Short- and long-term effects of platelet-rich plasma upon healthy equine joints: Clinical and laboratory aspects

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Abstract — This study aimed to verify whether transient inflammatory reactions incited by the administration of intra-articular platelet-rich plasma (PRP) affected joint components through short- and long-term in vivo evaluation of inflammatory biomarkers and extracellular matrix degradation products in synovial fluid. The effects of PRP were analyzed in a short phase protocol (SPP) and in a prolonged phase protocol (PPP), using saline-injected joints as controls. In the SPP, higher white blood cell counts and prostaglandin E2 and total protein concentrations were observed in the synovial fluid of PRP-treated joints \((P < 0.05)\). There were no differences between the interleukin-1 \(\beta\), interleukin-1 receptor antagonist protein, tumor necrosis factor-\(\alpha\), chondroitin sulfate, or hyaluronic acid concentrations between PRP and saline injected joints. In the PPP, there were no differences in evaluated parameters between groups. PRP injection elicits a mild and self-limiting inflammatory response shortly after administration, without long-term deleterious effects on joint homeostasis.

Résumé — Effets à court et à long terme de le plasma riche en plaquettes sur les articulations sains chez les équines : aspects cliniques et laboratoires. Cette étude a pour but de vérifier si les réactions inflammatoires passagères induites par l’administration intra articulaire de Plasma Enrichi en Plaquettes (PRP) affectent les composants articulaires. Les bio-marqueurs de l’inflammation et les produits de dégradation de la matrice extracellulaire ont été évalués in vivo, dans le liquide synovial, à court et long terme. Les effets du PRP ont été analysés lors d’un protocole court terme et lors d’un protocole long terme et les articulations contrôles ont été injectées avec du liquide physiologique. Le protocole court terme a révélé des comptages de globules blancs et de prostaglandine E\(_2\), ainsi que des concentrations en protéines totales plus élevés dans le liquide synovial des articulations traitées au PRP \((P < 0.05)\). Cependant, aucune différence de concentration en interleukine-1 \(\beta\), en protéine antagoniste des récepteurs à l’interleukine-1, en facteur de nécrose tumorale alpha, en chondroitine sulfate et en acide hyaluronique n’a été notée entre les articulations injectées au PRP et les articulations contrôles. Le protocole long terme n’a démontré aucune différence des paramètres évalués entre les deux groupes. L’injection de PRP provoque une réponse inflammatoire légère et auto-limitante rapidement après l’administration, sans effet délétère sur l’homéostasie de l’articulation à long terme.

(Traduit par les auteurs)

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Introduction

The increasing participation of equine athletes in various equestrian activities with high levels of performance has been accompanied by an increase in the occurrence of inflammatory disorders of the equine appendicular skeleton, which hinders performance and requires long and expensive treatments (1). These therapeutic approaches often result in healing, but the newly formed tissues have unpredictable strength, tensile, and mechanical properties, which may compromise the horse’s function, ability to return to athletic activities, and long-term health.

Articular cartilage damage often results from trauma, sports injury, or aging. It usually progresses to osteoarthritis, a more severe form of articular disease in which the ability to repair is limited, and lesions culminate in cartilage degeneration (2). To
achieve better quality healing of tissue in cartilage repair, autologous biologic therapies have been employed. These therapies have gained increasing popularity over the last decade because they are considered to be safe, easy to obtain, and not subjected to extensive testing that is normally required prior to drug use approval (3). Because it is not considered doping and has the appeal of an innocuous, autologous therapy, platelet-rich plasma (PRP) remains a current option in equine orthopedics.

In equine medicine, experimental and clinical studies have evaluated PRP’s effects on tendons and ligaments (4–7). However, reports on PRP’s efficacy in joints and on the in vivo effects on anabolic and catabolic events in the intra-synovial environment are scarce. Despite promising results (8–12), intra-articular PRP treatments have been employed without strong scientific evidence to support their use. Well-designed, randomized controlled clinical studies that prove PRP’s therapeutic efficacy in equine arthropathies are still lacking, as are studies that evaluate the in vivo articular responses to PRP administration.

Recent experimental research has shown that the in vivo intra-articular administration of PRP into healthy equine joints elicits a mild to moderate inflammatory response in synovial fluid that lasts for at least 24 h (13). Simultaneously, studies in human and animal models have suggested that positive results associated with the intra-articular use of PRP are mainly attributed to its anti-inflammatory properties (14) and not to the anabolic effects of growth factors on cartilage and joint components (15). These seemingly contradictory results likely reflect the complex and diversified nature of PRP and its bioactive factors, which are still not fully known.

The aim of this study was to use in vivo evaluation of biomarkers of inflammation and extracellular matrix degradation to verify if the previously reported, transient inflammatory reaction incited by intra-articular PRP administration affects joint components. This evaluation was carried out during a short observation period and also through a more extended evaluation protocol, in which weekly intra-articular PRP injections were administered to mimic a common prescription model employed in the clinical scenario. It was hypothesized that the acute intra-articular inflammatory response associated with PRP injection was not of a long duration nature, nor of deleterious consequences to articular homeostasis.

Materials and methods

Animals

The experimental protocol used in this study was approved by the Institutional Animal Care and Use Committee (protocol number: 2264/2011; date of approval: 08/17/2011). Eight clinically healthy horses (6 males and 2 females), without any history of articular disease, ranging in age from 4 to 10 y were selected.

In vitro and in vivo study

During the in vitro arm of this study, the horses served as blood donors for production of PRP, which was analyzed for platelet and white blood cell (WBC) count, total protein (TP), interleukin-1β (IL-1β), IL-1 receptor antagonist protein (IL-1ra), prostaglandin E₂ (PGE₂), and tumor necrosis factor alpha (TNF-α) concentrations. Plasma from these horses was used as control.

The in vivo arm of the study was divided into short phase and prolonged phase protocols. During the short phase, 8 horses were subjected to a single 4.0 mL injection of PRP in a randomly assigned metacarpophalangeal joint. After 15 d, the contralateral joint was injected with 4.0 mL of saline as control. Synovial fluid samples were collected immediately prior to each injection and at 3, 6, 24, 48, and at 168 h post-injection to determine the WBC counts and the TP, IL-1β, IL-1ra, PGE₂, TNF-α, and glycosaminoglycan (GAG) levels.

The prolonged phase protocol was started after a 30-day rest period. Eight horses received weekly intra-articular injections of PRP in a randomly assigned metacarpophalangeal joint for 3 consecutive weeks, as recommended in previous studies for the administration of PRP (9,10,16), and were observed over a 28-day period. After 15 d, the contralateral limbs were treated with intra-articular saline injections, following the same protocol, serving as controls. Synovial fluid samples were collected immediately prior to each PRP or saline injection on days 0, 7, 14, 21, and 28 and were analyzed by the same techniques employed in the short phase protocol.

Synovial fluid collection

In both protocols of the in vivo study, arthrocentesis was achieved through the lateral collateral sesamoidean ligament, as described by Misheff and Stover (17). The retrieved synovial fluid was centrifuged at 4°C for 15 min at 1960 × g, and the supernatant was stored at −80°C until further analysis.

PRP preparation

Acquisition of PRP began with aseptic blood collection, via the left jugular vein, into 9 tubes containing sodium citrate. One tube was used for the complete red and white blood cell (WBC) counts and TP concentration determination. The 8 remaining tubes were centrifuged at 150 × g for 5 min at 24°C, and the supernatant plasma was again centrifuged at 800 × g for 5 min at 24°C for the PRP acquisition. The PRP was left to rest for 1 h and was then homogenized for 1 h. The entire procedure was conducted under a laminar flow hood to ensure sterility of the final product.

Synovial fluid analysis

The WBC counts in the synovial fluid were done using freshly obtained aliquots in a Neubauer chamber. Differential counts were done using smears stained with May-Grünwald-Giemsa

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<th>Table 1. Composition of plasma and platelet-rich plasma (PRP) samples (mean concentration ± standard error)</th>
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<td><strong>PRP</strong></td>
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* Differences statistically significant as compared to plasma (P < 0.05).
dye. The TP levels in the synovial fluid and PRP were measured using the biuret method with an automated biochemical analyzer (Randox, Crumlin, UK).

Cytokines IL-1β, IL-1ra and TNF-α were measured by enzyme-linked immunosorbent assay (ELISA), using commercial kits. For IL-1β quantification, the USCn Life Science kit (Wuhan, China, E90569Eq) was used. This is a sandwich enzyme immunoassay, specific for equine IL-1β. IL-1ra was measured using the Quantikine MRA00 kit (R&D Systems, Minneapolis, Minnesota, USA), which is also a quantitative sandwich enzyme immunoassay validated for horses (18). TNF-α was measured by the equine-specific DuoSet ELISA system (DY1814; R&D Systems).

Eicosanoids in the synovial fluids were measured by ELISA using the 514010 Prostaglandin E2 EIA Kit (Cayman Chemical, Ann Arbor, Michigan, USA), as previously reported in equine species (19,20).

Hyaluronic acid (HA) and chondroitin sulphate (CS) concentrations were determined as previously described (21). In brief, synovial fluid samples (100 μL) were subjected to proteolysis, debris was removed by centrifugation, and the supernatant was freeze-dried and resuspended in water (50 μL). Aliquots (5 μL) were submitted to agarose gel electrophoresis, and stained with Toluidine blue in 1% acetic acid, and then in sodium acetate buffer, pH 5.0. These compounds were quantified by densitometry of the agarose gel slabs.

To compensate for the possible dilution of the synovial fluid samples, urea concentrations were measured by urease-glutamate dehydrogenase, using an automated biochemical analyzer (Randox) (20–22).

**Physical examination**

Before and after each procedure, the metacarpophalangeal joints were evaluated for effusion, enlargement, heat, response to palpation, and response to passive and active flexion tests. Lameness, if present, was graded according to Baxter and Stashak (23), on a scale from 0 (no lameness) to 5 (non-weight bearing lameness).

**Statistical analysis**

The data were analyzed for normality using the Kolmogorov-Smirnov test. Afterwards, paired t-tests, an analysis of variance (ANOVA), and Tukey’s post-test were used to compare the PRP group with the saline group. GraphPad Instat 3 software was used to perform the statistical analysis. Statistical significance was set at $P < 0.05$. 

**Figure 1.** Mean concentrations and standard errors of the nucleated cell counts (cells/μL), the prostaglandin E$_2$ (PGE$_2$/urea) and total protein (protein/urea) levels from PRP and saline groups during the short phase (A, B, C).

* Differences statistically significant compared with saline group ($P < 0.05$).

† Differences statistically significant compared with baseline values ($P < 0.05$).
Results

In vitro study

The mean PRP platelet concentration was $423 \times 10^3$ platelets/$\mu$L. The leukocyte counts remained unchanged ($8.36 \times 10^3$/mL), meaning that the platelets were concentrated after processing without a concomitant rise in the WBC counts.

Table 1 shows the results obtained for TP, TNF-α, IL-1β, IL-1ra, and PGE$_2$ concentrations in plasma and in PRP samples. Only IL1-ra concentrations were different in PRP compared with those found in plasma.

In vivo study

Short phase protocol. The WBC counts were increased in the saline group at 6, 24, and 48 h and in the PRP group at 3, 6, 24, and 48 h ($P < 0.05$). The groups differed at 3, 6, 24, and 48 h, with increased counts in the synovial fluid of PRP-treated joints ($P < 0.05$) (Figure 1A).

The PGE$_2$ was increased after 3, 6, 24, and 48 h in the PRP group and after 6 h in the saline group ($P < 0.05$). When comparing PRP and saline groups, higher PGE$_2$ concentrations were found at 3 and 6 h in the PRP group ($P < 0.05$) (Figure 1B).

Higher TP concentrations were found in the saline group at 6, 24, and 48 h and in the PRP group at 3, 6, and 24 h ($P < 0.05$). Comparisons between groups yielded significant increases in TP in the PRP group at 3, 6, 24, and 48 h ($P < 0.05$) (Figure 1C).

Detectable concentrations of IL-1β were inconsistently found only in the synovial fluid from PRP-treated joints, resulting in insignificant differences compared with those in the saline group.

The TNF-α concentration did not differ between the PRP and saline groups (Figure 2A). IL-1ra concentration was increased in saline and PRP groups at 24 h ($P < 0.05$) (Figure 2C), and there was no difference when the groups were compared.

The HA values decreased in the PRP group at 3, 6, 24, 48, and 168 h ($P < 0.05$). However, no differences were found when intergroup comparisons were performed (Figure 3A). Interestingly, the same decrease was observed for the CS concentrations at 3 and 6 h in the PRP and saline groups. Furthermore, in both groups, the CS concentrations were increased at 24 h (Figure 3C).

Throughout the experiment, no changes were observed during the physical evaluations for both groups.

Figure 2. Mean concentrations and standard errors of the tumor necrosis factor-α (TNF-α/urea) and interleukin 1 receptor antagonist protein (IL1-ra/urea) levels from PRP and saline groups during the short phase (A, C) and prolonged phase (B) protocols.

* Differences between PRP and saline groups statistically significant ($P < 0.05$).

† Differences statistically significant compared with baseline values ($P < 0.05$).
Prolonged phase protocol. The WBC counts were increased in the saline group at 7, 14, 21, and 28 d and in the PRP group at 7, 14, and 21 d (P < 0.05). There was no difference when the groups were compared (Figure 4A).

Over the 28 d observation period, no differences were found intra- and inter-groups in PGE\(_2\), TP (Figures 4B, 4C), TNF-\(\alpha\) (Figure 2B), IL-1\(\beta\), IL-1ra, HA and CS concentrations (Figures 3B, 3D). Similar results were obtained for the physical evaluations.

Discussion

In tendons and ligaments, several experimental and clinical studies have demonstrated a positive effect on the quality of healing tissues associated with PRP treatments (4–6). As a therapeutic option for equine articular lesions, PRP (among other hemoderivatives) has recently received much attention and is advocated as a therapeutic tool to enhance healing through newly formed higher quality tissue that withstands athletic demands over shorter time periods. Despite some experimental work yielding promising results (24), intra-articular PRP treatments have been employed without strong evidence to support its use (25). Well-designed, randomized, controlled clinical studies that prove PRP’s therapeutic efficacy in equine arthropathies are still lacking, as are studies that evaluate the in vivo articular responses to PRP administration.

To add information to this topic, PRP was obtained by an easy and repeatable technique, and the effects of this hemoderivative on normal equine joints were observed. The PRP, prepared as described, concentrates platelets in sufficient amounts to elicit a therapeutic response, without a concomitant increase in leukocytes. Although the effects of leukocyte concentration on the efficacy of PRP are not completely understood, some undesirable aspects have been attributed to their presence in the final product. For example, white blood cells release matrix metalloproteinases and produce reactive oxygen species that can lead to further damage or inhibit healing in certain tissues or at certain phases of recovery (26). Others have reported a positive correlation between increasing leukocyte concentrations in PRP preparations and elevated levels of the inflammatory cytokine IL-1\(\beta\) and the degrading extracellular matrix enzyme metalloproteinase-9 (27). Increased pain has also been reported after injecting leukocyte-rich preparations (28). Given the low WBC counts in the product employed here, these undesirable events were less likely to occur. In fact, we found no clinical signs of inflammation associated with intra-articular PRP injection.
Additionally, the choice to not employ platelet activators may have played a part in the absence of clinical signs of inflammation after PRP injection. As previously shown (13), increased effusion and flexion scores and periarticular signs of inflammation accompanied PRP intra-articular injections, particularly when bovine thrombin was used as an activator. Nonetheless, synovial effusion and periarticular signs of inflammation were detected shortly after injection of the resting and the calcium-activated PRP, likely because of the 1.9-fold increase in WBC concentration in that product.

Although the physical examination was unremarkable and the role of activators and leukocytes was not evident, a laboratory analysis of synovial fluid in the short phase protocol demonstrated an inflammatory response associated with the PRP injection. This result was demonstrated by an increase in PGE$_2$ concentration in the synovial fluid of PRP-treated joints within 6 h of administration, and by a rise in TP concentration and WBC counts in the synovial fluid of PRP-treated joints until 48 h post-injection, compared with the saline group. This latter response was also observed in a previous study (13), in which the early inflammatory response to PRP was characterized by a significant cellular influx with prominent neutrophilia and increased TP in the synovial fluid.

Other authors have encountered synovial reactions after PRP intra-articular injections. In New Zealand white rabbits, they were described as focal, non-invasive, and non-deleterious to articular cartilage. Although the authors performed the histological evaluation 2 wk after the injection procedure, there was synovial villous hyperplasia and marked chronic synovitis (29). Spaková (30) studied the effects of PRP on human knee osteoarthritis compared with hyaluronic acid treatment and documented worsening of knee pain after PRP injections in 6 of 60 cases. The reaction was self-limiting and disappeared spontaneously in 2 d.

The synovial fluid obtained after the intra-articular treatment with PRP had detectable levels of IL-1β, that were not significantly increased compared with the concentrations found in synovial fluid from saline injected joints. The increase in IL-1β was not mirrored by an increase in IL-1ra in PRP-treated joints, likely because the IL-1β increase was not significant. Increased levels of IL-1ra were observed at the 24-hour observation time in the SPP in both groups, likely as a local response to the short-gapped, repeated punctures.

Effects of seriated punctures also have to be taken into consideration, as already described (13,31–33). Indeed, saline injection into joints in the short phase protocol resulted in significantly increased WBC counts in synovial fluid at 24 and 48 h post-injection and increased TP concentration at 24 h post-injection compared with baseline values. However, in this protocol, the injection of PRP resulted in significant increases in WBC counts, PGE$_2$ and TP concentrations compared to the saline group, demonstrating that repeated arthrocentesis plays...
a lesser role in the inflammatory response observed after PRP injection.

Other findings likely related to the seriated arthrocentesis were the elevated concentrations of CS and diminished concentrations of HA in the saline and PRP-treated joints in the short phase protocol. The CS is an important biomarker of ECM degradation (34). In the present study, increased CS concentrations were attributed to serial punctures, in agreement with the findings of Van den Boom et al (33), as there were no differences between the groups. Nonetheless, we observed a trend for higher CS levels in the short phase protocol after PRP treatment. As previously shown, PRP contains not only platelets and bioactive factors that influence anabolic and anti-inflammatory pathways, but also components that favor catabolic and proinflammatory events which may have contributed to its short-lived deleterious effects on ECM metabolism (35).

Decreased HA concentrations were observed after intra-articular PRP injection at 3, 6, and 168 h post-injection, although there was no difference between the groups. During acute inflammation, degradation outpaces synthesis, and the concentrations of HA tend to decrease, indicating articular catabolism (36–38). On the other hand, in vitro studies have demonstrated PRP’s beneficial effects upon HA (39), proteoglycans (40) and type II collagen synthesis (41,42). However, this positive effect was not demonstrated in this study.

After demonstrating that PRP intra-articular administration into healthy equine metacarpophalangeal joints elicited a transient inflammatory reaction, whether through the activation of synovial cellular components or repeated punctures, we investigated the long-term effects of this response. Interestingly, all indicators of synovial inflammation that were observed in response to PRP 24 to 48 h after intra-articular injection were absent when the effects of weekly injections performed over a 28-d period were observed. Furthermore, unchanged concentrations of the cartilage’s turnover biomarkers (CS and HA) strongly suggested that ECM degradation was not occurring and that joint homeostasis was not compromised. These results indicate that the early inflammatory response associated with intra-articular PRP injection is self-limiting, does not adversely affect intra-synovial media, and has no cumulative effects when repeated weekly injections are administered. A comparison of the inflammatory (i.e., IL-1β and TNF-α) and anti-inflammatory (i.e., IL-1ra) synovial fluid cytokine profiles among the PRP group, saline group and baseline levels also failed to indicate ongoing articular catabolism, infectious, or inflammatory events during this extended observation period. These weekly evaluations demonstrate that the joint is capable of extinguishing the initial inflammatory reaction elicited by PRP injection, with no further compromise to articular homeostasis. Although verifying the beneficial effects of intra-articular PRP administration was not the primary goal of this study, we expected to identify favorable properties, which have already been well demonstrated in vitro. Our results, however, are in agreement with the inconsistent results obtained in PRP clinical trials, compared with the positive results obtained in experimental in vitro studies. We speculate that these incongruences could be explained with further research, not only in clinical trials but also in in vivo experimental models. Others have found that in vitro results tend to deviate from in vivo observations (15,43–45), favoring positive outcomes. The interaction between the unique, diverse, and complex composition of PRP with the dynamic and multi-faceted scenario in the intra-synovial environment may account for these differences. The lack of uniformity in acquisition methods, and therefore in PRP’s composition, is a major obstacle in the evaluation of its efficacy (27,35).

Furthermore, a positive correlation has been shown in the human literature between well-designed clinical studies and a lack of evidence of PRP’s beneficial effects on selected outcomes (5), as well as a lack of correspondence of the promising results verified in basic research and animal studies with the ones obtained in clinical trials (47). Therefore, such clinical studies are needed to generate sufficient evidence for PRP use in the healing of musculoskeletal injuries and to support the potentially beneficial effects derived from current basic research (44,48).

This study demonstrated that PRP is a safe therapeutic option for intra-synovial administration, with no long-term adverse effects to joint homeostasis, despite an early inflammatory response to its intra-articular injection. Although relevant, this early phenomenon associated with PRP injections should not distract us from the vital questions regarding its use and its efficacy in orthopedic therapeutics, which remain unanswered. Why are the results from in vivo experimental studies and clinical trials less encouraging than those obtained in the in vitro studies? How can we predict and reproduce the effects of a singular and autologous preparation whose composition can vary greatly among different donors and different acquisition methods? Utilizing in vivo models, clinically and experimentally, may aid in the clarification of these aspects, for they put the wide spectrum of variables present in the intra-synovial milieu into play.

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