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ORIGINAL ARTICLE

Effects of two platelet-rich gel supernatants at two concentrations on healthy cartilage explants from horses

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ABSTRACT. Platelet-rich plasma is used as a treatment of arthropathies in horses. However, there is lack of *in vitro* information about the mechanism of action of this substance and its effects on healthy cartilage of these animals. The aims were: (1) to evaluate the effects at 48 and 96 h of two concentrations (25 and 50%) of leukocyte- and platelet-rich gel (L-PRG) and pure platelet rich gel (P-PRG) supernatants on the production/degradation in normal cartilage explants (CEs) of platelet-derived growth factor isoform BB, transforming growth factor beta-1, tumour necrosis factor alpha, interleukin (IL) 4 (IL-4), and IL-1 receptor antagonist (IL-1ra); and (2) to study possible correlations of these molecules with their respective PRG supernatant treatments. CEs from six horses were cultured for 96 h with L-PRG and P-PRG supernatants at 25 and 50% concentrations, respectively. CE culture media were changed each 48 h and used for determination, by ELISA, of the molecules. L-PRG and P-PRG supernatants at 25 and 50% concentrations influenced the molecular anti-inflammatory profile of CE groups cultured with these substances. 50% L-PRG supernatant produced the most robust pro-inflammatory effects when compared to the CE control group and the CE group treated with the other PRG supernatant concentrations. In general, PRG supernatants induced pro-inflammatory effects in normal CEs.

Key words: cartilage, cytokines, growth factors, horse, platelet-rich plasma.

RESUMEN. El plasma rico en plaquetas es usado como tratamiento de artropatías en caballos. Sin embargo, existe escasa información *in vitro* acerca del mecanismo de acción de esta sustancia y sus efectos en el cartílago sano de estos animales. Los objetivos del estudio fueron: (1) evaluar los efectos a las 48 y 96 h de dos concentraciones de sobrenadantes de gel rico en plaquetas y leucocitos (L-PRG) y gel rico en plaquetas puro (P-PRG) sobre la producción/degradación de factor de crecimiento derivado de plaquetas isoforma BB, factor de crecimiento transformante beta-1, factor de necrosis tumoral alfa, interleucina (IL) 4 (IL-4) y antagonista del receptor de la IL-1 (IL-1ra) en explantes de cartílago (EC) equino normales; y (2) estudiar posibles correlaciones de estas moléculas con los tipos de tratamientos evaluados. Se cultivaron EC de seis caballos clínicamente sanos durante 96 h con sobrenadantes de L-PRG y P-PRG en concentraciones del 25 y 50%, respectivamente. Los medios de cultivo se cambiaron cada 48 h y se utilizaron para la determinación, por ELISA, de las moléculas. Los sobrenadantes de L-PRG y P-PRG en ambas concentraciones influyeron en el perfil antiinflamatorio molecular de los grupos de EC cultivados con estas sustancias. El sobrenadante de L-PRG al 50% produjo los efectos proinflamatorios más robustos en comparación con el grupo control y los grupos de EC cultivados con los demás tratamientos. Los sobrenadantes de ambos geles indujeron efectos proinflamatorios en EC normales.

Palabras clave: caballo, cartílago, citocinas, factores de crecimiento, plasma rico en plaquetas.

INTRODUCTION

There is an increased interest in the development and clinical use of cells and proteins for the treatment of acute and chronic musculoskeletal disease, such as osteoarthritis (OA) in humans and animals (Maia *et al* 2009, Jayabalan *et al* 2014, Arnhold *et al* 2015, Docheva *et al* 2015, Labusca *et al* 2015, Monteiro *et al* 2015). The main goal of these novel therapies is to arrest the catabolic/inflammatory process related with the course of the disease and possibly to modify the pathological changes with amelioration of the clinical signs associated (Labusca *et al* 2015). This new medical paradigm is known as regenerative medicine (Daar 2013).

Currently, platelet-rich plasma (PRP) is a popular "regenerative therapy" for the medical treatment of diverse acute and chronic diseases of the musculoskeletal system, such as traumatic arthritis, OA and tenodesmopathies (Maia *et al* 2009, Carmona *et al* 2013, de Vos *et al* 2014, Jayabalan *et al* 2014, Jeong *et al* 2014, Monteiro *et al* 2015). Several PRP preparations have been clinically used for the medical treatment of OA in humans (Jang *et al* 2013, Say *et al* 2013), horses (Carmona *et al* 2007, Pichereau *et al* 2014, Monteiro *et al* 2015) and dogs (Silva *et al* 2013). However, there are contradictory results regarding the beneficial effects of PRP in patients with naturally occurring musculoskeletal disease. The discrepancies in these results are related with several confounding factors that mask, and do not allow an evaluation of, the precise effects of these substances in clinical conditions (Moraes *et al* 2013, Brossi *et al* 2015, Giraldo *et al* 2015, Grambart 2015).

The main problems that bias the actual validation of PRP as a regenerative therapy for joint disease, amongst other variables, are: (1) the type of PRP used, because there are PRP preparations poor or rich in leukocytes. In this sense, there are basic and clinical controversial data regarding the ideal leukocyte concentration in a PRP preparation; (2) the absence of controlled clinical studies evaluating this and other regenerative therapies, such as stem cells; 3) the scarce and fragmented information

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about a PRP preparation from basic studies (i.e.: *in vitro* systems, animal models and preclinical research) to the definitive clinical use (Moraes *et al* 2013, Brossi *et al* 2015, Grambart 2015).

To our knowledge, there are few studies describing the use of a PRP preparation as a treatment for joint disease in the horse (Carmona *et al* 2007, Carmona *et al* 2009, Pichereau *et al* 2014). However, there are several *in vitro* studies in horses that show the positive effect of leukoreduced PRP (also known as pure PRP [P-PRP]) preparations in musculoskeletal tissues in contrast to leukoconcentrated PRP (L-PRP) preparations (Kisiday *et al* 2012, McCarrel *et al* 2012, Boswell *et al* 2014, Sundman *et al* 2014).

Recently, the anti-inflammatory and anabolic effects of two platelet-rich gel (PRG) supernatants from L-PRP and P-PRP preparations, activated with calcium gluconate on normal (Rios *et al* 2015[c]) and lypopolysaccharide (LPS)-challenged (Rios *et al* 2015[a]) synovial membrane explants (SMEs) from horses were described. The results from these studies suggest that these platelet-leukocyte preparations can induce different anti-inflammatory and anabolic profiles on these tissues, and these biological responses depend on whether the SMEs were challenged or not with an inflammatory stimuli.

Furthermore, an independent study that used a technology similar to that used for SME (Rios *et al* 2015[a], Rios *et al* 2015[c]) evaluated the anti-inflammatory and anabolic effects of PRG supernatants (L-PRG and P-PRG) in cartilage explants (CEs) from horses challenged with LPS (Rios *et al* 2015[b]). The results of the study showed that 50% L-PRG supernatant produced a more sustained concentration of growth factors and anti-inflammatory cytokines than the other haemoderivatives evaluated (Rios *et al* 2015[b]). However, it is unclear whether normal CEs from horses show different biological response to PRP preparations than CEs challenged with LPS, such has been observed in SME (Rios *et al* 2015[a], Rios *et al* 2015[c]).

The aims of this study were to measure and to compare the temporal anti-inflammatory effect of two types of PRG supernatants (L-PRG and P-PRG) at two concentrations (25 and 50%) in the culture media of normal CEs from horses cultured over 96 h. The production and degradation of platelet-associated growth factors (GFs) (platelet-derived GF (PDGF-BB) and transforming growth factor beta 1 (TGF-β_1)), pro-inflammatory (tumour necrosis factor alpha (TNF-α)) and anti-inflammatory cytokines (interleukin 4 (IL-4) and interleukin 1 receptor antagonist (IL-1ra)) are presented. Further, a correlation analysis between the variables studied was performed.

We evaluate the hypothesis that both platelet-gel supernatants at different concentrations should produce different growth-factor and cytokine concentrations in the culture media of normal CE from horses and that the biologic response of the normal CEs to these PRG supernatants induce an inflammatory profile in comparison to LPS-challenged CEs (Rios *et al* 2015[b]), in which these substances induce an anti-inflammatory response.

MATERIAL AND METHODS

This study was approved by the Ethical Committee for Animal Experimentation of the authors' institution.

ANIMALS AND SAMPLES

Cartilage samples from the metacarpophalangeal joints from 6 mature horses with a mean age of 7.6 (± 1.4) years were included. The samples were taken from horses free from musculoskeletal disease and euthanized by a pentobarbital intravenous overdose for other medical reasons. All the joints were radiographed and macroscopically evaluated to exclude horses with OA joint associated changes. The horses sampled in this study were different from those used in previous studies (Rios *et al* 2015[a], Rios *et al* 2015[c], Rios *et al* 2015[b]). However, the haemoderivatives evaluated were from the same animal used in the aforementioned studies (Rios *et al* 2015[a], Rios *et al* 2015[c], Rios *et al* 2015[b]), but obtained independently for each experiment.

L-PRP AND P-PRP PREPARATION

Venous blood from 1 adult clinically healthy mare was used to avoid the great variability in the GF, cytokine concentrations in the PRGs supernatants used in the experiments. L-PRP and P-PRP were obtained by a manual double centrifugation tube method (Giraldo *et al* 2013), previously validated and used clinically in horses with OA (Carmona *et al* 2007). Briefly, blood was drawn from jugular venipuncture and deposited in 4.5 mL tubes with sodium citrate solution (BD Vacutainer®, Becton Drive, Franklin Lakes, NJ, USA). After centrifugation at 120 *g* for five minutes, the first 50% of the top supernatant plasma fraction, adjacent to the buffy coat, was collected. This fraction was then centrifuged at 240 *g* for five minutes and the bottom fourth fraction was collected. This fraction was considered L-PRP, and the upper plasma fraction was considered as P-PRP. Whole blood and both PRP were analysed for PLT and WBC concentration using an impedance-based hematology device (Celltac-α MEK 6450, Nihon Kodhen, Japan).

Both PRP were activated with calcium gluconate (ratio 1:10) and remained in incubation at 37 ˚C for 1 h until clot retraction. L-PRG and P-PRG supernatants were always used fresh during each culture media changing at 1 and 49 h. Aliquots of both PRG supernatants obtained at every time point were frozen at -86 ˚C for later quantification of the molecules of interest.

CULTURE AND STUDY DESIGN

Cartilage slices were obtained aseptically with a scalpel blade. The slices were cut in circular 4 mm diameter explants by using a disposable biopsy punch (KAI Medical, Solingen, Germany). CEs were washed in phosphate buffered saline. The design of the study included the evaluation of five experimental groups, as follows: 1 CE control group (without addition of any PRG supernatant) and 4 CE groups cultured with L-PRG or P-PRG supernatants at two different concentrations, 25% and 50%.

CEs were stabilised in Dulbecco's Modified Eagle Medium (DMEM) (high glucose, 4500 mg/L) with L-glutamine and sodium bicarbonate and free of sodium pyruvate (DMEM, Lonza Group Ltd, Basel, Switzerland) and supplemented with streptomycin (100 μg/mL) and penicillin (100 μg /mL) without the addition of serum. Cultures were incubated in a 5% CO_2 and water saturated atmosphere for 24h and then replaced with fresh culture media. All CE groups were cultured during 48 h and the culture media was changed and replaced by fresh culture media and fresh PRG supernatants and incubated for other additional 48 h. Culture media obtained at 1, 48, 49 and 96 h were aliquoted and frozen at -86 ˚C for later determination of PDGF-BB, TGF-β_1, TNF-α, IL-4 and IL-1ra. All the culture experiments were performed by triplicate.

ELISA ANALYSIS

L-PRG and P-PRG supernatants and culture media alone or with PRG supernatants obtained at 1, 48, 49 and 96 h were used to determine the concentration of PDGF-BB, TGF-β_1, TNF-α, IL-4 and IL-1ra via ELISA by duplicate. All proteins were assayed using commercial ELISA development kits from R&D Systems (Minneapolis, MN, USA). PDGF-BB (Human PDGF-BB DuoSet, DY220) and TGF-β_1 (Human TGF-β1 DuoSet, DY240E) were determined using human antibodies because there is a high homology between these proteins in humans and horses (Penha-goncalves *et al* 1997, Donnelly *et al* 2006). Furthermore, these kits have been used for the same purposes in other equine PRP studies (Giraldo *et al* 2013). TNF-α (Equine TNF-alpha DuoSet, DY1814), IL-4 (Equine IL-4 DuoSet, DY1809) and IL-1ra (Equine IL-1ra/IL-1F3 DuoSet, DY1814) were assayed with equine-specific antibodies. The standards provided for each ELISA kit were used in preparing each standard curve according to the manufacturers' instructions. Readings were performed at 450 nm.

STATISTICAL ANALYSIS

The statistical analysis was performed using the software SPSS 19.0 (IBM, Chicago, IL, USA). A Shapiro-Wilk test was used to assess the fit of the data set to a normal distribution (goodness of fit). Both PLT and WBC counts in whole blood and both PRP preparations, and PDGF-BB, TGF-β_1, TNF-α, and IL-4 concentrations in all the evaluated groups showed a normal distribution (P>0.05). IL-1ra concentrations showed a non-parametric distribution (P<0.05), even after attempting several mathematical transformations.

Platelet and WBC counts in whole blood, L-PRP and P-PRP were evaluated by a one-way analysis of variance (ANOVA), followed by a Tukey test. PDGF-BB, TGF-β_1, TNF-α and IL-4 concentrations from both PRG supernatants were evaluated by a t-non-paired test; furthermore, IL-1ra concentrations were compared by a Mann-Whitney U test. PDGF-BB, TGF-β_1, TNF-α and IL-4 concentrations from culture media obtained at 48 and 96 h from all CE groups were analysed by a generalised linear model (GLM) followed, when necessary, by a Tukey test. IL-1ra concentrations were evaluated by using a Kruskal-Wallis test followed, when necessary, by Mann-Whitney U test pairwise comparisons.

PDGF-BB, TGF-β_1, TNF-α, IL-4 and IL-1ra concentrations in fresh culture media with PRG supernatants at 1 and 48 h were also compared with the concentrations for these molecules in the culture media from CE groups obtained at 48 and 96 h using a t-paired test (or a Wilcoxon test for the case of IL-1ra). A correlation analysis was performed to determine the Pearson correlation coefficient (r) (or Spearman correlation coefficient [ρ] for the case of IL-1ra) between the variables evaluated in the study. A P<0.05 value was accepted as statistically significant for all tests. Data are presented as mean ± mean standard deviation.

RESULTS

CELL, GROWTH FACTOR AND CYTOKINE IN L-PRP/L-PRG AND P-PRP/P-PRG

Platelet counts were significantly (P<0.05) different between whole blood, L-PRP and P-PRP, with the lowest concentration for P-PRP (98.7 ± 4.6 PLT/μL [mean ± s.d]), followed by whole blood (125.9 ± 3.4 PLT/μL) and L-PRP (312.8 ± 19.6 PLT/μL). WBC counts were also significantly different between the evaluated groups, with a higher concentration for L-PRP (35.1 ± 3.5 WBC/μL), followed by whole blood (8.3 ± 3.7 WBC/μL) and P-PRP (0.11 ± 0.04 WBC/μL).

TGF-β_1 concentration was similar between L-PRG (1672.2 ± 314.3 pg/mL) and P-PRG (1366.7 ± 20.9 pg/mL). PDGF-BB had a significantly (P<0.05) higher concentration in L-PRG (3064. 8 ± 1256.7 pg/mL) when compared with P-PRG (382.7 ± 80.4 pg/mL). TNF-α concentrations were similar between L-PRG (61 ± 0.2 pg/mL) and P-PRG (58 ± 1.7 pg/mL) supernatants. IL-4 concentrations were similar between L-PRG (74.9 ± 9.7 pg/mL) and P-PRG (62.3 ± 1.4) supernatants. IL-1ra concentration was significantly (P<0.05) higher in L-PRG (161.5 ± 64 pg/mL) when compared to P-PRG (59.5 ± 3.4 pg/mL).

PRODUCTION/DEGRADATION OF GROWTH FACTORS AND CYTOKINES IN CULTURE MEDIA OF CES TGF-β_1

Initial TGF-β_1 concentrations obtained at 1 and 49 h in the culture media were significantly (P<0.05) lower when compared with every homologous PRG supernatant treatment at 48 and 96 h, respectively (figure 1). This GF was released from the CE control group, and its concentration at 48 h was significantly (P<0.05) lower to those TGF-$\beta_1$concentrations measured in the culture media from the CE treated with all PRG supernatants. The CE groups cultured with both 50% PRG supernatants presented the highest (P<0.05) concentration for this protein when compared with the CE control group and those CE groups treated with both 25% PRG supernatants (figure 1). At 96 h, a similar statistical behaviour was observed (figure 1).

PDGF-BB

Platelet-derived growth factor-BB concentration was significantly (P<0.05) higher in culture media from all CE groups treated with different L-PRG supernatant concentrations at 1 and 49 h when compared with those PDGF-BB concentrations measured in the same groups at 48 and 96 h, respectively (figure 2). At 48 h, a significant diminution of PDGF-BB concentration was noticed for CE groups treated with both L-PRG supernatant concentrations. At this time point, culture media from CE group treated with 50% of L-PRG supernatant presented slight significant higher PDGF-BB concentrations in comparison with the rest of the CE groups evaluated (figure 2).

At 96 h, PDFG-BB concentration from culture media of the CE treated with 50% of L-PRG supernatant was significantly (P< 0.05) higher when compared with all the CE groups and the same group at 48 h (figure 2).

TNF-α

TNF-α concentrations from culture media of all CEs treated with all PRG supernatants were significantly lower at 1 and 49 h in comparison to 48 and 96 h, respectively. At 48 h, the concentration for this cytokine in the CE control group and the groups treated with both 25% PRG concentrations was significantly different (P<0.05) when compared to the CE cultured with both 50% PRG supernatants (figure 3). At 96 h, TNF-α concentration was significantly (P<0.05) lower in the culture media of the CE control group in comparison with the other CE treated groups. The CE groups cultured with higher PRG concentrations presented significantly (P<0.05) increased TNF-α concentrations when compared to the CE groups cultured with both 25% PRG supernatants (figure 3).

IL-4

IL-4 concentrations from culture media of all CEs treated with all PRG supernatants were significantly lower at 1 and 49 h in comparison to 48 and 96 h, respectively. At 48 h, IL-4 concentration was significantly (P<0.05) lower in culture media from the CE group treated with 25% P-PRG concentration in comparison to the CE control group and the CE groups treated with the other haemoderivatives at

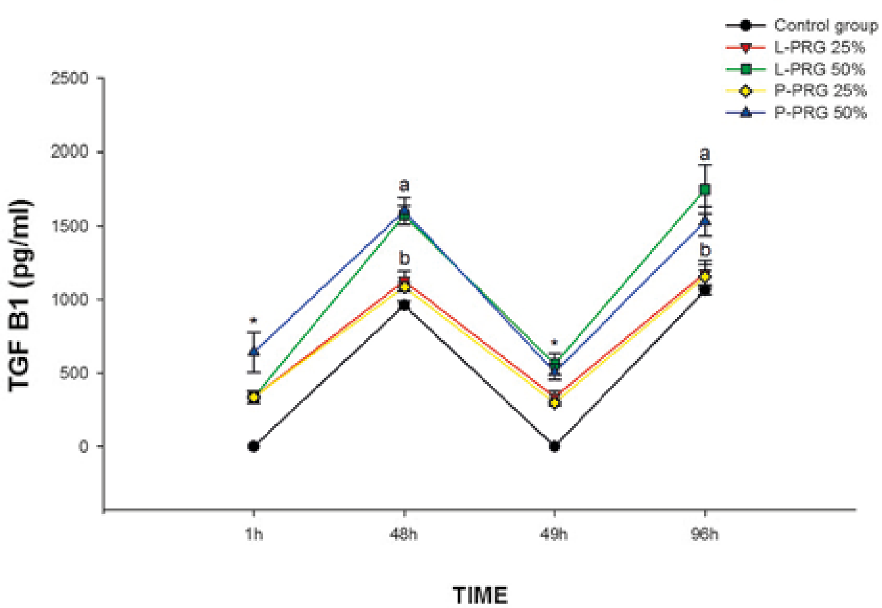

Figure 1. TGF-β_1 concentrations obtained in culture media of the cartilage explant (CE) groups over time.[a-b] Lowercase letters denote significant (P<0.05) differences between groups by Tukey test at 48 h: significantly different with (SDW) a: L-PRG 25%, P-PRG 25% and control group. b: L-PRG 50%, P-PRG 50% and control group; and 96 h: SDW a: L-PRG 25% and P-PRG 25% and, control group. b: L-PRG 50% and P-PRG 50%. *Denotes significant differences (P<0.01) between the same variable at 1 and 48 h and at 49 and 96 h by t-paired test.

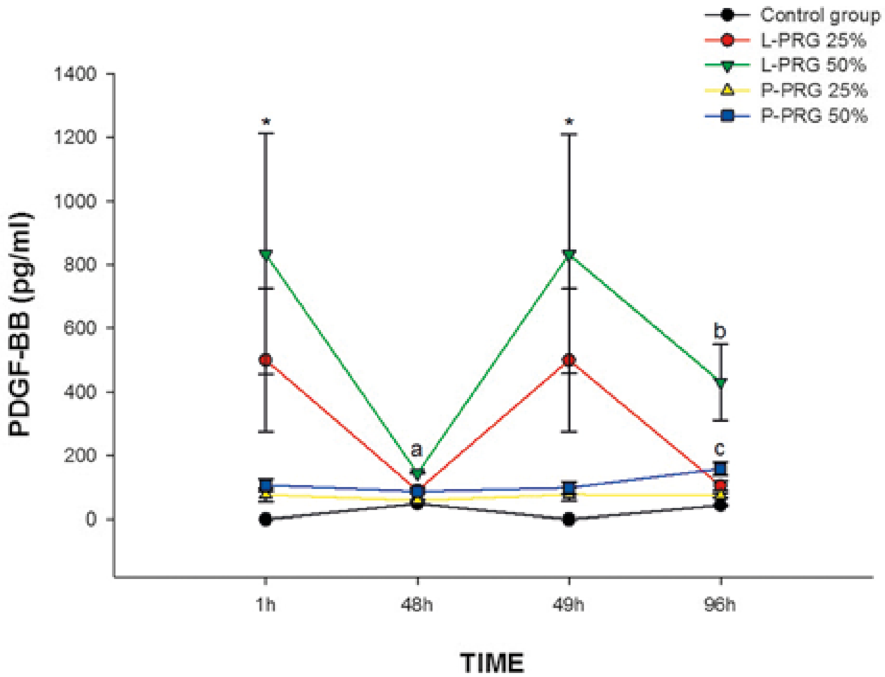

Figure 2. PDGF-BB concentrations obtained in culture media of the CE groups over time.[a-b] Lowercase letters denote significant (P<0.05) differences between groups by Tukey test at 48 h: SDW a: all CE groups; and 96 h: SDW b: all CE groups evaluated and L-PRG 50% at 48 h. *Denotes significant differences (P<0.01) between the same variable at 1 and 48 h and at 49 and 96 h by t-paired test.

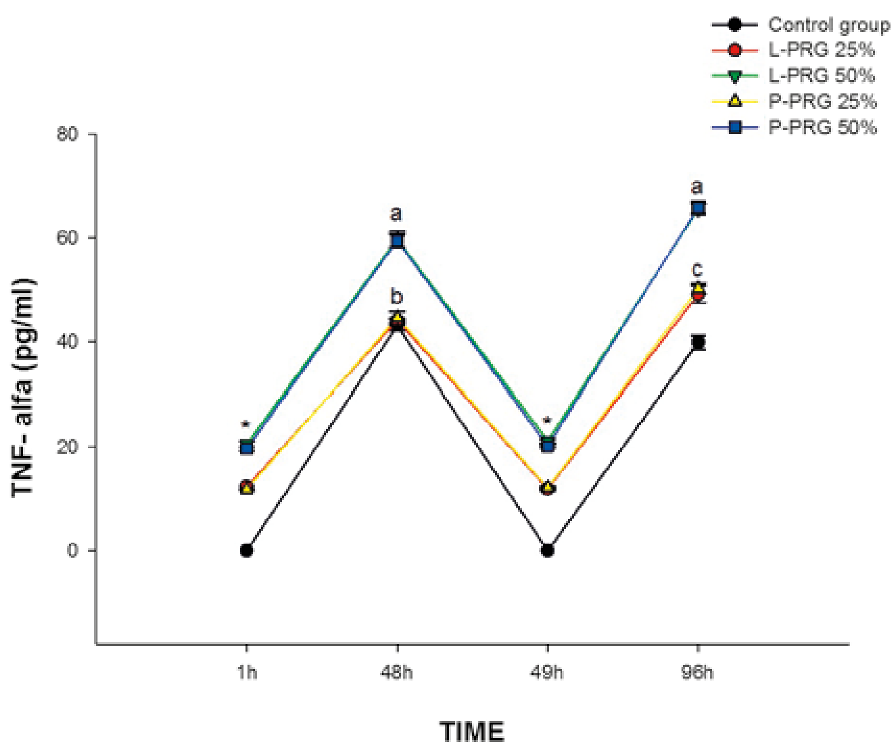

Figure 3. TNF-α concentrations obtained in culture media of the CE groups over time.[a-b] Lowercase letters denote significant (P<0.05) differences between groups by Tukey test at 48 h: SDW a: CE control group, L-PRG 25% and P-PRG 25%. b: L-PRG 50% and P-PRG 50%; and 96 h: SDW a: L-PRG 25% and P-PRG 25%. b: control group. *Denotes significant differences (P<0.05) between the same variable at 1 and 48 h and at 49 and 96 h by t-paired test.

different concentrations (figure 4). At 96 h, IL-4 concentration was significantly different for (P<0.05) each CE group evaluated with an higher concentration for 50% P-PRG, 50% L-PRG, 25% P-PRG, 25% L-PRG and control group, respectively. Furthermore, at 96 h, there was a significant decrease of IL-4 concentrations in the CE control group and the CE group treated with 25% L-PRG supernatant in comparison with the same CE groups at 48 h. Conversely, there was a significant increase of IL-4 concentrations in the CE groups treated with 25% P-PRG and both 50% PRG supernatants when compared with the same CE groups at 48 h.

IL-1RA

At 48 and 96 h, IL-1ra concentration was significantly (P<0.05) higher in the CE group treated with 25 and 50% L-PRG supernatants in comparison to the culture media from all the CE groups evaluated (figure 5). However, these last CE groups treated were significantly (P<0.05) different. Furthermore, IL-1ra concentrations were significantly (P<0.05) higher in the CE groups cultured with 25 and 50% L-PRG supernatants at 96 h when compared with the same group at 48 h.

CORRELATIONS

Significant correlations (P<0.05) were found between TGF-β_1 and TNF-α concentrations (r = 0.72) and between PDGF-BB and IL-1ra concentrations (ρ= 0.70).

DISCUSSION

All the haemoderivatives evaluated in this study produced a different GF and pro- and anti-inflammatory cytokine release profile when compared individually or with the CE control group. In a general sense, both 50% PRG supernatants produced the greatest release of TGF-β_1, TNF-α and IL-4, and both L-PRG concentrations induced the highest release of IL-1ra. Notably, 50% L-PRG supernatant increased the final PDGF-BB concentration at 96 h. This phenomenon is quite interesting because this GF is rapidly denatured by joint tissues (Textor *et al* 2013) and its local production is diminished in the absence of external factors, such as the addition of PRP.

In a general fashion, the temporal release pattern or concentration of TGF-β_1, PDGF-BB and TNF-α of CE from horses treated with different concentrations of PRG supernatants is very similar to those observed for normal

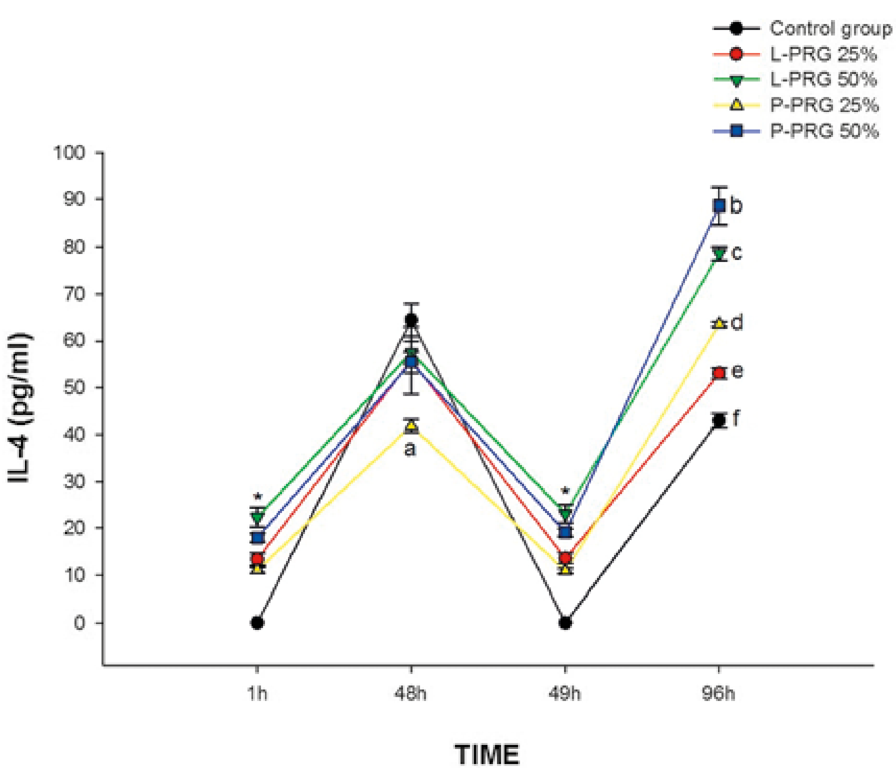

Figure 4. IL-4 concentrations obtained in culture media of the CE groups over time.[a-b] Lowercase letters denote significant (P<0.05) differences between groups by Tukey test at 48 h: SDW a: all groups; and 96 h: SDW b: all groups and P-PRG 50% at 48h. c: all groups and L-PRG 50% at 48 h. d: all groups. e: all groups. f: all groups and control group at 48 h. *Denotes significant differences (P<0.05) between the same variable at 1 and 48 h and at 49 and 96 h by t-paired test.

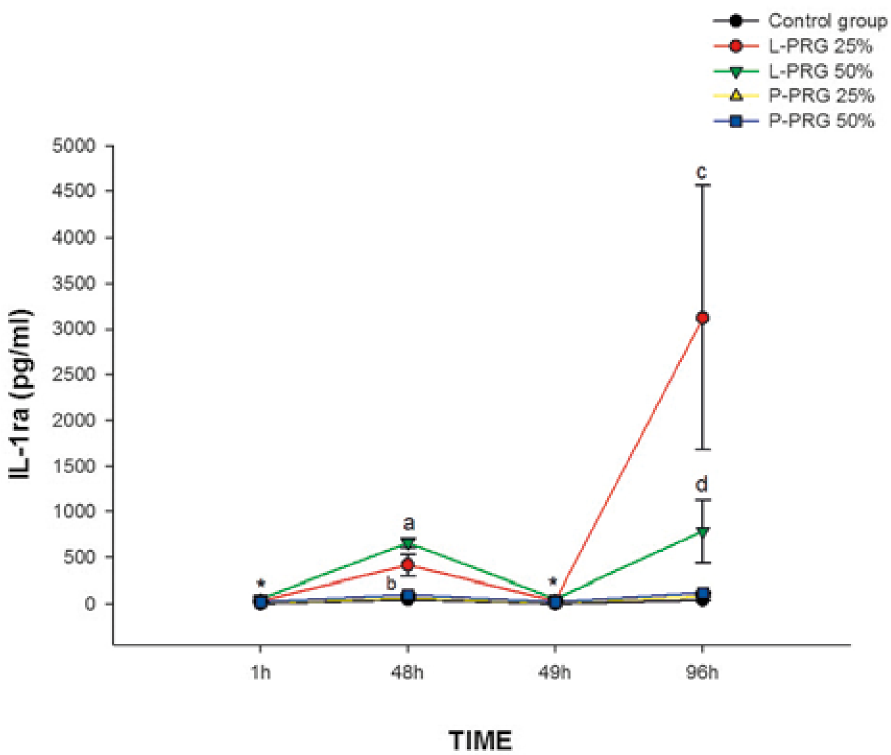

Figure 5. IL-1ra concentrations obtained in culture media of the CE groups over time.[a-b] Lowercase letters denote significant (P<0.05) differences between groups by Mann-Whitney U test at 48 h: SDW a: all groups. b: all groups; and 96 h: SDW c: all groups and L-PRG 25% at 48h. d: all groups. *Denotes significant differences (P<0.05) between the same variable at 1 and 48 h and at 49 and 96 h by Wilcoxon test. Note that 25% L-PRG supernatant promoted a strong anti-inflammatory cytokine IL1-ra release on normal equine cartilage explants.

synovial membrane explants treated with homologous haemoderivatives (Rios *et al* 2015[c]). However, some differences were noticed when compared to the same tissues challenged with LPS (Rios *et al* 2015[a], Rios *et al* 2015[b]).

As observed for normal (Rios *et al* 2015[c]) and LPS-challenged SMEs from horses (Rios *et al* 2015[a]), TGF-β_1 is also released from CEs, but in a lower fashion when compared to normal SMEs (Rios *et al* 2015[c]). Notably, the addition of LPS to the culture media of CEs produced a slight depression in the concentration of this GF to the culture media (Rios *et al* 2015[b]) when compared to the normal CEs of this study. These results could indicate that possibly inflamed joints are less responsive to the exogenous stimuli of PRP to increase the secretion of anti-inflammatory and anabolic GFs, such as TGF-β_1 (Rios *et al* 2015[a], Rios *et al* 2015[b]).

The present results indicate the possibility that L-PRG supernatant at higher concentrations could induce the novo PDGF-BB synthesis by CEs or diminish the intake or denaturing of this key protein by joint tissues. However, this phenomenon could be affected in cases of joint inflammation because CEs challenged with LPS showed a similar degradation/release pattern, but this was reduced in 50% (particularly for 50% L-PRG supernatant at 96 h) (Rios *et al* 2015[b]) in comparison with the healthy CE of this experiment treated with the same hemoderivative. Notably, this apparent consumption of PDGF-BB also has been observed in normal horses after intra-articular PRP injection at 48 hours (Textor *et al* 2013).

In general, normal CEs from this study treated with PRG supernatants released a greater quantity of TNF-α in comparison to the same tissue treated with LPS (Rios *et al* 2015[b]). This finding is very important; because it indicates that the exogenous addition of PRP preparation to healthy joints could induce an inflammatory state. Conversely, the injection of this substance could diminish the inflammation mediated by this cytokine in joints affected by inflammatory/degenerative process. This biological phenomenon was apparently similar in SMEs from horses (Rios *et al* 2015[a], Rios *et al* 2015[c]). However, the local production of TNF-α in CEs challenged with LPS was more dramatically affected in comparison to the rest of the joint tissues evaluated (Rios *et al* 2015[a], Rios *et al* 2015[c], Rios *et al* 2015[b]).

Conversely, the temporal release pattern of IL-4 and IL-1ra is affected by PRG supernatants in CE and synovial membrane explants in a different fashion. For example, IL-4 exhibited a biphasic release pattern in the CE control group, which was characterised by a higher release at 48 h followed by a significant and dramatic (50%) decrease in the culture media concentration at 96 h. However, all the haemoderivatives evaluated (except 25% L-PRG) in this study reversed this phenomenon and increased the final concentration of this cytokine at 96 h. A similar situation was only observed for synovial membrane explants cultured with 50% L-PRG supernatant (Rios *et al* 2015[c]).

Notably, IL-4 concentration in CEs was affected negatively by the addition of LPS (Rios *et al* 2015[b]). This finding was unique for cartilage, because the release of this cytokine was not affected by the addition of LPS in SMEs treated with PRP preparations when compared with non LPS-challenged SMEs (Rios *et al* 2015[a], Rios *et al* 2015[c]). Our results demonstrate that IL-4 production is selectively affected in inflamed cartilage and possibly the exogenous PRP addition could induce the local production of this important chondroprotective cytokine (Wojdasiewicz *et al* 2014).

The IL-1ra pattern release was different in CEs from our study in comparison to synovial membrane explants cultured with different PRG supernatant concentrations. Notably, both L-PRG supernatants, but more markedly the 50% concentration, induced an increased release of this protein from normal CEs. A situation that differed from those observed for synovial membrane explants, which were better stimulated by 25% L-PRG supernatant (Rios *et al* 2015[c]).

However, it is important to note that the CEs challenged with LPS and treated L-PRG supernatants showed a dramatic release (approximately, 2 fold at any time) of this anti-inflammatory cytokine (Rios *et al* 2015[b]) when compared to the normal CEs evaluated in this study. This finding suggests that most of the anti-inflammatory effect of the PRP preparations in patients with arthropaties could be produced by the stimulation of the secretion of IL-1ra.

The correlations obtained in this study were not observed for normal equine synovial membrane explants cultured with the same haemoderivatives (Rios *et al* 2015[c]). Although a correlation does not always equate to a causation, the results are interesting because both platelet-associated growth factors were correlated with mixed pro- (TGF-β_1 and TNF-α) and anti-inflammatory (PDGF-BB and IL-1ra) mechanisms, which could indicate a final pro-inflammatory role of PRP preparations on normal joint tissues. However, when joint tissues (either cartilage or synovial membrane) were challenged with LPS and treated with PRP preparations, a more complex web of GF and cytokine correlations was noticed (Rios *et al* 2015[a], Rios *et al* 2015[b]). Logically, these correlations are an indirect proof that under inflammation conditions the responses of joint tissues are apparently better and different to PRP preparations than in healthy joint tissues.

This *in vitro* study had several biases which have also been recognised in our previous studies (Rios *et al* 2015[a], Rios *et al* 2015[c], Rios *et al* 2015[b]), these are: (1) the haemoderivatives evaluated were obtained from one only horse for culturing the CE of different horses; this situation is not ideal in clinical conditions, but it is advantageous in *in vitro* studies, because the cellular product evaluated can be standardised for cell and protein concentrations, which reduces the variability in the biological response of the tissues treated experimentally. (2) *In vitro* studies do not show the whole biological events that happen in horses with degenerative joint disease or traumatic arthritis, because they are not useful to assess the role of the immunologic system, because they do not include leukocytes and globulins, which also modified the response of the cells or tissues involved in the system (Andia and Maffulli 2014).

It is concluded that both PRG supernatants at 25 and 50% concentrations influenced the release pattern of GF and cytokines of CE groups cultured. 50% L-PRG supernatant produced the most robust pro-inflammatory effects when compared to the CE control group and the CE group treated with the other PRG supernatant concentrations. In general, PRG supernatants induced pro-inflammatory responses in normal CEs. These results could indicate that PRP preparations should be used only for the treatment of adequately diagnosed arthropathies and not for preventive use in healthy athlete equines. Further studies using cartilage and synovial membrane explants in a co-culture system might guarantee to find the exact role of PRP preparations under normal and inflammatory conditions.

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