

Up to date, two techniques for obtaining equine PRP have been described: apheresis and buffy coat methods (8, 24). An additional technique in order to improve the efficiency of collection equine platelets after using a convectional method has also been reported (24). The aforementioned techniques are good, but they present technical and economical restrictions for many equine practitioners. For example, the apheresis method can only be performed in a specialized laboratory. On the other hand, the buffy coat method is very expensive. We think that the double centrifugation tube method presented in this study, represents a good alternative method in comparison to the other ones previously described.

In this study a lower concentration of platelets (250 ± 71.8 x 10⁶ platelets/ml) was obtained in comparison with the equine values reported for apheresis method
by Carter et al (8) (490 x 10^6 platelets/ml) or by Sutter et al (24) (855 x 10^6 platelets/ml) or for buffy coat method (1472 x 10^6 platelets/ml) (24). However, we obtained higher TGF-\(\beta_1\) levels (12515 ± 2443 pg/ml) than those reported by the apheresis method by Carter et al (8) (7480 ± 1315 pg/ml), but lower TGF-\(\beta_1\) levels in comparison with Sutter et al (24) (23600 pg/ml). Using the buffy coat method, Sutter et al (24), reported a total of 1472 x 10^6 platelets/ml and 15300 pg/ml of PRP. Again, if we compare the results obtained with our technique, it is possible to note that the platelet number is higher in that study (24) in relation with our results. However, TGF-\(\beta_1\) levels obtained with both techniques were very similar. It is important to note that many not well defined factors can influence the final GFs levels of platelet concentrates obtained by different techniques (8, 24).

Ideally one would try to minimize the number of leukocytes injected into joints. With our technique an inferior number of leukocytes (8.68 ± 3.78 cells x 10^6/ml) was obtained in comparison with the results described by Sutter et al (24) for apheresis (33.7 leukocytes x 10^6/ml) and buffy coat (32.5 leukocytes x 10^6/ml) methods. However, the importance of leukocyte type and number in platelets concentrates remains not well understood and actually researchers do not known if the presence of these inflammatory cells is responsible or not of the some beneficial and detrimental effects of PRP in the tissues. Another important factor to consider in the use of PRP is the method used for activation. In our study calcium chloride was used to stimulate platelet activation. In another study (8) platelets were activated with thrombin, which although not specified, was most likely to be bovine in origin. The use of this protein can be associated with allergic reactions
In this sense the method described here is safer and less costly than in other methods where purified bovine thrombin is utilized.

Concentrations of GFs in PRP are high when compared to the levels required to stimulate equine cartilage anabolism \textit{in vitro}. Concentrations of 5 ng/ml of TGF-\(\beta\) produce chondrocyte mitosis and stimulate ECM synthesis (11, 14, 29). This same action has been observed with doses of 200-500 ng/ml of IGF-I (13, 23) and IGFII in concentrations of 25-50 ng/ml (10). One must remember that GFs \textit{in vitro} could act very differently to what is observed \textit{in vivo} (20). Perhaps supraphysiological concentrations of GFs, (e.g.: TGF-\(\beta_1\)) can be found in these PRP, could control catabolic and inflammatory processes occurring in joint diseases, like OA. Recently, in another study (Argüelles et al., unpublished data) it was found that the top plasma fraction (50\%) obtained during the first centrifugation to concentrate platelets presents a similar concentration of TGF-\(\beta_1\) than the fraction (PRP) used in this study. The number of leukocytes present in this platelet fraction was lower in comparison with the number of leukocytes present in the platelet fraction used in this study. We hypothesize that this fraction may be better for treatment of joints, since the leukocytes could be responsible of the transient joint effusion observed in some of the patients of this study. However, this hypothesis should be further validated.

The short and limited effect of the vast majority of therapeutic agents used to combat joint disease and particularly OA is a very important problem (9, 18). Horses suffering OA need periodic treatment. Very few agents modify the course or progression of the disease and some may actually accelerate joint destruction
(7, 9, 18). The sum of the analgesic plus the seemingly prolonged antiinflammatory effect observed in these horses treated with PRP in the joint make us think this could be a remission inductive therapy (16). However, our study had serious limitations that cannot lead us to conclude that PRP may really being used for the treatment of equine joint disease. It should be considered that number of the cases that we evaluated was small and not representative. Furthermore, we did not have age and disease matched controls. It is known that rest and time usually diminish clinical signs of joint disease (6, 7, 18) but it may be worthy to note that our study the patients we included had had clinical signs of joint disease for at least 3 months and up to a year prior to treatment and except in 2 cases the horses went back to the same level of activity that they had prior to treatment.

Nevertheless, from our positive clinical observations we think that Intraarticular PRP injection may be a promising new instrument in the treatment of joint disease in the horse. However, this therapy can not be recommended until \textit{in vitro} studies had been performed and more clinical cases have been assessed. Ideally a prospective randomised double blind clinical study would be necessary to validate these initial clinical observations.
References


24. Sutter WW, Kaneps AJ, Bertone AL. Comparison of hematologic values and transforming growth factor-β and insulin-like growth factor concentrations in


Table 1. Signalment and clinical signs of the horse of this study.

<table>
<thead>
<tr>
<th>Horse No</th>
<th>Breed</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Aptitude</th>
<th>Joints affected</th>
<th>Diagnosis</th>
<th>Previous treatment</th>
<th>PRP (ml/art)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Arabian</td>
<td>Male</td>
<td>7</td>
<td>Enduracing</td>
<td>Fetlocks</td>
<td>OA</td>
<td>IA steroids</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>Haflinger</td>
<td>Male</td>
<td>15</td>
<td>Dressage</td>
<td>Left Coffin joint</td>
<td>OA</td>
<td>IA steroids and HA</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>French mountain</td>
<td>Female</td>
<td>8</td>
<td>Carriage</td>
<td>Left tibiotal and proximal intertarsal joints</td>
<td>OA</td>
<td>NSAIDs</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>Warmblood</td>
<td>Male</td>
<td>6</td>
<td>Jumping</td>
<td>Right medial femorotibial joint with a medial meniscal lesion</td>
<td>OA</td>
<td>NSAIDs</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>Hannoverian</td>
<td>Filly</td>
<td>1.4</td>
<td>Intended use, jumping</td>
<td>Stifles</td>
<td>OCD</td>
<td>Arthroscopic Surgery IA steroids and HA</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>Spanish Sport</td>
<td>Filly</td>
<td>1.2</td>
<td>Intended use, jumping</td>
<td>Stifles</td>
<td>OCD</td>
<td>Arthroscopic Surgery</td>
<td>20</td>
</tr>
<tr>
<td>7</td>
<td>Holsteiner</td>
<td>Male</td>
<td>3.5</td>
<td>Jumping</td>
<td>Left tibiotal joint</td>
<td>OCD</td>
<td>No previous therapy</td>
<td>15</td>
</tr>
</tbody>
</table>

OCD: Osteochondrosis. OA: Osteoarthritis. HA: Hialuronic acid. IA: intraarticular
Table 2. Lameness degree and joint distension scores of the horses of the study.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
</tr>
<tr>
<td>Degree of lameness</td>
<td>1.1 a, Range 0.5-2</td>
</tr>
<tr>
<td>Degree of joint effusion</td>
<td>2.25 a, Range 2-3</td>
</tr>
</tbody>
</table>

a-c : Values with different letters in a row are significantly different statistically (P<0.05).
Table 3. Description of synovial fluid parameters from the joints of some of the horses treated in this clinical study.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Initial (n:3)</th>
<th>Before 2nd injection (n: 4)</th>
<th>Before 3rd injection (n:3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Protein (g/dl)</td>
<td></td>
<td>1.73 ± 0.5</td>
<td>1.4 ± 0.37</td>
<td>1.73 ± 0.4</td>
</tr>
<tr>
<td>Leukocytes (x10³ cel/µl)</td>
<td></td>
<td>0.57 ± 0.06</td>
<td>0.78 ± 0.2</td>
<td>0.53 ± 0.06</td>
</tr>
</tbody>
</table>

Values are expressed as means ± standard deviation. n: number of joints.
Figure 1. Ultrasonic longitudinal sections of the dorsomedial recess of the tibiotarsal joint (2cm below the medial malleolus) of the Horse number 7 with osteochondrosis. Note the decreasing of the synovial effusion. a) Image before the treatment with platelet rich plasma. b) Image after the third injection.
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