Anti-inflammatory effects of two platelet-rich gel supernatants in an *in vitro* system of ligament desmitis

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SUMMARY
Background. There is not even a knowledge of what is the perfect concentration of cells that should be present in platelet-rich plasma (PRP) to induce an effect for treatment of desmitis. Our aims were determining the temporal effects (48 and 96 h) of leukocyte-concentrated platelet-rich gel supernatant (Lc-PRGS) and leukocyte-reduced platelet-rich gel supernatant (Lr-PRGS) of ligament explants (LEs) challenged with lipopolysaccharide (LPS). The production and degradation of growth factors, proinflammatory and anti-inflammatory cytokines were measured.
Methods. LEs from horses were challenged with LPS and cultured for 96 hours with Lc-PRGS and Lr-PRGS at both 25% and 50% concentrations. Culture medium was changed every 48 h and used for determination, by ELISA, of platelet-derived growth factor isoform BB (PDGF-BB), transforming growth factor beta-1 (TGF-β1), interleukin 1 beta (IL-1β) , tumor necrosis factor alpha (TNF-α), interleukin 4 (IL-4) and interleukin 1 receptor antagonist (IL-1ra).
Results. LEs cultured with both concentrations of Lc-PRGS showed the lowest release of IL-1β in comparison with the other LE groups at 48 and 96 hours, whereas 50% Lr-PRGS induced the lowest TNF-α release from LEs at 96 hours. IL-1ra was mainly released from LEs cultured with both concentrations of Lc-PRGS at 48 and 96 hours, whereas IL-4 was mainly produced in LEs cultured with 50% Lr-PRGS at 48 and 96 hours.
Conclusions. The anti-inflammatory effect of Lc-PRGS in LEs challenged with LPS seems to be related with heightened concentrations of PDGF-BB that apparently induced a higher release of IL-1ra. On the other hand, the anti-inflammatory action of Lr-PRGS could be associated with a higher release of IL-4 that may reduce the release of TNF-α). Although we observed significant correlations between some mediators evaluated in this study, these findings should be considered with caution because a correlation does not necessary mean a causal relationship. Additional *in vivo* studies which evaluating the effects of both Lc-PRP and Lr-PRP in either animal models or in human patients are necessary, for determination in clinical future application`s. It will be easy, safe, rational and cheaper in cases for desmopaties.

KEY WORDS
anti-inflammatory effect; desmitis; desmopathy; platelet-rich gel supernatants; platelet-rich plasma

BACKGROUND

Ligaments are dense, fibrous connective tissues, mainly composed of type I collagen, that connect bone to bone and transmit the mechanical forces that stabilize the skeleton, allow for body movement, and prevent overextension of joints (1). These anatomic structures can be acutely damaged by a direct trauma (desmitis) or chronically affected by a degenerative/inflammatory process (desmopathy). Notably, any ligament of the body can suffer a traumatic injury or a degenerative/inflammatory disease. However, the ligaments most commonly affected are the anterior cruciate ligament (2); collateral ligaments of the knee, ankle, elbow, and hand (2,3); and medial patellofemoral ligament (4). On the other hand, the plantar fascia is also considered to be a ligament (5), which can be affected by a degenerative/inflammatory disease known as plantar fasciitis (6).

The suspensory ligament (SL) (*m. interosseus medius*) is a specialized anatomical structure of the suspensory apparatus of the horse that supports the fetlock and prevents excessive extension of that joint during the weight-bearing or stance phase of the stride (7). From an anatomic comparative point of view, the SL of the equine hind limb could be comparable to the human plantar fascia, although, the biomechanics of both structures are quite different because, in the horse, the SL prevents fetlock hyperextension (8), whereas, in humans, the plantar fascia maintains the arch of the foot (5). However, the plantar fascia and SL may both be affected by apparently similar pathophysiological mechanisms that produce ligament inflammation and degeneration (desmopathy) with subsequent presentation of chronic pain (lameness) and reduction of the functional capability of the individuals affected by these musculoskeletal disorders (9).

Platelet-rich plasma (PRP) is an emerging "regenerative" biologic therapy for humans with desmitis (10) and plantar fasciitis (11) and horses with SL desmopathy (12). Furthermore, some *in vitro* studies (13) support the use of PRP in patients with ligament disease. However, there are many PRPs with different concentrations of cells, growth factors (GFs), and cytokines that yield variable results when used under clinical conditions (14,15).

PRPs can be classified into two groups, as follows: leukocyte-concentrated PRP (Lc-PRP) and leukocyte-reduced PRP (Lr-PRP), also known as pure platelet-rich plasma (16). Lc-PRP presents detectable numbers of leukocytes (i.e., white blood cells (WBCs) in relation to basal cell counts in whole blood, whereas Lr-PRP exhibits low WBC counts (16). Once PRP preparations are mixed with calcium salts or thrombin, they polymerize in a platelet-rich gel (PRG) and release a supernatant (PRGS) that is rich in GFs and cytokines and other key molecules that regulate wound healing and inflammation (17).

Recent *in vitro* evidence gleaned from research conducted in tendons (18-20) and ligaments (20) suggests that Lr-PRPs could be more suitable for the clinical management of musculoskeletal disease versus Lc-PRPs because lower platelet (PLT) and WBC concentrations in Lr-PRPs could induce less tissue catabolism/inflammation and more tissue anabolism as compared with PLT- and WBC- rich preparations (18,19). However, to our knowledge, there are no published *in vitro* studies at this time comparing the anti-inflammatory effects of either Lr-PRP or Lc-PRP in ligament explants (LEs) challenged with a proinflammatory stimuli produced by lipopolysaccharide (LPS) to establish the perfect PRP hemoderivative for the treatment of patients with desmitis or desmopathies. Thus, in the present study, we investigated the hypothesis that PRGSs at different concentrations should produce different GF and cytokine concentrations with respect to normal LEs and those cultured with LPS. Therefore, we designed an *in vitro* study to compare the time-related effects (at one, 48, 49, and 96 hours) of two concentrations (25% and 50%) of Lc-PRGS and Lr-PRGS in normal LEs challenged with LPS. For comparison purposes, we described the production and degradation of platelet-associated GF platelet-derived GF isoform BB (PDGF-BB) and transforming GF beta-1 (TGF-β1), proinflammatory cytokines interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α), and anti-inflammatory cytokines interleukin 4 (IL-4) and interleukin 1 receptor antagonist (IL-1ra). Additionally, we performed a correlation analysis between the studied variables.

MATERIALS AND METHODS

This study was approved by the institutional committee of animal experimentation. An informed consent authorization was signed by the owner of the horses.

Samples

Samples from the body of the suspensory ligament (*m. interosseus medius*) from the hind limbs of six horses, aged four to seven years old, were included in this study. The samples were taken from animals apparently free from musculoskeletal disease and euthanized by way of a pentobarbital intravenous overdose for other medical reasons. Prior the euthanasia, all hind limbs of the horses were radiographed and ultrasonographically evaluated in a standing position for the purpose of excluding animals with SL desmopathy–associated changes.

Blood collection

Six clinically healthy horses (three geldings and three mares), between six and nine years of age were used as blood donors.

All of these horses were stabled, fed, and managed in a similar fashion. These animals were selected after an extensive physical examination together with a complete blood cell count and a general blood chemistry panel. Only clinically healthy horses with platelet counts higher than $100 \times 10^3/$ µL were used. Whole blood from each horse was obtained by jugular venipuncture using a 21-gauge butterfly catheter.

Platelet-rich plasma/platelet-rich gel supernatant preparation

Both PRPs were obtained by way of a manual double-centrifugation tube method that was previously validated and used clinically in horses with SL desmopathy. Blood was deposited in 4.5 mL tubes with sodium citrate solution (Vacutainer®; Benton Dickson, 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 further centrifuged at 240 g for five minutes and then the bottom fraction was collected. This fraction was considered to be Lc-PRP, while the upper plasma fraction was considered to be Lr-PRP (**figure 1**). Whole blood and both PRP products were analyzed regarding platelet and leukocyte counts (Celltac-α MEK 6450; Nihon Kohden, Tokyo, Japan).

Additionally, both PRPs were activated with calcium gluconate (Ropsohn Therapeutics Ltda., Bogotá, Colombia) (ratio of 1:10) and incubated at 37°C for one hour until clot retraction occurred. Only fresh Lc-PRGS and Lc-PRGS samples were used for the experiments. Aliquots of both PRGSs were frozen at −80°C for later quantification of GFs and cytokines as a control.

Ligament explant culture

Suspensory ligament samples were obtained aseptically. Specifically, they were sectioned with a scalpel blade in rectangular ($5 \times 3 \times 3$ mm) explants with 70 mg ± 4 mg of weight. A total of 36 LEs were obtained from each horse. All of the explants were washed in phosphate-buffered saline and stabilized in Dulbecco's Modified Eagle medium (DMEM) (high glucose, 4,500 mg/L) with L-glutamine and sodium bicarbonate, being free of sodium pyruvate (Lonza Group Ltd., Basel, Switzerland) and supplemented with streptomycin (100 µg/mL) and penicillin (100 µg/mL) without the addition of fetal bovine serum. Cultures were incubated in a 5% CO_2 and water saturated atmosphere for 24 hours and then replaced with fresh culture media. At this time point, a portion of the tissue samples was challenged with 100 ng/mL of LPS (Sigma-Aldrich, St. Louis, MO, USA) to induce inflammatory/catabolic damage of the tissue explants.

Study design

Six experimental groups (with six explants in each) were included. Tissue cultures were performed in six-well plates (Corning® Costar® TC-Treated Multiple Well Plates; Merck KGaA, Darmstadt, Germany) with a total volume of 2.5 mL per well considering the final concentration of the PRGS assayed in the culture media. The study included the evaluation of two LE control groups (one with the addition of LPS and one without LPS) without the addition of any PRGS as well as four LE groups cultured with Lc-PRGS and Lr-PRGS at the two concentrations of 25% and 50%. All LE groups were cultured during 48 hours and the culture media was changed and replaced by fresh culture media and fresh PRGS and incubated for another additional 48 hours. Samples of culture media (0.3 mL) were obtained at one, 48, 49, and 96 hours. Culture media were obtained, aliquoted, and frozen at −80 ºC for later determination of GFs and cytokines. **Figure 1** summarizes the study's design and methodology.

Enzyme-linked immunosorbent assay analysis

The concentration of cytokines and GFs were measured via enzyme-linked immunosorbent assay (ELISA) in duplicate samples in both PRGS and culture media alone or mixed with PRGS obtained at different time points. Cytokines and GFs were assayed using commercial ELISA development kits from R&D Systems (Minneapolis, MN, USA). IL-1β (Equine IL-1 beta/IL-1F2 DuoSet, DY3340), 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 measured separately using equine antibodies. PDGF-BB (Human PDGF-BB DuoSet, DY220) and TGF-β1 (Human TGF-β1 DuoSet, DY240E) were determined separately using human antibodies because there is a high homology between these proteins in humans and horses. To note, these ELISA antibodies have been used for the same purposes previously in other equine PRP studies. The standards provided in each ELISA kit were used to construct each standard curve according to the manufacturer´s instructions. Readings were performed at 450 nm.

Statistical analysis

The Shapiro–Wilk test was used to assess the fit of the data set to a normal distribution (goodness of fit) of each one of the evaluated variables, including the hematological variables in whole blood and both PRPs as well as cytokine and GF concentrations in both PRGSs and culture media at one, 48, 49, and 96 hours. All the parameters presented
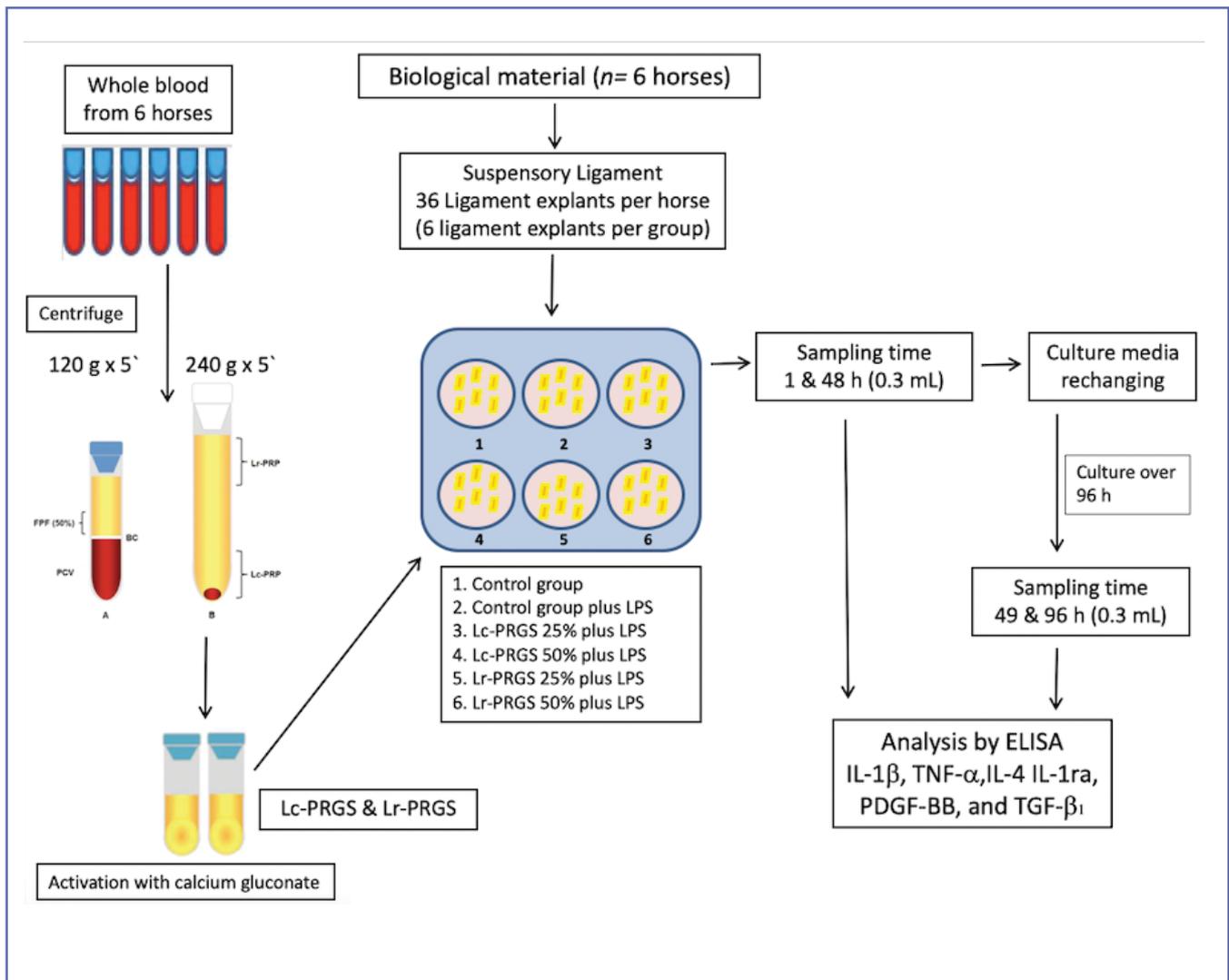
Figure 1. Schematic workflow of the experiments of the study. CM, culture medium; Lc-PRP, leukocyte-concentrated platelet-rich plasma; Lr-PRP, leukocyte-reduced platelet-rich plasma; PRG, platelet-rich gel; Lc-PRGS, leukocyte-concentrated platelet-rich gel supernatant; Lr-PRGS, leukocyte-reduced platelet-rich gel supernatant; IL-1β, interleukin 1 beta; TNF-α, tumor necrosis factor alpha; IL-4, interleukin 4; IL-1ra; interleukin 1 receptor antagonist; PDGF-BB, platelet-derived growth factor isoform BB; TGF-β1, transforming growth factor beta-1.

a normal distribution (P > 0.05). Furthermore, the Levine test was performed to establish variance homogeneity of each one of the variables for each experimental group. Only hematological variables exhibited variance homogeneity (P > 0.05). The rest of the evaluated variables (i.e., cytokines and GFs in both PRGSs and culture media at different time points) failed the Levine test (P < 0.05). Platelet and WBC counts in whole blood and both PRPs were compared with the one-way analysis of variance (ANOVA) followed, when necessary, by the Tukey test.

Cytokine and GF concentrations in PRGSs were compared by a t-test for unpaired samples. Cytokine and GF concentrations in the culture media of each independent group at one, 48, 49, and 96 hours were also compared with a repeated measures two-way ANOVA and a *post-hoc* Games–Howell test where appropriate. Correlation analyses were performed in order to determine the Pearson correlation product (*r*) between the evaluated variables in the study for each experimental group. A P-value of < 0.05 was accepted as statistically significant for all tests.

RESULTS

Cell concentration in whole blood, leukocyte-concentrated platelet-rich plasma, and leukocyte-reduced platelet-rich plasma

Platelet counts were significantly (P < 0.05) different between whole blood, Lc-PRP, and Lr-PRP. Lc-PRP presented the highest platelet concentration, followed by whole blood and Lr-PRP (**table I**). WBC counts were significantly (P < 0.01) different between the hemoderivatives evaluated, with whole blood having the highest concentration, followed by Lc-PRP and Lr-PRP (**table I**).

Concentration of growth factors and cytokines in leukocyte-concentrated platelet-rich gel supernatant and leukocyte-reduced platelet-rich gel supernatant

Our study results indicated PDGF-BB, TGF-β1, and IL-1ra concentrations were significantly (P < 0.001) higher in Lc-PRGS versus in Lr-PRGS, whereas TNF-α and IL-4 concentrations were not significantly different between the PRGSs. On the other hand, IL-1β concentrations were not detected in any hemoderivative (**table I**).

Concentration of growth factors and cytokines in the culture medium of ligament explants at different time points

PDGF-BB concentrations were detected only in those LE groups cultured with PRGS at one and 49 hours, respectively. At these time points, the concentrations for this GF were significantly (P < 0.001) different between the LE groups cultured with both PRGSs with the highest polypep-tide concentrations for LEs cultured with 50% Lc-PRGS, followed by 25% Lc-PRGS, 50% Lr-PRGS, and 25% Lr-PRGS. PDGF-BB concentrations were significantly (P < 0.001) different between the same hemoderivatives, with the exception of the LEs cultured with 50% Lr-PRGS at one, 48, 49, and 96 hours, with the lowest concentration of this GF present at 48 and 96 hours (**figure 2**).

At 48 hours, PDGF-BB concentrations were significantly (P < 0.001) different between the LEs of the control group, those LEs of the control group challenged with LPS, and those cultured with both PRGSs at the two concentrations. At this time point, LEs of the control group challenged with LPS produced the lowest concentration of this polypeptide in comparison with the rest of the groups (**figure 2**).

At 96 hours, PDGF-BB concentrations were significantly (P < 0.001) different between the groups in the study with a significant (P < 0.001) diminution in the release of this GF in LEs of the control group when compared with the same group at 48 hours. On the other hand, at 96 hours, the PDGF-BB concentrations in LEs cultured with both Lc-PRGSs were significantly (P < 0.001) higher in comparison with the concentrations of this GF with the same hemoderivatives at 48 hours (**figure 2**).

TGF-β1 concentrations were detected at one and 49 hours in the culture medium of LEs from groups cultured with both PRGSs at the two concentrations. At these time points, the concentrations of this GF were significantly (P < 0.001) higher in the culture medium of LEs cultured with 50% Lc-PRGS, followed by 50% Lr-PRGS, 25% Lc-PRGS, and 25% Lr-PRGS (**figure 3**).

At 48 hours, TGF-β1 concentrations were similar between the groups, while, at 96 hours, a similar GF concentration pattern was observed, although, the TGF-β1 concentrations of culture medium of LEs of the control group were signifi-

Table I. Concentrations of platelets, white blood cells, growth factors and cytokines in whole blood, both platelet rich plasma and both platelet rich gel supernatants. *

Variable	Fluid		
	Whole blood	**Lc-PRP**	**Lr-PRP**
Platelet x10³/mL	119.6 ± 5.6[a]	321.7 ± 19.3[b]	98.7 ± 4.3[c]
WBC x10³/m L	8.6 ± 3.8[a]	4.2 ± 0.6[b]	0.13 ± 0.03[c]
		Lc-PRGS	**Lr-PRGS**
PDGF-BB (pg/mL)		1710.6 ± 498.6[a]	253.3 ± 56.9[b]
TGF-β1 (pg/mL)		2160.8 ± 516.1[a]	1350.7 ± 160.1[b]
TNF-α (pg/mL)		60 ± 0.5	59 ± 1.4
IL-4 (pg/mL)		63.8 ± 6.28	55.4 ± 2.7
IL-1ra (pg/mL)		1195.5 ± 431.9[a]	68.9 ± 7.6[b]

[a-b] Lowercase letters denote significant differences (p < 0.01) between groups in the same row by Tukey test. ND: no determined. * Data are presented as means (s.d.).
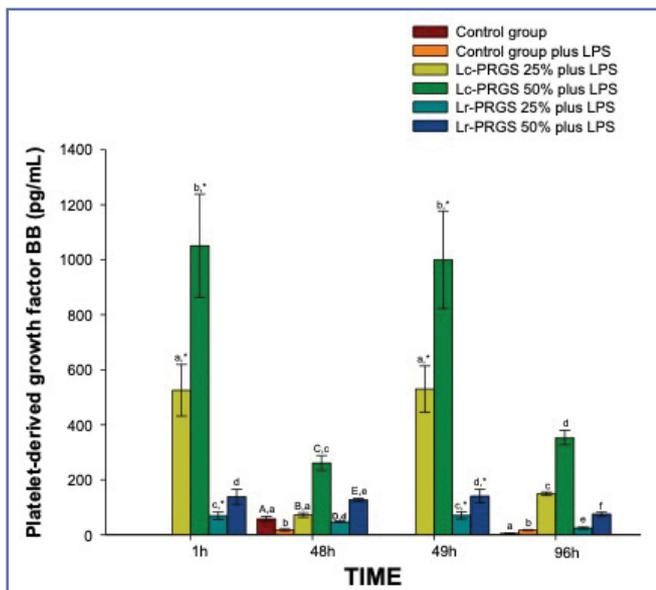
Figure 2. PDGF-BB concentrations observed in the culture media of LE groups at different time points. Capital letters denote significant (P < 0.01) differences between the same group at 48 and 96 hours by Games–Howell test. Lowercase letters denote significant (P < 0.01) differences between groups at the same time by Games–Howell test. *Denotes significant differences (P < 0.01) between the same group at one, 48, 49, and 96 hours by Games–Howell test. Data are presented as means standard deviations (SDs).
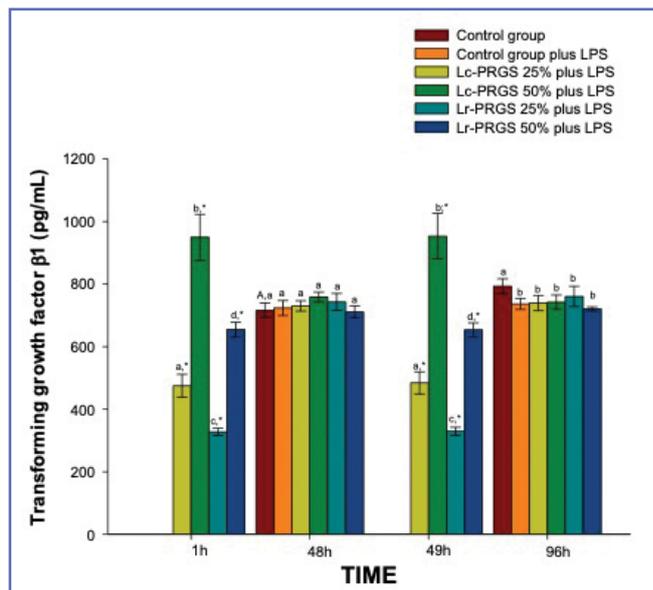

Figure 3. TGF-β1 concentrations observed in the culture media of LE groups at different time points. Capital letters ([A–E]) denote significant (P < 0.01) differences between the same group at 48 and 96 hours by Games–Howell test. Lowercase letters ([a–f]) denote significant (P < 0.01) differences between groups at the same time by Games–Howell test. *Denotes significant differences (P < 0.01) between the same group at one, 48, 49, and 96 hours by Games–Howell test. Data are presented as means (SDs).

cantly higher in comparison with the GF concentrations of the same group at 48 hours. On the other hand, at 96 hours, the LEs of the control group presented significantly (P < 0.001) higher TGF-β$_1$ concentrations when compared with the rest of the groups (**figure 3**).

IL-1β concentrations were not detected in the culture media of the LE groups at one and 49 hours. At 48 hours, the concentrations of this cytokine were significantly (P < 0.001) higher in the culture medium of LEs cultured with 50% Lr-PRGS, followed by in that of LEs from the control group. The culture media of LEs from control group challenged with LPS and those cultured with 25% Lc-PRGS exhibited the lowest significant (P < 0.001) IL-1β concentrations when compared with the rest of the groups, although the LE groups cultured with 50% Lc-PRGS and 25% Lr-PRGS presented a significantly (P < 0.001) lower IL-1β concentration in comparison with the LEs of the control group and those cultured with 50% Lr-PRGS (**figure 4**).

At 96 hours, IL-1β concentrations were significantly (P < 0.001) higher in the culture medium of LEs of the control group challenged with LPS, followed by in the culture media of those LE groups cultured with 50% Lr-PRGS and

25% Lc-PRGS. On other hand, LEs cultured with 25% Lr-PRGS presented a significantly (P < 0.001) lower IL-1β concentration in comparison with the rest of the LE groups, though the culture media of LEs of the control group and those explants cultured with 50% Lc-PRGS presented apparently similar IL-1β concentrations that were significantly (P < 0.001) lower than the concentrations for this cytokine in the culture media of LEs from the control group challenged with LPS as well as those explants cultured with 25% Lc-PRGS and 50% Lr-PRGS (**figure 4**).

IL-1β concentrations were significantly different between the same groups at 48 and 96 hours. In general, at 48 hours, the concentrations of this cytokine were significantly higher in the case of LEs from the control group and in those cultured with 50% Lr-PRGS when compared with the other groups at 96 hours. However, at 96 hours, a contrary situation was observed regarding LEs of the control group challenged with LPS, where there was a significant (P < 0.001) increase in IL-1β concentration versus in the same group at 48 hours (**figure 4**).

TNF-α concentrations were only detected at one and 49 hours in culture medium of LEs from groups cultured with both PRGSs at the two concentrations. At these same time
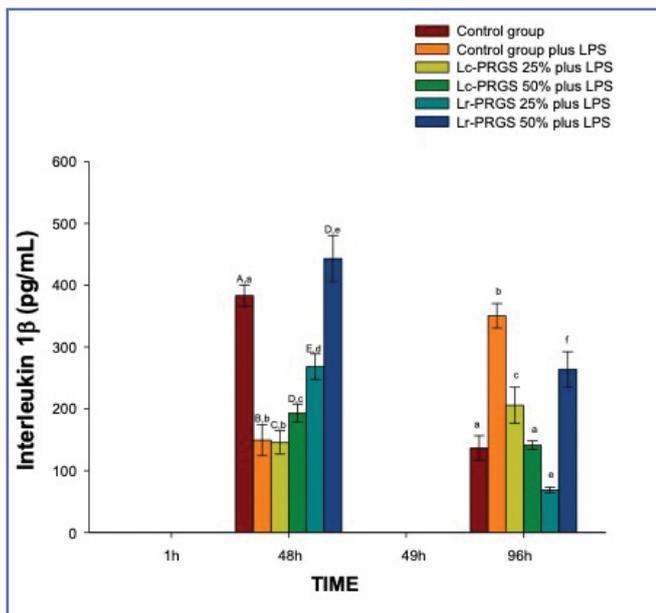
Figure 4. IL-1β concentrations observed in the culture media of LE groups at different time points. Capital letters ([A–E]) denote significant (P < 0.01) differences between the same group at 48 and 96 hours by Games–Howell test. Lowercase letters ([a–f]) denote significant (P < 0.01) differences between groups at the same time by Games–Howell test. *Denotes significant differences (P < 0.01) between the same group at one, 48, 49, and 96 hours by Games–Howell test. Data are presented as means (SDs).
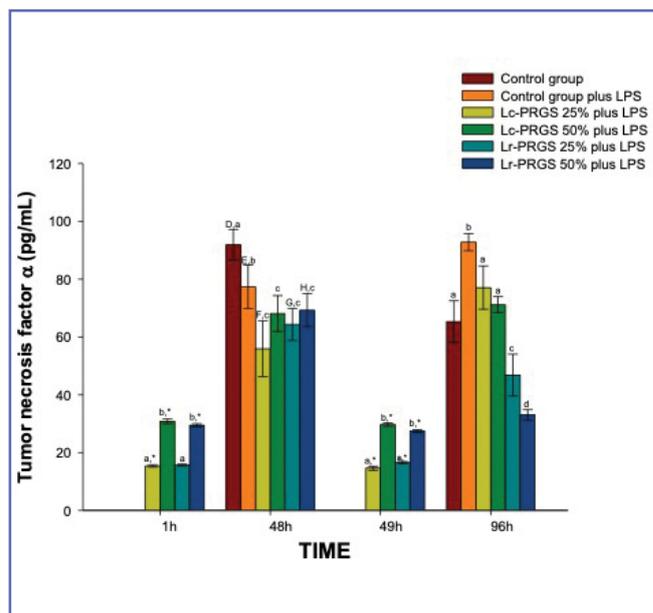

Figure 5. TNF-α concentrations observed in the culture media of LE groups at different time points. Capital letters ([A–E]) denote significant (P < 0.01) differences between the same group at 48 and 96 hours by Games–Howell test. Lowercase letters ([a–f]) denote significant (P < 0.01) differences between groups at the same time by Games–Howell test. *Denotes significant differences (P < 0.01) between the same group at one, 48, 49, and 96 hours by Games–Howell test. Data are presented as means (SDs).

points, the concentrations of this cytokine were significantly (P < 0.001) different between the LE groups cultured with both PRGSs at 25% and both PRGSs at 50%. On the other hand, the concentrations of this cytokine were significantly different between the same groups at one, 48, 49, and 96 hours (**figure 5**).

At 48 hours, TNF-α concentrations were significantly higher (P < 0.001) in the culture media of LEs of the control group and those of the control group challenged with LPS. The TNF-α concentrations of this last group were similar to those observed in LE groups cultured with 50% Lc-PRGS and Lr-PRGS at both concentrations. On the other hand, LEs of the group cultured with 25% Lc-PRGS showed a significantly (P < 0.001) lower TNF-α concentration when compared with both control groups, though the amount was not significantly different as compared with the cytokine concentration obtained in the culture media of the LEs cultured with the rest of the hemoderivatives (**figure 5**).

At 96 hours, significantly (P < 0.001) higher TNF-α concentrations were observed for LEs of the control group challenged with LPS, followed by LEs cultured with 25% L-PRGs, and those LEs of the control group and cultured with 50% Lc-PRG, respectively. However, these last three experimental groups were not significantly different from one another regarding the concentration of this cytokine. On the other hand, at 96 hours, significantly (P < 0.001) low TNF-α concentrations were observed in the LE group cultured with 50% Lr-PRGS, followed by 25% Lr-PRGS, when compared with the rest of the LE groups. TNF-α concentrations were significantly (P < 0.001) different between the LEs of both control groups and those LE groups cultured with 25% Lc-PRGS and both Lr-PRGSs at 48 and 96 hours (**figure 5**). IL-4 were detected in low concentrations at one and 49 hours in the culture medium of LEs from groups cultured with both PRGSs at the two concentrations. To note, IL-4 was not detected at this time in SLEs from both control groups. At these same time points, the concentrations of this cytokine were significantly (P < 0.001) different between the LE groups cultured with both PRGSs at 25% and both PRGSs at 50%. Also, the concentrations of this cytokine were significantly different between the same groups at one, 48, 49, and 96 hours (**figure 6**).

At 48 hours, IL-4 concentrations were significantly (P < 0.001) higher in the culture medium of LEs of the control group when compared with in the culture media of the rest of the groups. At this time point, the culture medium of LEs of the control group challenged with LPS showed significantly (P < 0.001) lower IL-4 concentrations, followed by LEs cultured with 25% Lc-PRGS, when compared with the rest of the groups. On the other hand, LEs cultured with 50% Lc-PRGS and both Lr-PRGSs presented higher IL-4 concentrations, but there were no significant differences between these groups (**figure 6**).

At 96 hours, IL-4 concentrations were significantly diminished in the culture media of LEs from control group and those LEs cultured with 50% Lc-PRGS and 25% Lr-PRGS. At this time point, the culture media of LEs of control group challenged with LPS and those LEs cultured with 50% Lr-PRGS showed significantly (P < 0.001) higher IL-4 concentrations when compared with the rest of evaluated groups. Separately, IL-4 concentrations were significantly higher in the LE group cultured with 25% Lc-PRGS when compared to the LEs of the control group and those LEs cultured with 50% Lc-PRGS. In general,

IL-4 concentrations were significantly different between the same LE experimental groups at 48 and 96 hours, with a sensitive lowering observed in their concentrations in the culture media of LEs of the control group and those LEs cultured with 50% Lc-PRGS and 25% Lr-PRGS (**figure 6**).

IL-1ra concentrations were only detected at one and 49 hours in the culture medium of LEs from groups cultured with both PRGSs at the two concentrations. At these same time points, the concentrations of this cytokine were significantly (P < 0.001) different between the LE groups cultured with 25% Lc-PRGS, 50% Lc-PRGS, and both Lr-PRGSs, although there was no difference in the concentrations of this cytokine between these last two groups (**figure 7**).

IL-1ra concentrations were significantly different between the LE groups at 48 and 96 hours, with significantly (P < 0.001) higher concentrations observed in the LEs of the group cultured with 50% Lr-PRGS, followed by in those cultured with 25% Lc-PRGS. In general, IL-1ra concentrations were significantly different for the same experimental groups at 48 and 96 hours. However, we noted that, at 96 hours, the concentrations of this cytokine tended to increase
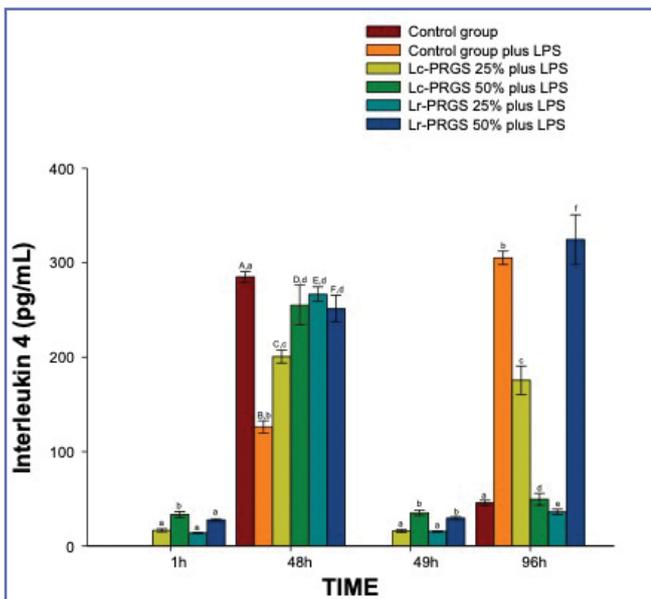

Figure 6. IL-4 concentrations observed in the culture media of LE groups at different time points. Capital letters ([A–E]) denote significant (P < 0.01) differences between the same group at 48 and 96 hours by Games–Howell test. Lowercase letters ([a–f]) denote significant (P < 0.01) differences between groups at the same time by Games–Howell test. *Denotes significant differences (P < 0.01) between the same group at one, 48, 49, and 96 hours by Games–Howell test. Data are presented as means (SDs).
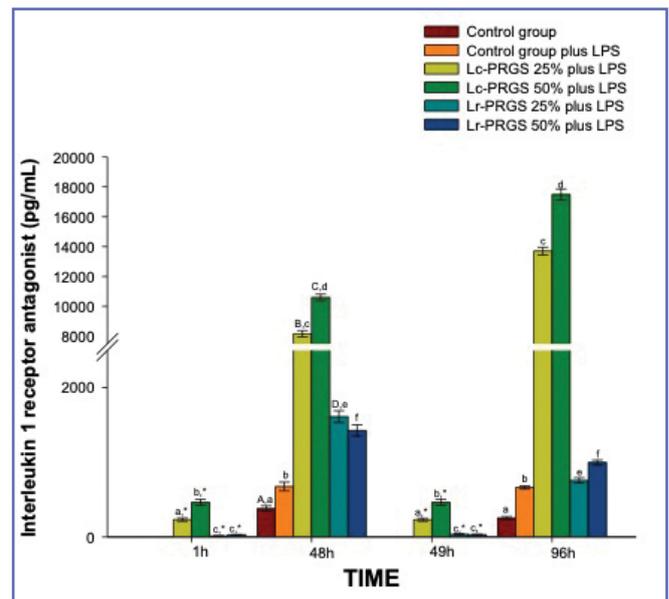
Figure 7. IL-1ra concentrations observed in the culture media of LE groups at different time points. Capital letters ([A–E]) denote significant (P < 0.01) differences between the same group at 48 and 96 hours by Games–Howell test. Lowercase letters ([a–f]) denote significant (P < 0.01) differences between groups in the same time by Games–Howell test. *Denote significant differences (P < 0.01) between the same group at one, 48, 49, and 96 hours by Games–Howell test. Data are presented as means (SDs).

in the LE groups cultured with Lc-PRGS but showed a decrease in LE groups cultured with Lr-PRGS (**figure 7**).

CORRELATIONS

At 48 hours, PDGF-BB and IL-1ra (r = 0.71, P = 0.0001) were found to be significantly correlated. At 96 hours, IL-1β and IL-4 (r = 0.80, P = 0.0001), PDGF-BB and IL-1ra (r = 0.89, P = 0.0001), and IL-1ra and TNF-α (r = 0.70, P = 0.0001) were also significantly correlated.

DISCUSSION

Lc-PRGS and Lr-PRGS at different concentrations produced mixed anti-inflammatory responses in LEs challenged with LPS mediated by either diminution in the concentrations of IL1β or TNF-α or by increasing the concentrations of IL-1ra or IL-4 in the culture media of LE groups. The anti-inflammatory effect of Lc-PRGS in LEs challenged with LPS seems to be related to higher concentrations of PDGF-BB that apparently induce a more significant release of IL-1ra and, consequently, IL-1 blockage. On the other hand, the anti-inflammatory action of Lr-PRGS could be associated with a higher release of IL-4 that possibly could diminish the release of TNF-α.

The GFs (PDGF-BB and TGF-β1) and proinflammatory (IL-1β and TNF-α) and anti-inflammatory (IL-4 and IL-1ra) cytokines evaluated in this study were selected due to their role as anabolic, proliferative, and anti-inflammatory polypeptides released from PRP or on account of how they are implicated in the catabolic and anabolic ways of musculoskeletal soft tissue degenerative pathologies [21]. PDGF-BB and TGF-β1 are two important GFs contained in PRP [11,18]. PDGF-BB promotes ligament cell (fibroblast) proliferation and increases the expression of stem cell markers while also accelerating the maturation of collagen chains [22,23], whereas TGF-β1 signaling is critical in the development of ligaments, in increasing collagen expression and synthesis [24], and in inducing stem cell differentiation to ligament cells [25. IL-1β and TNF-α are key cytokines implicated in the genesis and perpetuation of musculoskeletal degenerative/inflammatory disease, such as osteoarthritis [25], tendinopathy [21], and desmopathy [26], because they induce cell apoptosis and matrix metalloproteinase expression, which are processes that are detrimental for these connective tissues [21]. IL-4 is an anti-inflammatory cytokine that increases collagen and extracellular matrix production by fibroblasts, induces fibroblast proliferation 27, and controls chemokine production [28], whereas IL-1ra directly blocks IL-1 receptors and avoids the catabolic effects of this last cytokine in tissues. IL-1ra also enhances ligament repair by reducing the number of myofibroblasts and increasing type I procollagen synthesis during ligament injury [29].

Some of the limitations of this research were that an *in vitro* system study only can evaluate some components implicated in desmopathy. In line with this, our study only evaluated the interaction of two components: LEs and PRGS. This last component is only plasma with higher concentrations of some GFs and which is free from fibrin, platelets, and leukocytes. Thus, the basic information retrieved for this *in vitro* research could be useful to evaluate some molecular responses of LEs challenged with LPS to soluble components contained in PRGS and to design more rationale treatments to be evaluated in animal models of desmopathy or in patients with naturally-occurring disease.

The cellular receptors for LPS and IL-1β are closely interlinked and described as the toll-like receptor (TLR) IL-1 receptor (IL-1R) superfamily of receptors. When activated, they induce a series of intercellular signaling pathways that converge on the activation of transcription nuclear factor kappa B (NFκB), resulting in proinflammatory cytokine expression [30] It is important to clarify that TLR4 and TLR2 are also activated in contact with LPS [31]. Of note, the interaction between LPS and TLR4 unchain the intracellular activation of signaling complexes—such as Toll/interleukin-1-receptor-domain–containing adaptor protein (TIRAP), amongst others—which also leads to the nuclear translocation of NFkB [31,32]. Thus, other limitation of our study was that the expression of TLRs was not evaluated, which prevents us from explaining part of the mechanism of action by which PRP produced a late (96 hours) diminution in the release of proinflammatory cytokines. However, the apparent inhibitory effect of PRGS on the release of IL-1β and TNF-α by LEs challenged with LPS could represent an indirect reflex in the potential capacity of these hemoderivatives to induce TLR downregulation.

We previously performed an *in vitro* study in normal LEs and tendon explants in which we evaluated the release of the same mediators measured in this research over 48 hours [20]. The results from that study revealed that LEs cultured without PRGS produced higher concentrations of IL-1β when compared with LEs cultured with PRGS concentrations of 25% and 50%. Furthermore, we observed a diminished TNF-α release of LEs of the control group in comparison with the LEs cultured with both PRGSs at these two concentrations [20]. However, in the present study, we observed a depression in the release of these catabolic cytokines in the LE groups that were challenged with LPS over first 48 hours, with exception of the LE group that was cultured with 25% Lr-PRGS with respect to IL-1β release. In general, the two PRGSs evaluated at the two aforementioned concentrations exhibited important anti-inflammatory effects when they

were compared to the LEs of the control group challenged with LPS. However, 50% Lc-PRGS and 25% Lr-PRGS stimulated the lowest release of IL-1β versus the other hemoderivatives, having as a comparative cutoff a mean IL-1β release of LEs of control group at 96 hours. On the other hand, all hemoderivatives evaluated at 25% and 50% produced lower TNF-α concentrations in comparison to the release of this cytokine in the culture medium of LEs of control group challenged with LPS. At this time point, the better hemoderivatives were 25% and 50% Lr-PRGS, because the TNF-α release here was less than the release for this cytokine in the culture medium of LEs of the control group.

The anti-inflammatory findings from our study could be seen as contradictory to the results from other *in vitro* studies that support the use of Lr-PRP preparations, because these hemoderivatives induce scarce matrix metalloproteinase expression and lower proinflammatory cytokine release in musculoskeletal soft tissue explants than do Lc-PRP preparations (7,18). However, we believe that one of the main limitations in those studies evaluating the effects of PRP on tissue explants and cells or in animal models or clinical conditions are related with the diversity of the PRP preparations evaluated (14,15) and with the absence of the use of a standard (i.e., universal) and simple classification aimed as classifying PRP preparations according to a range of platelet and leukocyte counts. We classified the PRP preparations used in this study according to Dohan Ehrenfest et al.'s classification (16); however, not all previous authors classified their PRP preparations according to this method. For instance, the PRP preparations in the studies by McCarrel et al. (18) and Cross et al. (19) that were considered to be Lr-PRP preparations and which produced better biological effects on tissues were quite similar to our Lc-PRP preparation in the present study. In line with this, we believe that the presence of lower numbers of leukocytes concentrated in PRP could be more clinically useful that a completely leukoreduced PRP preparation or a PRP with higher leukocyte concentrations.

At 96 hours, we found a significantly higher (0.80) correlation between IL-1β and IL-4. Interestingly, we observed that higher IL-4 concentrations were also accompanied by higher IL-1β concentrations; this phenomenon was evident in the culture media of LEs of the control group plus LPS and those LEs cultured with 50% Lr-PRGS. These findings could suggest that IL-4 is produced by cell residents in LEs to counteract the catabolic effect of IL-1. Further, this anti-inflammatory cytokine is able to diminish the production of TNF-α mediated by LPS stimuli (33). Possibly, this was the reason for why 50% Lr-PRGS presented higher concentrations of IL-4 accompanied by lower TNF-α concentrations.

IL-1ra was strongly correlated with PDGF-BB in this study at both 48 and 96 hours. We have noticed that this cytokine is released from platelets and, possibly, its synthesis is stimulated in LEs by a direct effect of PDGF-BB. This fact could be corroborated in our study because LE groups cultured with Lc-PRGS presented higher PDGF-BB concentrations together with higher IL-1ra concentrations. In light of these results, both Lc-PRGSs could be more useful in treating ligament injury, because this cytokine produces a direct block of IL-1 and consequently induces collagen synthesis (29).

Finally, we observed significant correlations between some mediators evaluated in this study, these findings should be considered with caution because a correlation does not necessary mean a causal relationship. Thus, further studies are necessary to clarify the value of the correlations observed in this study, where should be included *in vivo* studies evaluating the effects of both Lc-PRP and Lr-PRP in either animal models or in human patients are necessary.

CONFLICT OF INTERESTS

All authors declare no conflict of interest.

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