Original Article
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Autologous processed plasma: cytokine profile and effects upon injection into healthy equine joints

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This experimental controlled study was performed to evaluate the composition of autologous processed plasma (APP), and the effects of APP intra-articular injection into healthy equine metacarpophalangeal joints. The effects on joints were analysed with a short-phase protocol and a prolonged-phase protocol using saline-injected joints as controls. For the short protocol, horses received one intra-articular APP injection. Synovial fluid samples were collected prior to the injection and 3, 6, 24, 48, and 16 h after treatment. For the prolonged protocol, the joints received three weekly injections of APP, and samples were collected at 0, 7, 14, 21, and 28 days before APP administration. IL1-ra level was found to be increased in APP compared to plasma. Upon intra-articular administration of APP, transient (up to 24 h) increases in white blood cell (WBC) counts along with elevated protein and prostaglandin E_2 (PGE_2) concentrations were observed in the treated joints. Over the 28-day observation period, APP did not elicit changes relative to baseline levels, but WBC counts, PGE_2 and chondroitin sulphate concentrations were lower than those found in the control. In conclusion, APP intra-articular injection induced a mild and transitory inflammatory response but no inflammation reaction was observed over a longer period of treatment and observation.

Keywords: autologous processed plasma, equine, inflammatory markers, joint, synovial fluid

Introduction

The growing participation of equine athletes in different sports has been accompanied by an increasing incidence of inflammation in the appendicular skeleton, particularly in the joints. In fact, osteoarthritis is a common finding in sport horses [31]. The demand for therapeutic options to shorten the disease course and reduce related sequelae has similarly increased [35]. It is now well recognized that arthropathies are caused by disruption of the balance between anabolic and catabolic events with a dominance of catabolism during the most advanced stages of the disease [9].

There is evidence indicating that interleukin-1β (IL-1β) and tumor necrosis factor (TNF) are the most important pro-inflammatory cytokines in joint diseases [24]. IL-1β and TNF can stimulate their own production and induce chondrocytes to produce other cytokines, prostaglandin E_2 (PGE_2) and also matrix metalloproteases (MMP-1 and MMP-3), leading to the digestion of the articular cartilage extracellular matrix [6]. In cases of osteoarthritis, the equilibrium between IL-1β and its natural antagonist interleukin-1 receptor antagonist (IL-1ra) could favor IL-1β because little IL-1ra is produced [24]. The synovial fluid of patients with early osteoarthritis contains more IL-1β and TNF than individuals with advanced osteoarthritis [3]. Therefore, the introduction of agents that could block degenerative and inflammatory processes is of great clinical relevance.

Different cell types present in the osteoarthritic synovium could be responsible for the observed inflammation. These include macrophages, synovial fibroblasts, and mononuclear cells (as reviewed in [34]). The synovial membrane is thus a promising target to prevent cartilage catabolism and treat clinical symptoms [4]. In addition, several molecules can counteract the activities of IL-1β by binding to the cytokine itself or its receptor, thereby limiting transcription or acting locally at regulatory sites (as reviewed in [14]). As already mentioned, IL-1ra is a natural inhibitory cytokine [1] that competes for occupation of surface IL-1 receptors [10] and acts

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in a dose-dependent fashion. Meijer *et al.* [25] demonstrated that physico-chemical stimulation of blood in a commercial syringe containing treated glass beads, incubated at 37°C for 24 h, and centrifuged results in serum enriched with growth factors and anti-inflammatory cytokines, particularly IL-1ra. This haemoderivative known as autologous conditioned serum (ACS) and sold under the trade names Orthokine and irap (Orthogen Veterinary, Germany) has been used in equine and human orthopaedic therapies for treating musculoskeletal injuries [35].

A recent study comparing two commercial methods for producing ACS from equine blood (irap and IRAPII) has shown that the addition of heparin to total blood prior to incubation (processing) results in plasma with IL-1ra levels that are increased 1.5-fold compared to ACS [17]. Brossi *et al.* [7] have also shown that plasma obtained after blood is incubated at 37°C for 24 h and centrifuged in regular heparinized tubes is, in fact, a haemoderivative with antioxidant properties called autologous processed plasma (APP). This reagent can elicit an antioxidant effect equivalent to that of ACS on stimulated equine synovial fluid cells. Thus, mounting evidence has indicated that the processing of plasma, similarly to ACS, might result in a haemoderivative with therapeutic activities that are useful for the management of musculoskeletal injuries in horses.

The present study was designed to evaluate the composition of APP. Additionally, the effects exerted by intra-articular injection of APP on healthy equine metacarpophalangeal joints and synovial fluid inflammatory markers were investigated. Total protein contents, nucleated cell counts, inflammatory and anti-inflammatory cytokines, and glycosaminoglycans (GAGs) were analysed.

Materials and Methods

Animals

The experimental protocol used for this study was approved by the Animal Care and Use Committee of School of Veterinary Medicine and Animal Science – University of São Paulo (FMVZ-USP) (protocol no. 2266/2011; date of approval: 06/22/2011). Ten male and female mixed breed horses of FMVZ-USP, weighing 350 to 400 kg and 4 to 10 years of age were used. All animals were clinically healthy and did not have a history of articular disease.

In vitro and *in vivo* studies

During the *in vitro* part of this study, the horses served as blood donors for the production of APP. The levels of total protein, IL-1β, Il-1ra, PGE_2, interleukin-10 (IL-10), and tumour necrosis factor α (TNF-α) in the APP were measured. These characteristics were compared to those of unprocessed plasma recovered from the same horse.

The experimental *in vivo* part of this study was divided in a short-phase period and prolonged phase period. During the short phase, or hour protocol, we subjected 10 horses to a single 4-mL injection of APP into one randomly assigned metacarpophalangeal joint. After 7 days, the contralateral joint was injected with 4 mL of saline as a control. Synovial fluid samples were collected immediately prior to each injection and 3, 6, 24, 48, and 168 h after injection to determine the white blood cell (WBC) counts along with the total protein, IL-1β, Il-1ra, PGE_2, TNF-α, and GAG levels.

The prolonged protocol, or week protocol, was started after a 30-day rest period and included six animals. The animals received weekly intra-articular injections of APP in a randomly assigned metacarpophalangeal joint for 3 consecutive weeks as previously recommended for the administration of ACS [13]. As a control, the contralateral limbs were treated with intra-articular saline injections.

Synovial fluid sample collection

Synovial fluid samples were collected immediately prior to each APP or saline injection on days 0, 7, 14, 21, and 28 and analysed in a similar fashion to that for the short phase protocol. In both phases (*i.e.*, the short and prolonged protocols) of the *in vivo* study, arthrocentesis was performed through the sesamoidean collateral lateral ligament using 21 G1.1/4" hypodermic needles (Becton, Dickinson and Company, USA) as previously described by Misheff and Stover [26]. The puncture site was aseptically prepared using povidone iodine scrubs (Riodeine; Rioquímica, Brazil) and 70% alcohol.

APP preparation

APP acquisition began with the aseptic collection of approximately 20 mL of blood via the left jugular vein into tubes containing sodium heparin (Vacutainer; Becton, Dickinson and Company). The samples were incubated at 37°C for 24 h in 5% CO_2. The plasma was then collected and transferred to a sterile Falcon tube (TTP Techno Plastic Products, Switzerland) and centrifuged at 300 × g for 10 min at 24°C to remove cellular debris. The supernatant was transferred to another sterile Falcon tube and further centrifuged at 900 × g for 10 min at 24°C. The APP was aspirated using a sterile needle and syringe, and immediately injected through a 0.22-μm Millipore microfilter (Millex-GV; Merck Millipore, Ireland). The entire procedure was performed under a laminar flow hood to ensure the sterility of the final product.

Synovial fluid analysis

WBC counts in the synovial fluid were determined using *in natura* aliquots in a Neubauer chamber (Belden, Germany). Differential counts were performed using smears stained with May-Grümwald-Giemsa dye. The total protein levels in the synovial fluid and APP were measured using the biuret method

with an automated biochemical analyser (Randox Laboratories, UK).

IL-1β, IL-1ra, and TNF-α levels were measured with an ELISA using commercial kits. For IL-1β quantification, the USCN Life Science (China) E90569Eq kit was used. This is a sandwich enzyme immunoassay specific for equine IL-1β. IL-1ra was measured with a Quantikine MRA00 kit (R&D Systems, USA) that is also a quantitative sandwich enzyme immunoassay previous validated for horses [13]. TNF-α was measured with an equine-specific DuoSet ELISA system produced by R&D Systems (DY1814).

Eicosanoids in the synovial fluid were quantified with an ELISA using a 514010 Prostaglandin E_2 EIA kit (monoclonal; Cayman Chemical Company, USA). In brief, the plates were pre-coated with goat polyclonal anti-mouse IgG, and incubated with tracer acetylcholinesterase-linked PGE_2, a monoclonal antibody specific for PGE_2, and sample containing free PGE_2. As the concentration of PGE_2 tracer is held at a constant level and the amounts of PGE_2 in different samples vary, the tracer bound to the specific antibody is inversely proportional to the PGE_2 concentration of the sample [2,30].

Hyaluronic acid (HA) and chondroitin sulphate (CS) concentrations were determined as previously described [23]. In brief, synovial fluid samples (100 μL) were subjected to proteolysis and debris was removed by centrifugation (3,000 × g for 15 min at 24°C). The supernatant was then freeze-dried and resuspended in water (50 μL). Aliquots (5 μL) were subjected to agarose gel electrophoresis. The gels were stained with toluidine blue (Quemis do Brasil, Brazil) in 1% acetic acid and then in sodium acetate buffer (pH 5). Band density was quantified by densitometry (VisionWorks LS; UVP, USA). To compensate for possible dilution of the synovial fluid samples, urea concentrations were measured by urease-glutamate dehydrogenase using an automated biochemical analyser (Randox) as previously described [2,15,23].

Statistical analysis

Data were evaluated for normality using the Kolmogorov-Smirnov test. Afterwards, paired *t*-tests and an ANOVA followed by Tukey test were used to compare the APP with the control group. GraphPad Instat 3 software (GraphPad, USA) was used to perform the statistical analyses. *P* values < 0.05 were considered significant.

Results

Analysis of plasma and APP

Table 1 shows the total protein, TNF-α, IL-1β, IL-1ra, IL-10, and PGE_2 concentrations in plasma prior to processing and in the APP samples. Only the IL-1ra concentration differed, which was increased in APP compared to the unprocessed plasma. IL-1β levels in both unprocessed plasma and APP as well as IL-1ra concentrations in plasma were below the detection limit of the assays.

Table 1. Composition of plasma prior to processing and autologous processed plasma (APP) samples

	Plasma	APP
Total protein (g/dL)	6.10 ± 0.44	6.00 ± 0.37
TNF-α (pg/mL)	456.1 ± 179.5	522.9 ± 197.6
IL-1β (pg/mL)	< 15	< 15
IL-1ra (pg/mL)	< 4	128.9 ± 52.7*
IL-10 (pg/mL)	675.2 ± 308.0	481.25 ± 263.5
PGE_2 (pg/mL)	38.71 ± 6.42	34.32 ± 1.34

*Statistically significant difference ($p < 0.05$).

In vivo effects of APP intra-articular administration

During the short-phase protocol, synovial fluid acquired from the joints that were injected with APP had increased WBC counts compared to the saline-injected joints at 3, 6, 24, and 48 h ($p < 0.05$) with a predominance of polymorphonuclear cells at 3 and 6 h. The APP-treated joints also exhibited significantly increased concentrations of PGE_2 and total protein at 3 and 6 h, and CS at 24 h (Fig. 1). In contrast, the HA concentrations did not vary between the groups (Fig. 2).

As shown in Figs. 1 and 3, APP-treated joints had increased WBC counts at 6 h compared to baseline levels. Additionally, protein and PGE_2 concentrations were increased at 3 and 6 h, and CS and IL-1ra levels were elevated at 24 h ($p < 0.05$). In the saline-injected joints, increases relative to the baseline were observed for WBC counts and CS concentrations at 24 and 48 h, IL-1ra at 24 h, and PGE_2 at 6 h. Protein concentrations did not significantly vary in the saline-injected joints ($p < 0.05$).

During the prolonged-phase protocol (28 days), synovial fluid samples from the APP-treated joints had lower protein concentrations and WBC counts with a predominance of mononucleated cells on days 14, 21, and 28 compared to saline-injected joints. Decreased PGE_2 concentrations were also noted on day 28 ($p < 0.05$). Furthermore, the CS concentrations were reduced on days 7, 14, and 21 ($p < 0.05$; Figs. 3 and 4). HA levels did not differ significantly between the two groups as observed during the short-phase protocol (Fig. 2).

Over the 28-day observation period, no changes in WBC counts or protein and PGE_2 concentrations relative to baseline levels were observed in the APP-treated joints. In contrast, WBC counts at 14, 21, and 28 days, and PGE_2 concentrations at 28 days ($p < 0.05$) were elevated in the saline-injected joints (Fig. 4). Compared to baseline levels, CS and HA concentrations did not vary in either APP-treated or saline-injected joints (Figs. 2 and 4). Likewise, no variations in TNF-α or IL-1β concentrations were observed in either the APP-treated or

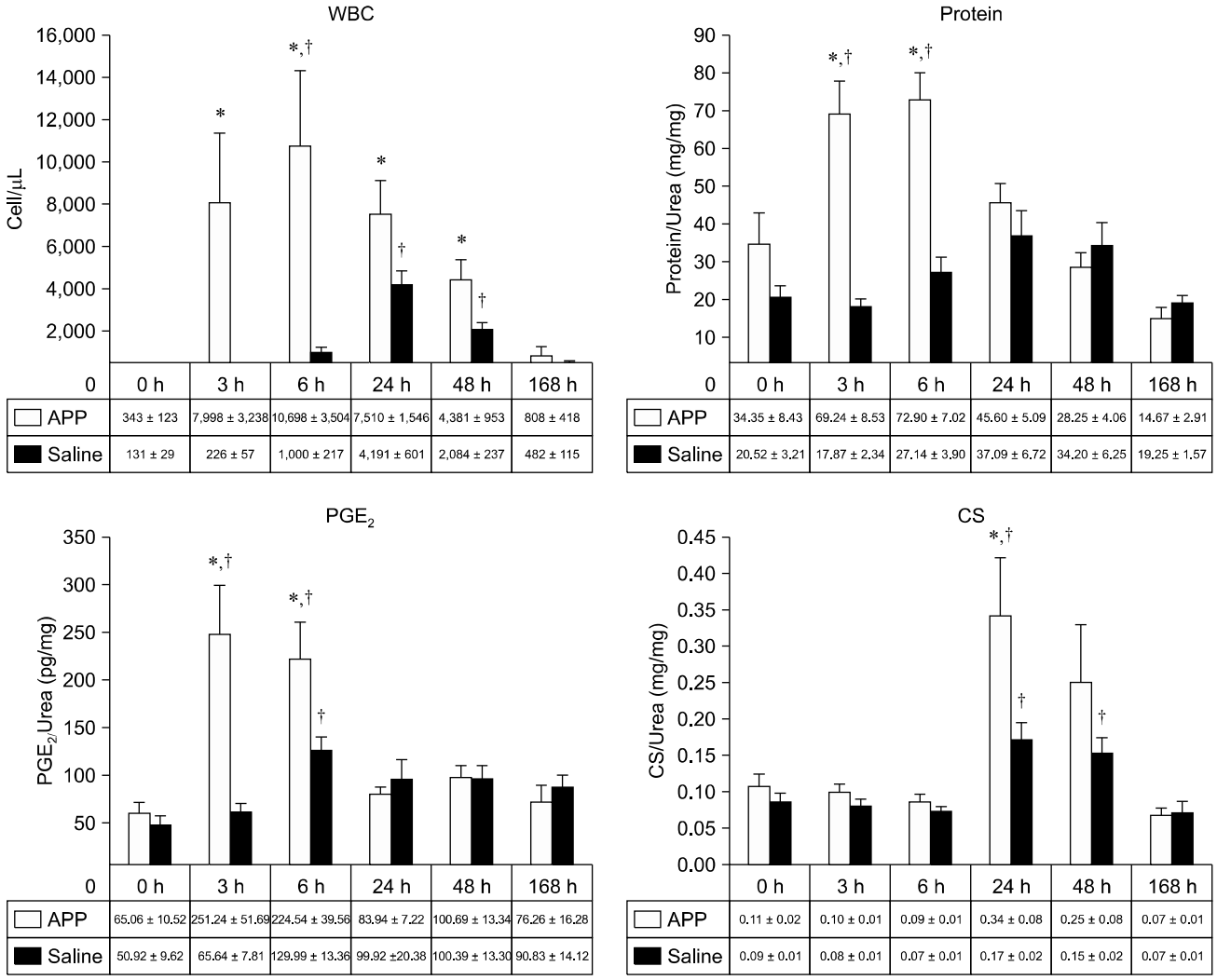

Fig. 1. Nucleated cell counts (WBC, cell/μL) and concentrations (expressed as average ± standard error [SE]) of total protein, prostaglandin E_2 (PGE_2), and chondroitin sulphate (CS) from APP-treated and saline-injected groups during the short-phase protocol (0～168 h). All concentrations are expressed as urea ratios to correct for possible fluid volume variations. *Statistically significant differences compared to the saline-injected (control) group ($p < 0.05$); †Statistically significant differences compared to the baseline levels for each group ($p < 0.05$).

saline-injected joints during the short- and long-term protocols (Fig. 3).

Discussion

Haemoderivatives enriched in anti-inflammatory cytokines and growth factors were introduced for treating joint diseases in both humans and animals. These preparations are usually administered by arthrocentesis, a routine procedure performed for both diagnostic and therapeutic purposes. Nonetheless, arthrocentesis *per se* requires perforation of the synovial membrane and causes cell rupture along with a local inflammatory reaction [39]. This trauma may lead to the release or activation of several biomarkers involved in inflammation and possibly cartilage degradation, thus altering the synovial fluid composition [40]. To avoid or minimize interference due to the arthrocentesis procedure itself, each APP-treated joint in the present study was compared to its contralateral joint that received saline injections and was used as a paired control.

Data from the present study showed that the only difference in composition between unprocessed plasma and APP was the IL-1ra concentration, which was increased more than 100 times. APP was free from inflammatory cytokines that might potentially cause adverse effects (see Table 1). Rutgers *et al.* [33] determined the cytokine profile of ACS and also found increased levels of anti-inflammatory cytokines, such as IL-1ra and IL-10, and pro-inflammatory cytokines after processing. Nevertheless, it should be noted that the ACS produced by

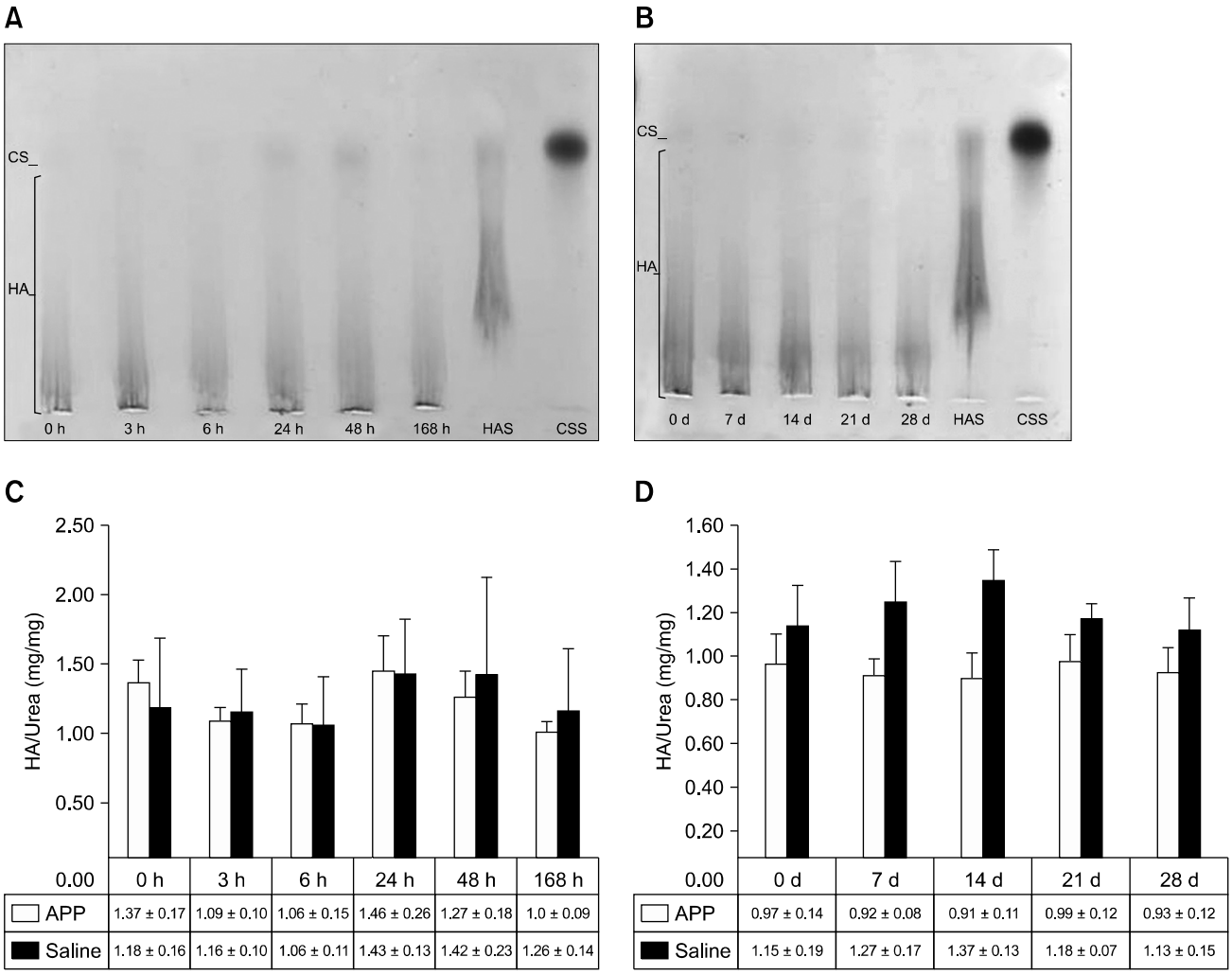

Fig. 2. Agarose gel electrophoresis of glycosaminoglycans isolated from equine synovial fluid samples collected during the short- and prolonged-phase protocols (A and B), and hyaluronic acid concentrations from APP-treated and saline-injected groups during the short- and prolonged-phase protocols (C and D). All concentrations are expressed as urea ratios to correct for possible fluid volume variations. CS: chondroitin sulphate, HA: hyaluronic acid, HAS: hyaluronic acid standard, CSS: chondroitin sulphate standard.

Rutgers was obtained with 6 h of incubation while our APP was acquired after a 24-h incubation of the blood sample. This possibly resulted in different products. As demonstrated by Hraha *et al.* [17], the incubation time is a key factor for producing molecules of therapeutic interest in ACS, and shortened incubation times can adversely affect the composition of the blood derivative.

APP intra-articular administration seemed to promote a mild, transient, inflammatory response since increased WBC counts along with elevated PGE_2 and total protein concentrations were observed within 3～6 h compared to saline-injected joints. These markers returned to baseline levels at 24～48 h. In contrast, the synovial fluid CS concentration increased significantly 24 h after arthrocentesis in both the APP-treated and saline-injected joints, but more so in the APP-treated joints. This response over time suggests that inflammatory mediators may stimulate cartilage catabolism, leading to CS increases in synovial fluid.

During the 28-days observation period with weekly injections of APP or saline, APP did not cause any increases in inflammatory or catabolism markers. WBC counts as well as PGE_2, total protein, and CS concentrations were low throughout the experimental period. On the contrary, saline injections induced mild inflammatory changes, as indicated by increased WBC and PGE_2 levels, while APP did not. It seems that this long-term, mild, inflammatory response induced by arthrocentesis and saline was inhibited by APP.

Inflammatory responses after saline injection have been previously been detected in equine synovial fluid [36]. Nevertheless, saline was preferred as a control instead of unprocessed plasma because the latter is a complex mixture of proteins and soluble factors, with a wide and not fully known

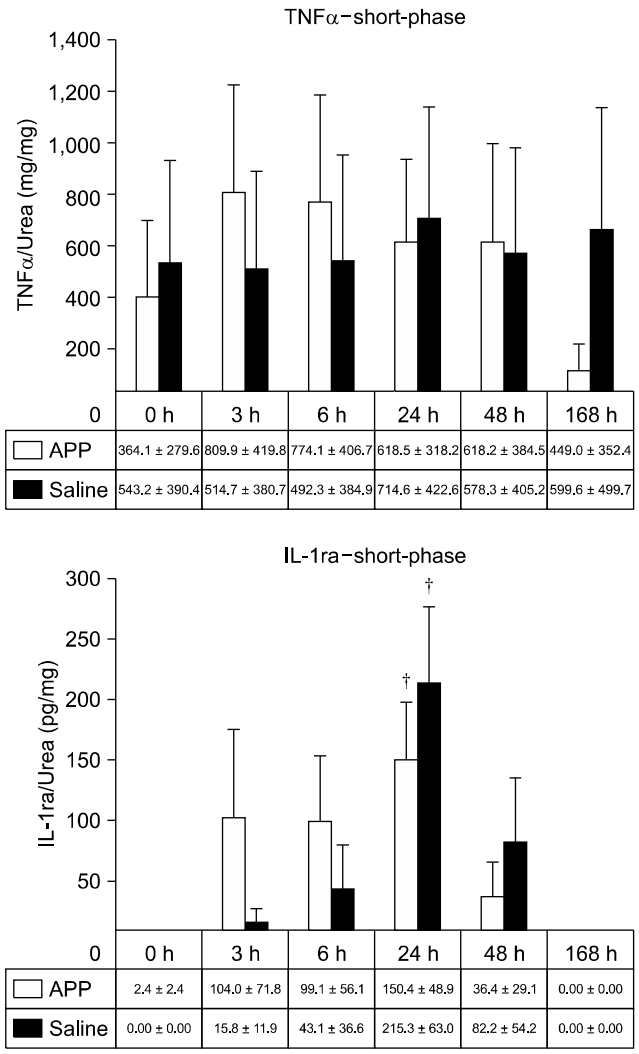

Fig. 3. Cytokine profiles of synovial fluid from the APP-treated and saline-injected groups collected during the short-phase and prolonged-phase protocols. All concentrations are expressed as urea ratios to correct for possible fluid volume variations. TNFα: tumor necrosis factor alpha, IL-1ra: interleukin 1 receptor antagonist, d: day. †Statistically significant differences compared to baseline values ($p < 0.05$).

range of therapeutic properties [20,29]. Thus, unprocessed plasma is a non-inert product that may be unsuitable for the desired purposes.

In the synovial fluid of healthy equine joints, an average of 50～500 WBC/μL with 90% mononuclear cells and 10% polymorphonuclear cells [9] is usually considered normal. Lower WBC counts have been reported for metacarpophalangeal synovial fluid (390 WBC/μL) in agreement with our results (initial 131～343 WBC/μL) [22]. In four out of ten animals, substantial increases in WBC counts (up to 29,000 WBC/μL) with 88% polymorphonuclear cells (average 51%) were observed 3～24 h after APP administration in our investigation. This profile is similar to that observed in cases of articular disorders, whether inflammatory or infectious in nature, especially the relative number of polymorphonuclear cells [16,37]. Comparable increases were previously reported by others within 8 h after injury [11,32] accompanied by increased PGE_2 levels, which is also in agreement with our findings. Nevertheless, at 24～48 h all these parameters were back to baseline levels and no further changes were observed during the prolonged-phase protocol.

Similar results were obtained for total protein. The initial concentration was in the normal range [22] expressed as urea ratios to compensate for possible fluid volume changes [19]. This level increased twice in 6 h before returning to baseline concentrations afterwards.

Carmalt *et al.* [8] reported TNF-α as one of the first cytokines to be secreted after joint injury with levels peaking in a few hours. On the other hand, Billinghurst *et al.* [5] found no difference in synovial fluid TNF-α concentrations of healthy and diseased joints. In the present study, no significant changes in TNF-α levels were observed after APP or saline administration. This finding supports the notion that TNF-α concentrations in synovial fluid do not always correlate with the presence of lesions, and corroborate the proposed low specificity of this biomarker [21].

IL-1β is a major cytokine that induces the synthesis of enzymes and inflammatory mediators. It is found at high

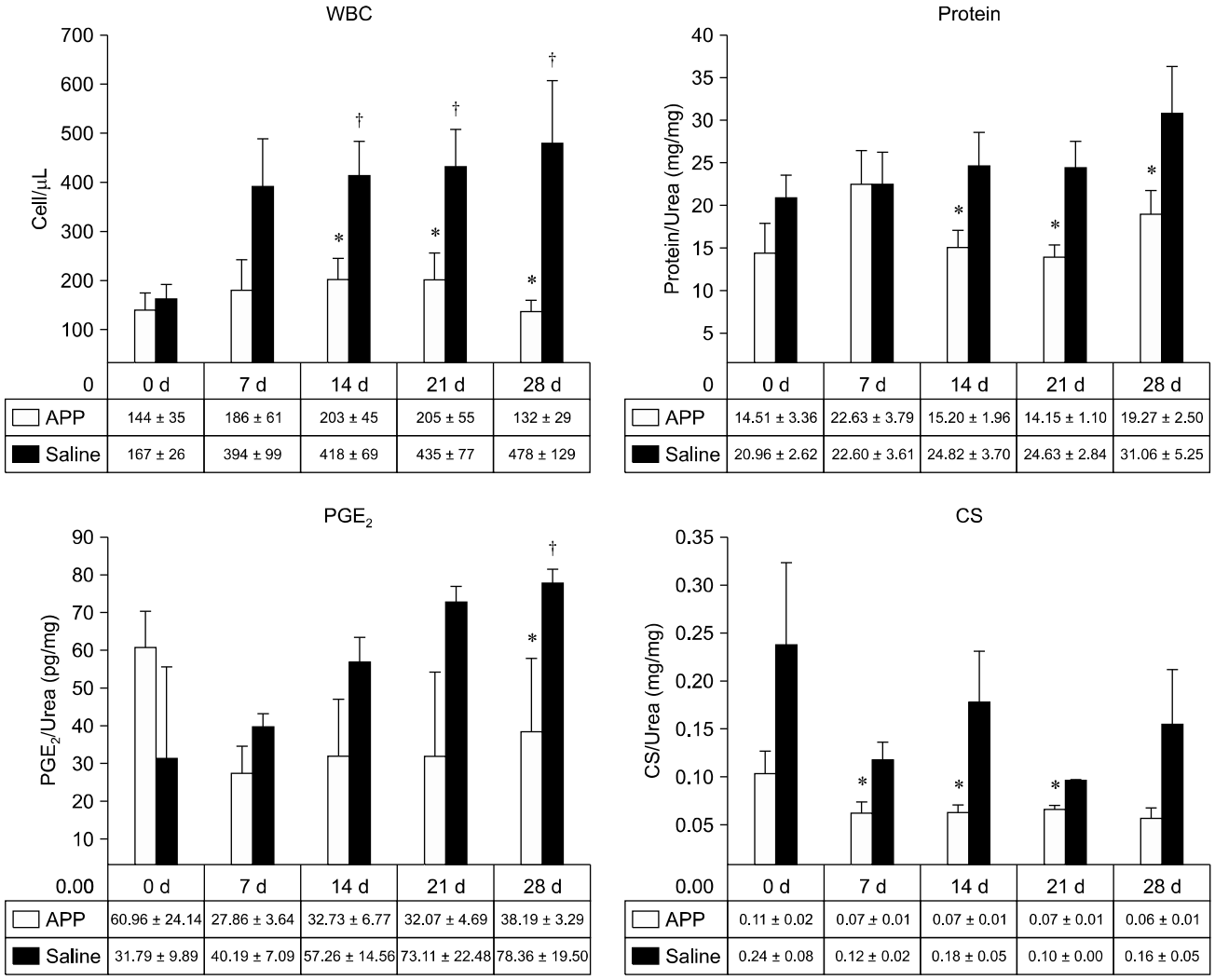

Fig. 4. Nucleated cell counts (WBC, cell/μL) and concentrations (expressed as average ± SE) of total protein, PGE_2, and CS for the APP-treated and saline-injected groups during the prolonged-phase protocol (0 ~ 28 days). All concentrations are expressed as urea ratios to correct for possible fluid volume variations. *Statistically significant differences compared to the saline-tinjected (control) group ($p < 0.05$). †Statistically significant differences compared to the baseline levels for each group ($p < 0.05$).

concentrations in the synovial fluid of inflamed joints [27]. We did not detect IL-1β in any of our samples. This is somewhat surprising. However, only healthy animals were enrolled in our study and the haemoderivative we used did not contain detectable levels of IL-1β after processing. Therefore, the absence of significant amounts of IL-1β suggests that no significant inflammatory reaction occurred. However, Kamm *et al.* [18] did not find IL-1β in synovial fluid, even in osteoarthritis-affected joints, and attributed this result to the low sensitivity of the kit that was used. This could also be the case in the present study.

As previously mentioned, IL-1ra is a physiological antagonist of IL-1 that competes for the IL-1 receptor. Haemoderivatives enriched in IL-1ra have been prepared by different procedures. Among the most frequently used for horses are irap and IRAP-II devices. Hrara *et al.* [17] reported increased IL-1ra levels in serum obtained after 24 h of blood clotting and irap (200 times) while IRAP-II products had IL-1ra levels that were increased 300 times. Moreover, these authors modified the ELISA kit recommendations to improve assay sensitivity. In the present study, we used an R&D Systems ELISA kit previously validated for horses to quantify IL-1ra levels. The IL-1ra concentration in unprocessed plasma was below the assay detection levels (< 4 pg/mL, Table 1). After plasma processing (to acquire APP), the IL-1ra concentration was ~130 pg/mL, representing an increase more than 100-fold. However, in contrast to IRAP-II processed serum in which IL-1β concentrations also increase [17], IL-1β levels in our preparations remained below the ELISA detection levels.

Curiously, APP-treated and saline-injected joints exhibited

increased IL-1ra concentrations at 24 h. This increase was initially observed in the APP group beginning at 3 h, but not for the saline group. This early increase could be due to exogenous IL-1ra present in the administered APP.

Rutgers *et al.* [33] also measured the cytokine levels in the synovial fluid 3 days after injection. This group failed to detect significant changes in cytokine concentrations despite repeated ACS administration. This finding is consistent with results from our study despite differences in the methods of blood derivative production.

In the present study, HA concentrations did not vary upon either APP or saline treatment throughout the experimental periods of both protocols. Others have also found a poor correlation between HA concentration and the presence of inflammation in joints [13,38]. In contrast, increased concentrations of sulphated GAGs in synovial fluid that accompanies articular disease is predominantly due to elevated CS levels [2,23,28]. Thus, CS is a useful marker for early diagnosis of joint disease. The incremental increase of CS concentration observed 24 h after intra-articular APP injection is consistent with previously reported increases by other groups during articular inflammation [2,12,23]. The low CS levels in the synovial fluid samples suggest that neither APP nor saline (or arthrocentesis *per se*) induced significant cartilage destruction.

In conclusion, findings from this study highlight the importance of combining short- and long-term observation protocols to achieve accurate evaluation of haemoderivatives currently used for equine orthopaedic therapeutics. APP has proven to be a safe option for treating equine articular diseases. Clinical trials are currently being conducted to further evaluate the effects of this haemoderivative.

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Conflict of Interest

There is no conflict of interest.

References

1. **Arend WP.** Interleukin-1 receptor antagonist: discovery, structure and properties. Prog Growth Factor Res 1990, **2**, 193-205.
2. **Baccarin RYA, Rasera L, Machado TSL, Michelacci YM.** Relevance of synovial fluid chondroitin sulphate as a biomarker to monitor polo pony joints. Can J Vet Res 2014, **78**, 50-60.
3. **Benito MJ, Veale DJ, FitzGerald O, van den Berg WB, Bresnihan B.** Synovial tissue inflammation in early and late osteoarthritis. Ann Rheum Dis 2005, **64**, 1263-1267.
4. **Berenbaum F.** Targeted therapies in osteoarthritis: a systematic review of the trials on www.clinicaltrials.gov. Best Pract Res Clin Rheumatol 2010, **24**, 107-119.
5. **Billinghurst RC, Fretz PB, Gordon JR.** Induction of intra-articular tumour necrosis factor during acute inflammatory responses in equine arthritis. Equine Vet J 1995, **27**, 208-216.
6. **Bondeson J, Wainwright SD, Lauder S, Amos N, Hughes CE.** The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis. Arthritis Res Ther 2006, **8**, R187.
7. **Brossi PM, Baccarin RYA, Massoco CO.** Do blood components affect the production of reactive oxygen species (ROS) by equine synovial cells *in vitro*? Pesqui Vet Bras 2012, **32**, 1355-1360.
8. **Carmalt JL, Bell CD, Tatarniuk DM, Suri SS, Singh B, Waldner C.** Comparison of the response to experimentally induced short-term inflammation in the temporomandibular and metacarpophalangeal joints of horses. Am J Vet Res 2011, **72**, 1586-1591.
9. **Caron JP.** Osteoarthritis. In: Ross MW, Dyson SJ (eds.). Diagnosis and management of Lameness in the Horse. 1st ed. pp. 572-594, Saunders, Philadelphia, 2003.
10. **Carter DB, Deibel MR Jr, Dunn CJ, Tomich CSC, Laborde AL, Slightom JL, Berger AE, Bienkowski MJ, Sun FF, McEwan RN, Harris PKW, Yem AW, Waszak GA, Chosay JG, Sieu LC, Hardee MM, Zurcher-Neely HA, Reardon IM, Heinrikson RL, Truesdell SE, Shelly JA, Eessalu TE, Taylor BM, Tracey DE.** Purification, cloning, expression and biological characterization of an interleukin-1 receptor antagonist protein. Nature 1990, **344**, 633-638.
11. **de Grauw JC, van de Lest CH, van Weeren PR.** Inflammatory mediators and cartilage biomarkers in synovial fluid after a single inflammatory insult: a longitudinal experimental study. Arthritis Res Ther 2009, **11**, R35.
12. **Frisbie DD, Al-Sobayil F, Billinghurst RC, Kawcak CE, McIlwraith CW.** Changes in synovial fluid and serum biomarkers with exercise and early osteoarthritis in horses. Osteoarthritis Cartilage 2008, **16**, 1196-1204.
13. **Frisbie DD, Kawcak CE, Werpy NM, Park RD, McIlwraith CW.** Clinical, biochemical, and histologic effects of intra-articular administration of autologous conditioned serum in horses with experimentally induced osteoarthritis. Am J Vet Res 2007, **68**, 290-296.
14. **Garlanda C, Riva F, Bonavita E, Mantovani A.** Negative regulatory receptors of the IL-1 family. Semin Immunol 2013, **25**, 408-415.
15. **Gough MR, Munroe GA, Mayhew IG.** Urea as measure of dilution of equine synovial fluid. Equine Vet J 2002, **34**,

76-79.
16. **Hawkins DL, Mackay RJ, Gum GG, Colahan PT, Meyer J.** Effects of intra-articularly administered endotoxin on clinical signs of disease and synovial fluid tumor necrosis factor, interleukin 6, and prostaglandin E_2 values in horses. Am J Vet Res 1993, **54**, 379-386.
17. **Hraha TH, Doremus KM, McIlwraith CW, Frisbie DD.** Autologous conditioned serum: the comparative cytokine profiles of two commercial methods (IRAP and IRAP II) using equine blood. Equine Vet J 2011, **43**, 516-521.
18. **Kamm JL, Nixon AJ, Witte TH.** Cytokine and catabolic enzyme expression in synovium, synovial fluid and articular cartilage of naturally osteoarthritic equine carpi. Equine Vet J 2010, **42**, 693-699.
19. **Kraus VB, Stabler TV, Kong SY, Varju G, McDaniel G.** Measurement of synovial fluid volume using urea. Osteoarthritis Cartilage 2007, **15**, 1217-1220.
20. **Lathrop JT, Anderson NL, Anderson NG, Hammond DJ.** Therapeutic potential of the plasma proteome. Curr Opin Mol Ther 2010, **5**, 250-257.
21. **Ley C, Ekman S, Elmén A, Nilsson G, Eloranta ML.** Interleukin-6 and tumor necrosis factor in synovial fluid from horses with carpal joint pathology. J Vet Med A Physiol Pathol Clin Med 2007, **54**, 346-351.
22. **Liberg P, Magnusson LE, Schougaard H.** Studies on the synovia in healthy horses with particular reference to the protein composition. Equine Vet J 1977, **9**, 87-91.
23. **Machado TSL, Correia da Silva LCL, Baccarin RYA, Michelacci YM.** Synovial fluid chondroitin sulphate indicates abnormal joint metabolism in asymptomatic osteochondritic horses. Equine Vet J 2012, **44**, 404-411.
24. **Martel-Pelletier J, Alaaeddine N, Pelletier JP.** Cytokines and their role in the pathophysiology of osteoarthritis. Front Biosci 1999, **4**, D694-703.
25. **Meijer H, Reinecke J, Becker C, Tholen G, Wehling P.** The production of anti-inflammatory cytokines in whole blood by physico-chemical induction. Inflamm Res 2003, **52**, 404-407.
26. **Misheff MM, Stover SM.** A comparison of two techniques for arthrocentesis of the equine metacarpophalangeal joint. Equine Vet J 1991, **23**, 273-276.
27. **Morris EA, McDonald BS, Webb AC, Rosenwasser LJ.** Identification of interleukin-1 in equine osteoarthritic joint effusions. Am J Vet Res 1990, **51**, 59-64.
28. **Palmer JL, Bertone AL, McClain H.** Assessment of glycosaminoglycan concentration in equine synovial fluid as a marker of joint disease. Can J Vet Res 1995, **59**, 205-212.
29. **Pascual C, Bredle D, Karzai W, Meier-Hellmann A, Oberhoffer M, Reinhart K.** Effect of plasma and LPS on respiratory burst of neutrophils in septic patients. Intensive Care Med 1988, **24**, 1181-1186.
30. **Rasera L, Massoco CO, Landgraf RG, Baccarin RYA.** Exercise induced apoptosis and necrosis in the synovial fluid cells of athletic horses. Pesqui Vet Bras 2008, **28**, 231-236.
31. **Riggs CM.** Osteochondral injury and joint disease in the athletic horse. Equine Vet Educ 2006, **18**, 100-112
32. **Ross TN, Kisiday JD, Hess T, McIlwraith CW.** Evaluation of the inflammatory response in experimentally induced synovitis in the horse: a comparison of recombinant equine interleukin 1 beta and lipopolysaccharide. Osteoarthritis Cartilage 2012, **20**, 1583-1590.
33. **Rutgers M, Saris DBF, Dhert WJA, Creemers LB.** Cytokine profile of autologous conditioned serum for treatment of osteoarthritis, *in vitro* effects on cartilage metabolism and intra-articular levels after injection. Arthritis Res Ther 2010, **12**, R114.
34. **Sellam J, Berenbaum F.** The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis. Nat Rev Rheumatol 2010, **6**, 625-635.
35. **Textor J.** Autologous biologic treatment for equine musculoskeletal injuries: platelet-rich plasma and IL-1 receptor antagonist protein. Vet Clin North Am Equine Pract 2011, **27**, 275-298.
36. **Textor JA, Tablin F.** Intra-articular use of a platelet-rich product in normal horses: clinical signs and cytologic responses. Vet Surg 2013, **42**, 499-510.
37. **Tulamo RM, Bramlage LR, Gabel AA.** Sequential clinical and synovial fluid changes associated with acute infectious arthritis in the horse. Equine Vet J 1989, **21**, 325-331.
38. **Tulamo RM, Heiskanen T, Salonen M.** Concentration and molecular weight distribution of hyaluronate in synovial fluid from clinically normal horses and horses with disease joints. Am J Vet Res 1994, **55**, 710-715.
39. **van den Boom R, Brama PAJ, Kiers GH, DeGroot J, Barneveld A, van Weeren PR.** The influence of repeated arthrocentesis and exercise on matrix metalloproteinase and tumour necrosis factor α activities in normal equine joints. Equine Vet J 2004, **36**, 155-159.
40. **van den Boom R, van de Lest CHA, Bull S, Brama PAJ, van Weeren PR, Barneveld A.** Influence of repeated arthrocentesis and exercise on synovial fluid concentrations of nitric oxide, prostaglandin E_2 and glycosaminoglycans in healthy equine joints. Equine Vet J 2005, **37**, 250-256.