Synovial fluid growth factor and cytokine concentrations after intra-articular injection of a platelet-rich product in horses

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Platelet-rich plasma (PRP) products may be useful for treatment of joint disease in horses, but may contain undesirable pro-inflammatory cytokines in addition to growth factors. This study investigated whether autologous PRP increases synovial fluid growth factor and cytokine concentrations when injected into normal equine metacarpophalangeal and metatarsophalangeal (fetlock) joints. Fetlock joints of seven healthy horses received one of four treatments: saline, resting PRP, CaCl2-activated PRP or thrombin-activated PRP. Synovial fluid was sampled prior to injection and at 6, 24, 48 and 96 h post-injection. Platelet-derived growth factor (PDGF-BB), transforming growth factor β1 (TGFβ1), interleukin (IL)-6 and tumor necrosis factor α (TNFα) concentrations in synovial fluid and PRP were measured by ELISA. Synovial fluid PDGF-BB, TGFβ1, IL-6, TNFα and IL-1 concentrations were also measured in vitro after incubation for 6 h with resting PRP only. Growth factor concentrations, but not cytokine concentrations, were significantly higher in activated PRP than in resting PRP samples. After intra-articular injection with resting or thrombin-activated PRP, synovial TGFβ1 increased significantly compared to baseline levels. TNFα and IL-6 were significantly increased in synovial fluid after thrombin-activated PRP injection. In vitro, growth factor concentrations increased significantly in synovial fluid after mixing with PRP, indicating that exogenous activation of PRP for intra-articular injection may be unnecessary, whereas cytokine levels did not. In conclusion, thrombin-activated PRP induced an inflammatory cytokine response in joints, whereas resting or CaCl2-activated PRP did not. Synovial growth factor levels were low overall; the reported benefits of intra-articular PRP may not be attributable to changes in local PDGF or TGFβ1 concentrations.

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Introduction

Platelet-rich plasma (PRP) is an autologous biological therapy used to enhance tissue healing in orthopedic injuries (Sampson et al., 2008; Taylor et al., 2011). PRP was initially used to treat tendon and ligament lesions in humans (Mishra and Pavelko, 2006; Paoloni et al., 2011) and horses (Waselau et al., 2008; Bosch et al., 2010), but is now also used intra-articularly. Beneficial effects of intra-articular PRP therapy have been reported for osteochondral lesions, early osteoarthritides, intra-articular fractures and lesions of intra-articular ligamentous tissues in humans (Filardo et al., 2011; Randelli et al., 2011; Mei-Dan et al., 2012; Wei et al., 2012) and laboratory animals (Saito et al., 2009; Sun et al., 2010). In horses, intra-articular PRP use is reported anecdotally or in abstract form only (Abellanet and Prades, 2009; Fortier, 2009), but has not yet been described in the published literature.

The therapeutic effect of platelet-based therapies derives from the growth factor content of platelets themselves (Marc, 2004). Platelet α granules contain at least 10 different growth factors (Nurden, 2011), of which platelet-derived growth factor (PDGF-BB) and transforming growth factor β1 (TGFβ1) are the most plentiful. When platelets undergo activation, α granule contents are released into the extracellular milieu. These contents include growth factors and cytokines (Semple et al., 2011). The pro-inflammatory cytokine content of PRP has received little attention in the literature and has been ascribed to the leukocytes in PRP rather than the platelets (Sundman et al., 2011; McCarrel et al., 2012), but further study of the source of these cytokines is required. To be of therapeutic value, the anabolic effects of PRP must outweigh any inflammatory effects (Sundman et al., 2011; McCarrel et al., 2012).

We hypothesized that intra-articular PRP injection would result in increased levels of growth factors and cytokines in synovial fluid, which would vary according to the activation status of the PRP. The aim of this study was to determine the PDGF-BB, TGFβ1, interleukin (IL)-6 and tumor necrosis factor α (TNFα) concentrations in three different preparations of a PRP product and in
synovial fluid for 5 days after intra-articular injection of PRP in horses. A corollary in vitro study evaluated the isolated effects on these same synovial growth factor and cytokine concentrations after short-term incubation with resting PRP. The main study hypothesis was that intra-articular (IA) PRP injection would result in increased levels of growth factors and cytokines in the synovial fluid, which would vary according to the activation status of the PRP.

Materials and methods

This experiment was part of a larger study published by Textor and Tablin (2013); the same horses, joints and PRP were used as described in that publication.

Animals

Seven healthy horses (four castrated males and three females) of various breeds were used as experimental subjects (mean age 12.7 years, median age 14 years, range 2–21 years). Animal use was approved by the Institutional Animal Care and Use Committee (protocol number 16054, date of approval 11 August 2010).

Preparation of platelet-rich product

Autologous PRP was prepared using a gravity filtration system (Equine Platelet Enhancement Therapy, E-PET, Pall Corporation).

Preparation of activators

Thrombin (0.2 mL of 0.2 μM-filtered bovine thrombin; Sigma–Aldrich) was added to 2.3 mL PRP to produce a final thrombin concentration of 10 U/mL (Textor et al., 2011). CaCl₂ (0.2 mL of 42.5 mg/mL 0.2 μM-filtered CaCl₂ 2H₂O; ACS grade, Fisher Chemicals) was added to 2.3 mL of PRP to produce a final CaCl₂ concentration of 3.4 mg/mL (23 mM) (Sanchez et al., 2008; Kon et al., 2011).

Treatment and sampling

One investigator performed all platelet preparations and arthrocenteses. At each time point, horses were examined and blood was collected; clinical, hematological and synovial cytological data are reported by Textor and Tablin (2013). All fetlocks of each horse were used concurrently: one of four treatments was randomly assigned per leg and the investigator was blinded to treatment until the completion of the study. The four treatments were: resting (R)-PRP, CaCl₂-activated (Ca)-PRP, thrombin-activated (T)-PRP and a 2% saline control (S). For the resting treatment, a placebo ‘activator’ (0.9% saline) was used to maintain blinding of the study. Each joint was sampled and then injected with its assigned treatment at time 0 and synovial fluid was again collected at 6, 24, 48 and 96 h.

Sample handling and analysis

Samples were processed within 2 h of collection; after centrifugation at 21,000 g for 10 min, the supernatants were collected and frozen at −20 °C. PDGF, TGF-β, IL-6, TNF-α concentrations in synovial fluid and PRP were determined by use of ELISAs (Human PDGF-BB, Human TGF-β, Human IL-6 Quantikine ELISAs, R&D Systems; Equine TGF-β ELISA, ThermoFisher Scientific), which have been reported previously in horses (Tablin et al., 2008; McFarlane and Holbrook, 2008; Bosch et al., 2010). Since only 2 mL synovial fluid were collected at any time point, there was insufficient sample volume to perform IL-1β analysis on synovial fluid, but IL-1β concentrations in PRP were quantified (Equine IL-1 Beta ELISA, Genway Biotech).

In vitro study

Six months after completion of the in vivo study, a corollary in vitro study was performed. Autologous PRP was prepared from the same seven horses using the E-PET system. Synovial fluid was collected from the intercarpal joint of each horse; all carp were considered to be clinically normal on examination. One millilitre of R-PRP was mixed with synovial fluid (1.5 mL) from the same horse and incubated for 6 h at 37 °C. Control samples consisted of 1 mL 2% saline (the vehicle for the PRP) mixed with 1.5 mL synovial fluid. Samples were then centrifuged at 21,000 g for 10 min and the supernatants were collected and frozen at −20 °C until the day ELISAs were performed. PDGF, TGF-β, IL-6, TNF-α and IL-1β concentrations were determined in R-PRP, the synovial fluid + PRP (SF + PRP) mixture and the synovial fluid + saline (SF + S) mixture using the methods described above.

Statistical analysis

Data were assessed for residual normality using Wilk–Shapiro tests; log transformation was performed if necessary to achieve normality. Normally distributed data were analyzed by repeated measures testing (Mixed Model analysis of variance, ANOVA). Non-parametric (Kruskal–Wallis) testing was employed for any remaining data. Post hoc analyses were performed with Tukey’s (for comparisons between PRP types) or Dunn’s (for comparisons among synovial fluid data) multiple comparisons tests. For the in vitro study, treated samples were compared to controls using paired t tests. Statistical significance was designated for all tests at P < 0.05. Analyses were performed using SAS version 9.3.

Results

Platelet, leukocyte, growth factor and cytokine concentrations in platelet-rich product

The mean ± standard deviation (SD) platelet concentration in PRP was 650.0 ± 242.6 × 10³/μL. The mean ± SD leukocyte concentration was 14.8 ± 3.84 × 10³/μL.

Growth factor concentrations were significantly different between PRP treatment groups (Fig. 1). PDGF concentrations in the activated PRP groups (Ca-PRP and T-PRP) were both significantly higher than the R-PRP group (P = 0.0005 and P < 0.0001, respectively). Mean ± SD values were 470 ± 870 pg/mL for R-PRP, 2271 ± 1754 pg/mL for Ca-PRP and 3811 ± 2942 pg/mL for T-PRP. The concentration of TGFβ was significantly higher in T-PRP than R-PRP (P = 0.0163). Mean ± SD values were 954 ± 1802 pg/mL for R-PRP, 1577 ± 939 pg/mL for Ca-PRP and 3830 ± 1910 pg/mL for T-PRP. In contrast, cytokine concentrations did not differ significantly among PRP, Ca-PRP and T-PRP (Fig. 2).

Growth factor concentrations in synovial fluid

There were no significant differences in baseline synovial growth factor concentrations between groups (Fig. 3). PDGF concentrations were minimally detected in synovial fluid before or after PRP injection. In contrast, TGFβ was detected in baseline (time 0) synovial fluid samples, as reported by Albro et al. (2012). Statistically significant increases in TGFβ were observed after R-PRP and
T-PRP injection at 6 h in comparison with the baseline value and again at 24 h for the T-PRP group. However, within any given time point, TGFβ concentrations in the treatment groups were not significantly different from the saline control group.

Cytokine concentrations in synovial fluid

Neither TNFα nor IL-6 was detected in baseline synovial fluid samples, but concentrations of both cytokines increased in PRP-treated joints at certain time points (Fig. 4). At 6 h after injection, TNFα increased significantly in the T-PRP group in comparison with the baseline value (0 pg/mL) and the 6 h control. Changes in IL-6 were also mainly in the T-PRP group; concentrations in the R-PRP and Ca-PRP groups were slightly but significantly increased at 6 h.

**In vitro growth factor and cytokine concentrations**

After PRP was mixed with synovial fluid for 6 h, PDGF concentrations in PRP were significantly higher than controls (P < 0.001) and the baseline concentration (P = 0.0008). A similar pattern was observed for TGFβ (P = 0.0052 for SF + PRP vs. PRP alone; P = 0.0007 for SF + PRP vs. control) (Fig. 5). In contrast, no significant differences were observed for IL-1β, TNFα or IL-6 between SF + PRP, SF + saline or PRP alone (Fig. 6).

**Discussion**

Intra-articular use of PRP has been described in experimental animal models (Saito et al., 2009; Sun et al., 2010) and in clinical treatment of humans and horses (Sanchez et al., 2008; Abellanet and Prades, 2009; Fortier, 2009; Sampson et al., 2010; Mei-Dan et al., 2012; Wei et al., 2012). This study was undertaken to investigate growth factor and cytokine concentrations in synovial fluid after intra-articular injection of a platelet-rich product, and also within the PRP itself after different methods of activation.

The growth factors and cytokines evaluated in this study were chosen because each one is either derived from or affects platelets, is involved in joint disease or demonstrates more than one of these characteristics. PDGF and TGFβ are involved in every stage of tissue healing (Barrientos et al., 2008) and platelets are a major source of these growth factors (Kaplan et al., 1979; Blakytny et al., 2004). The cytokines IL-1β, TNFα and IL-6 have been widely studied in equine joint disease (Bertone et al., 2001; van den Boom et al., 2004; Ley et al., 2007; Sutton et al., 2009; Kamm et al., 2010); IL-1β and TNFα induce a synergistic effect in cartilage degeneration and are characterized as purely catabolic cytokines (Goldring and Goldring, 2004). Platelets can synthesize IL-1β...
(Lindemann et al., 2001), but also inhibit certain IL-1β effects on chondrocytes (van Buul et al., 2011). Platelets may express TNFα in certain disease states (Limb et al., 1999). IL-6 is considered to be a modulatory cytokine, with both pro- and anti-inflammatory effects, and is rapidly produced after joint insult (Bertone et al., 2001; Ley et al., 2007). Platelets do not produce or contain IL-6, but platelet-IL-6 interaction occurs at several levels (Oleksowicz et al., 1994; Loppnow et al., 1998; Franchimont et al., 1999).

In this study, PDGF and TGFβ concentrations in PRP increased significantly in response to platelet activation, as expected from previous studies (Martineau et al., 2004; Tablin et al., 2008; Textor and Tablin, 2012), but no significant differences in IL-1β concentrations were observed. We expected that IL-1β concentrations in PRP would follow the same pattern as the growth factors, since human platelets are known to synthesize IL-1β upon activation (Lindemann et al., 2001). However, unlike PDGF and TGFβ, which are ready for immediate release upon activation, IL-1β synthesis does not begin until activation, in a process known as signal-dependent translation (Weyrich et al., 1998). Mature IL-1β has been detected at low levels in platelets within 1 h of thrombin treatment and continues to increase for 18 h (Lindemann et al., 2001). The initial quantification of IL-1β in PRP was performed on samples activated for only 1–2 h; however, during our vitro study, PRP was incubated with synovial fluid for 6 h and platelet activation was evident on the basis of increased growth factor levels in these samples. Had IL-1β synthesis occurred, we would have expected to observe significant differences during this time period.

In osteoarthritis, the net effect of catabolic, anti-catabolic and anabolic processes is determined by a complex interplay of cytokines and growth factors (Grimaud et al., 2002; Goldring and Goldring, 2004). Given the known anabolic and pro-inflammatory effects of platelets, we expected that PRP would add another layer of complexity to the synovial cytokine network, whilst also resulting in increased growth factor concentrations. Instead, we found little detectable growth factor in synovial fluid after intra-articular PRP injection. Over a 96 h observation period, the synovial PDGF or TGFβ concentrations were not different in the PRP-treated joints compared to the saline control joint.

We had hoped to determine whether spontaneous platelet activation would occur after intra-articular PRP injection by detecting increased growth factors within the R-PRP injected synovial fluid, but the growth factor levels were too low to draw meaningful conclusions. Intra-articular PRP injection resulted in joint effusion for 6 h (Textor and Tablin, 2013), which may have diluted PDGF and TGFβ below detectable limits. In contrast, the cytokines TNFα and IL-6 were significantly increased 6 h after PRP injection. We considered the potential sources for these cytokines to be the PRP itself, the resident cells of the joint (i.e. synoviocytes or chondrocytes) or the post-injection leukocyte influx into synovial fluid.

To attempt to clarify our in vivo results, we performed a follow-up study in which we incubated R-PRP with synovial fluid in vitro and again measured growth factor and cytokine concentrations. We chose a 6 h incubation period because the most dramatic clinical signs and synovial leukocyte infiltration were observed at this
The mechanism for platelet activation in synovial fluid is not immediately obvious. Although platelets are capable of responding to a range of both chemical and physical stimuli, exposure to the main platelet agonists (thrombin and type IV collagen) would not be anticipated in normal synovial fluid. Physical means of platelet activation may be a more likely explanation, since synovial fluid is highly viscous and is likely to exert different shear forces on platelets than those experienced in the bloodstream. During the in vitro study, the synovial fluid + PRP samples were incubated on a rocker to provide gentle continuous flow, since fluid stasis is known to promote platelet activation (Mammen, 1992). Despite our intention to prevent artefactual platelet activation, it is possible that the movement of platelets through a thick fluid may have induced shear forces sufficient to activate them. The platelet response to shear is not uniform; platelets are less stimulated by high shear conditions (i.e. rapid, laminar blood flow) than they are by low-shear or turbulent conditions (Yin et al., 2011). Albro et al. (2012) report that the latent TGFβ present in normal synovial fluid can be activated by shear alone. The authors of that study proposed that, under shearing conditions, the physical interactions between hyaluronic acid in synovial fluid and the latency-associated peptide (LAP) of TGFβ may be sufficient to break the non-covalent bonds linking the LAP to TGFβ, thereby liberating the active growth factor. An analogous effect could occur when platelets are mixed with synovial fluid, either in vitro or in vivo. However, it is worth noting that, since the viscosity of synovial fluid decreases in joint disease (Chen et al., 2012; Conrozier et al., 2012), the physical effects of synovial fluid on injected platelets could vary depending on disease state and may also differ for true platelet-rich plasma as opposed to platelets suspended in saline, as used in this study.

In studies of blood products intended for transfusion, the cytokines TNFα and IL-6 increase in stored platelet concentrates (Chaudhary et al., 2006; Shaigean et al., 2006). These substances have been determined to arise from leukocytes contained in the platelet product, since leukoreduction prior to storage prevents cytokine accumulation. Similarly, IL-1β and TNFα concentrations in leukocyte-rich PRP (containing four times the leukocytes of whole blood) have been correlated with leukocyte concentrations in the product (Sutton et al., 2009; Kamm et al., 2010; Sundman et al., 2011). Exposure of tendon explants to leukocyte-rich PRP induces higher mRNA expression of these two cytokines than that observed after treatment with leukoreduced PRP (McCarrel et al., 2012). The platelet-rich product used in this study contained an intermediate leukocyte concentration (two times whole blood WBC concentration).

When used in normal joints without thrombin activation, PRP did not increase synovial IL-1β or TNFα concentrations during a 5 day study. IL-6, a modulatory cytokine, increased slightly but significantly at 6 h. None of the cytokines increased after short-term in vitro incubation with synovial fluid. The results of this study suggest that platelet-rich products containing up to two times the systemic white blood cell concentration are unlikely to create significant increases in inflammatory cytokines, either within the product itself or the treated site.

**Conclusions**

On the basis of the results of this study, four conclusions can be drawn regarding intra-articular use of platelet-rich products in horses. Firstly, the inflammatory cytokine content of PRP with intermediate platelet and leukocyte concentrations is low. Secondly, this low cytokine content of PRP is not increased by platelet activation. Thirdly, activation is not necessary to induce growth factor release from platelets injected intra-articularly; the platelets appear to spontaneously release PDGF and TGFβ by virtue of exposure to synovial fluid alone. Fourthly, intra-articular injection of R-PRP with intermediate leukocyte concentration does not increase
synovial fluid levels of IL-1α or TNFα in normal joints. Overall, the results suggest minimal catabolic impact of PRP in joints, but also an uncertain anabolic contribution of the main platelet growth factors, which were only minimally detected after intra-articular injection. Future research is required to determine whether intra-articular platelet-rich products result in therapeutic benefits for horses with joint disease. If so, the interaction between cytokines already present in diseased joints and the platelets, growth factors and cytokines present in the PRP must also be characterized.

Conflict of interest statement

Six Equine Platelet Enhancement Therapy (E-PET) kits (of the 10 used throughout this study) were donated by Pall Corporation. None of the authors of this paper have any other financial or personal relationship with other people or organisations that could appropriately influence or bias the content of the paper.

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