

Evaluation of the Catabolic and Anabolic Gene Expression Effects and Histology Changes induced by Platelet-Rich Gel Supernatants in Equine Suspensory Ligament Explants Challenged with Lipopolysaccharide

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SUMMARY

Background. Platelet-rich plasma (PRP) has been used as treatment of ligament desmopathy. However, there is scarce *in vitro* information about the mechanism of action of leukocyte rich and leukocyte poor PRP related products. The aim of this study was to compare the anti-inflammatory, anabolic and anti-catabolic effects of a leukocyte-concentrate platelet-rich gel supernatant (Lc-PRGS) and a leukocyte-reduced PRGS (Lr-PRGS) of ligament explants (LEs) challenged with lipopolysaccharide (LPS).

Methods. LEs from 6 horses were cultured for 72 h with Lc-PRGS and Lr-PRGS at concentrations of 25 and 50%, respectively. Hyaluronic acid (HA) was measured by ELISA on culture media of experimental groups at 1 h and 72 h. At 72 h, LEs were used either for quantitative gene expression of nuclear factor kappa B (NFkB), type 1 collagen (COL1A1), cartilage oligomeric matrix protein (COMP), decorin (DCN), transforming growth factor beta 1 (TGF-β1), matrix metalloproteinase 3 (MMP3) and matrix metalloproteinase 13 (MMP13) or for histology analysis.

Results. All PRGS produced NFkB down-regulation. 25% Lr-PRGS produced up-regulation of COL1A1, DCN and TGF-β1. 25% Lc-PRGS induced MMP3 and MMP13 down-regulation in comparison to the rest of hemoderivatives. The general histology score was apparently better in LEs cultured with 25% Lr-PRGS.

Conclusions. These findings suggest that 25% Lr-PRGS is more suitable for SL desmopathy and that possibly the up-regulation of extracellular matrix (ECM) ligament anabolic genes is more important to induce SL healing than the down-regulation of ECM ligament catabolic genes. In order to determine the clinical relevance of the results found in this study, is necessary to test the effects of Lc-PRP and Lr-PRP in animal or human models. The use of Lc-PRP and Lr-PRP in desmopathy cases will be an easy-safe-low cost option for treatment.

KEY WORDS

Platelet-rich plasma; desmopathy; leukocytes; growth factors; extracellular matrix; gene expression.

BACKGROUND

The suspensory ligament (SL) (*m. interosseus medius*) is a collagen-rich tissue band containing variable amounts of muscle, which is the main component of the suspensory apparatus of the horse (1).

Anatomically, the equine hind limb SL can be compared with the human plantar fascia but biomechanically they differ because the equine SL prevents the fetlock hyperextension (2) and the human plantar fascia maintains the arch of the foot (3). However, both structures may be affected by comparable molecular mechanisms that lead to ligament inflammation and reduced functionality of the affected limb (4). This is why, studies performed in the horse with this type of musculoskeletal disorders can help the understanding of similar diseases in humans (5).

There are several medical and surgical treatments for the clinical management of horses with desmopathy, which produced variable results (1, 6). However, currently some desmopathy cases have been treated with diverse “regenerative” therapies that include the use of gene therapy (7), adult stem cells (8), bone marrow and several PRP related products (9, 10). To note, PRP treatment presents some technical and economical advantages in comparison with the other aforementioned regenerative therapies, which makes it more frequently used by equine practitioners (11). PRPs can be classified according the concentration of leukocytes (WBCs) in leukocyte-concentrated PRP (Lc-PRP) or leukocyte-reduced PRP (Lr-PRP). Lc-PRP presents detectable numbers of leukocytes (WBC) in relation to basal cell counts in whole blood, whereas Lr-PRP exhibits negligible WBC counts with respect to basal cell counts in whole blood (12). Once PRP preparations are mixed with calcium salts or thrombin, they polymerize in a platelet-rich gel (PRG) (12) and release a supernatant (PRGS) rich in growth factors (GFs) and cytokines, and other proteins that regulate wound healing and inflammation (13).

There are *in vitro* studies that suggest that Lr-PRPs could be more suitable for the clinical management of tendinitis than Lc-PRPs because of lower platelet and WBC concentrations that may induce less tissue catabolism/inflammation and more tissue anabolism (14). However, to the authors’ knowledge, this hypothesis has not been proved to be true in equine ligament explants (LEs).

Some *in vitro* studies evaluated several mechanisms related with the anabolic and anti-inflammatory effect of PRP on SL, in either fibroblasts (15) or explants (16). In general, in SL fibroblasts, PRP stimulated the release of COMP and increased the capacity of protein synthesis of these cells (15). On the other hand, PRP induced COL1A1, DCN and COMP gene up-regulation without inducing MMP-13 gene

expression, in suspensory LEs (16). However, is necessary to obtain further information that includes the evaluation of Lc-PRP and Lr-PRP at several concentrations in an *in vitro* system of ligament inflammation, because there is limited *in vitro* data about the response of SL under experimental inflammatory conditions.

In line with this, we propose a study in which equine LEs were challenged with lipopolysaccharide (LPS) and cultured with Lc-PRGS and Lr-PRGS at 25% and 50% over 72 h. We measured and compared the release of HA, the quantitative expression of some pro-inflammatory NFkB, extracellular matrix (ECM) anabolic (COL1A1, COMP, DCN and TGF-β1) and catabolic (MMP3 and MMP13) genes, which are potentially implicated in desmopathy. Furthermore, we evaluated by conventional histology whether PRGS could produce some structural protective effect in LPS-affected LEs.

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 SL from the hind limbs of 6 horses, 4 to 7 years of age, were included in this study. The samples were taken from animals apparently free from musculoskeletal disease and euthanized by a pentobarbital intravenous overdose for other medical reasons. All the hind limbs of the horses were radiographed and ultrasonographically evaluated for excluding animals with desmopathy associated changes.

Horses and blood collection and processing

Six clinically healthy horses (3 gelding and 3 mares), between 6-9 years of age were used as blood donors. All the horses were stabled, fed and managed in a similar manner. These animals were selected after an extensive physical examination together with a complete blood cell count and a general clinical chemistry panel. Only clinically healthy horses with platelet counts higher than $100 \times 10^3/\text{mL}$ were used.

Lc-PRP/Lc-PRGS and Lr-PRP/Lr-PRGS preparation

Whole blood from each horse was obtained by jugular puncture using a 21G butterfly catheter. Both PRPs were obtained through a manual double centrifugation tube method that was previously validated and used clinical-

ly in horses with SL desmopathy (17). Blood was stored in eight, 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 centrifuged at 240 g for five minutes, and then the bottom fourth of the fraction was collected. This fraction was considered as Lc-PRP. The upper plasma fraction was considered as Lr-PRP (**figure 1**). Whole blood and both PRP products were analyzed for platelet and leukocyte counts (Celltac- α MEK 6450, Nihon Kodhen, Japan).

Both PRPs were activated with calcium gluconate (Ropsohn Therapeutics Ltda®, Bogotá, Colombia) (ratio 1:10) and incubated at 37 °C for 1 h until clot retraction occurred. Aliquots of both PRGS obtained were frozen at - 80 °C for later quantification of platelet-derived growth factor BB (PDGF-BB) and TGF- β 1.

Ligament explant culture

SL samples were obtained aseptically and sectioned with a scalpel blade in rectangular (5 x 3 x 3 mm) explants with 70 \pm 4 mg of weight. A total of 36 LEs were obtained from each

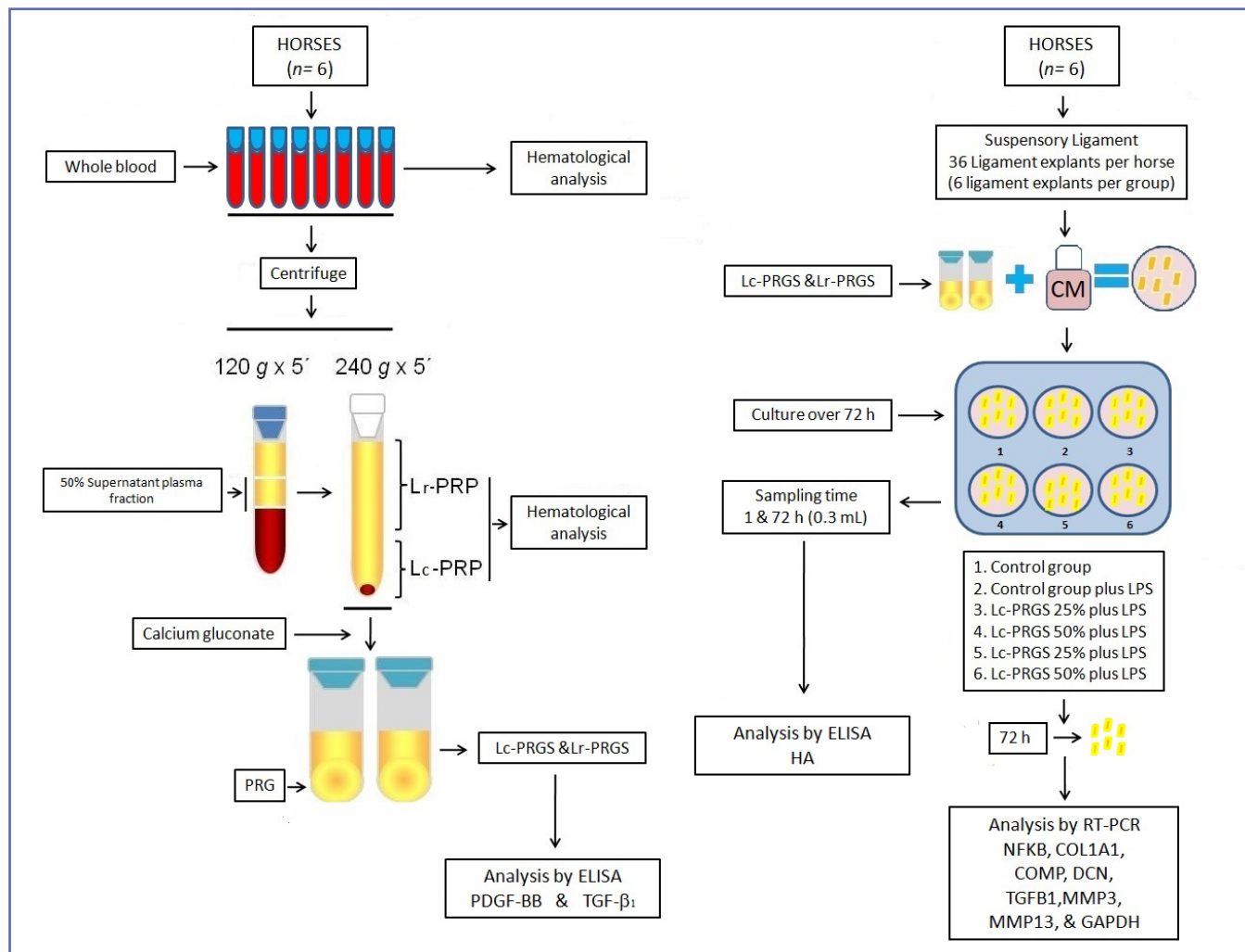


Figure 1. Schematic representation of the study's design and methodology. Lc-PRP, leukocyte-concentrated platelet-rich plasma; Lr-PRP, leukocyte-reduced platelet-rich plasma; PRG, platelet-rich gel; Lc-PRGS, leukocyte-concentrated platelet-rich gel supernatant; Lr-PRGS, leukocyte-reduced platelet-rich gel supernatant; PDGF-BB, platelet-derived growth factor BB; HA, hyaluronic acid, TGF- β 1, transforming growth factor beta 1; COMP, cartilage oligomeric matrix protein; COL1A1, type 1 collagen; DCN, decorin; MMP-3 & -13, matrix metalloproteinase 3 and -13; NFKB, nuclear factor kappa B; CM, culture medium.

horse. All the explants were washed in phosphate-buffered saline and stabilized 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% CO₂ and water saturated atmosphere for 24 h and then replaced with fresh culture media. At this time point, a part of the tissue samples was challenged with 100 ng/mL of LPS (Sigma-Aldrich, St Louis, MO, USA) to induce an inflammatory/catabolic damage of LE.

Study design

Six experimental groups (with 6 explants each one), were included. Tissue cultures were performed in 6 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 2 LE control groups (1 with addition of LPS and 1 without LPS) without addition of PRGS and 4 LE groups cultured with Lc-PRGS and Lr-PRGS at 2 concentrations, 25% and 50%. All SLE groups were cultured at 72 h. Samples of culture media (0.3 mL) were obtained at 1 h and 72 h, aliquoted and frozen at - 80 °C for later determination of HA.

Furthermore, LEs were deposited in buffered formaldehyde for histological analysis or in an RNA conserving solution (RNAlater, Life Technologies, Carlsbad, CA, USA) for quantitative gene expression of NFκB, DCN, COL1A1, COMP, TGF-β1, MMP3, MMP13, and GAPDH (glyceraldehyde 3-phosphate dehydrogenase), the primers were designed and validated by us, with the support in the Colombian Centre for Bioinformatics and Computational Biology (BIOS Manizalez-Minciencias). **Figure 1** summarizes the study, design and methodology.

Growth factors and HA measurement

PDGF-BB and TGF-β1 concentrations were measured in Lc-PRGS and Lr-PRGS, whereas HA concentrations were measured in culture media of SLE groups obtained at 1 h and 72 h. These molecules were determined via ELISA in 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-β1 (Human TGF-β1 DuoSet, DY240E) concentrations were determined using human antibodies because there is a high sequence homology between these proteins in

humans and horses. Furthermore, similar ELISA antibodies have been used for the same purposes in other equine PRP studies. HA (Hyaluronan, DuoSet, DY3614) was determined using a multispecies detection ELISA kit. Standards provided for each ELISA kit were used to construct each standard curve according the manufacturers' instructions. Absorbance readings were performed at 450 nm.

Histology evaluation

The LEs samples were dehydrated in serial alcohol concentrations, fixed in wax blocks, cut at 3 µm of thickness and stained with either haematoxylin and eosine (H & E) or alcian blue stains. The first stain was used to evaluate the roundness of the nuclei of the ligament fibroblasts, cell density, vascularization, and the pattern of the collagen fibers. The alcian blue stain was used to determine the deposition of ground substance in the ligaments. All of the preparations were analyzed using a light microscope (Zeiss®, Axio Imager, Upright Microscope) in a 40x magnification by a modified Bonar's semi-quantitative score (**table I**).

Five histological evaluated parameters were used: 1 roundness of the nuclei of the ligament fibroblasts, 2 cell density, 3 ground substance, 4 collagen fibers arrangement, and 5 vascularity. These variables were quantified using a 0–3 scale, with 0 being normal and 3 being abnormal (**table I**). Thus, a totally normal ligament would score 0 and a maximally abnormal ligament would score 15. Three sections randomly selected from each sample were blindly evaluated and the average score was used for comparison.

Relative Gene expression measurement

SLEs were pulverized in liquid nitrogen and mixed with TRIzol reagent (Life Technologies, Carlsbad, CA, USA) for 5 min. The samples were centrifuged for 10 minutes at 10.000 g, and the supernatant was mixed with 20% chloroform (volume/volume) and then centrifuged for 15 minutes at 12.000 g. The aqueous phase of the samples was removed and transferred to special columns (PureLink RNA Mini Kit, Life Technologies, Carlsbad, CA) for RNA extraction according to the manufacturer's instructions. RNA concentrations were measured using a spectrophotometer (Nanodrop 2000, Thermo Scientific, Wilmington, DE, USA). Samples were diluted to a concentration of 5 ng/mL of RNA.

The samples were assayed in duplicate for quantitative gene expression levels in a quantitative RT-PCR device (StepOnePlus Real-Time PCR System, Life Technologies, Carlsbad, CA, USA) using a SuperScript III platinum SYBR Green One-Step qRT-PCR kit (Life Technologies, Carlsbad,

Table I. Bonar's modified semi-quantitative score for suspensory ligament lesion determination.

Structure	Grade			
	0	1	2	3
Ligament fibroblasts	Inconspicuous elongated spindle shaped nuclei with no obvious cytoplasm at light microscopy.	Increased roundness: the ovoid nucleus becomes more round with clear cytoplasm.	Increased roundness and size; the nucleus is round, slightly enlarged and a small amount of cytoplasm is visible.	Nucleus is round, large with abundant cytoplasm and lacuna formation (chondroid change).
Cell density	Normal pattern.	Slightly increase.	Moderately increase.	Severely increase.
Ground substance (Alcian blue stain)	No stainable ground substance.	Stainable mucin between fibers but bundles still discrete.	Stainable mucin between fibers with loss of clear demarcation or bundles.	Abundant mucin through-out with inconspicuous collagen staining.
Collagen (with and without polarized light)	Collagen arranged in tightly cohesive well demarcated bundles with a smooth dense bright homogeneous polarization pattern with normal crimping.	Diminished fiber polarization; separation of individual fibers with maintenance of demarcated bundles.	Bundle changes; separation of fibers with loss of demarcation of bundles giving rise to expansion of the tissue overall and clear loss of normal polarization pattern.	Marked separation of fibers with complete loss of architecture.
Vascularity	Inconspicuous blood vessels coursing between bundles.	Occasional cluster of capillaries, less than 1 per 10 high power fields.	1-2 clusters of capillaries per 10 high power fields.	Greater than 2 clusters per 10 high power fields.

CA, USA). Primers for NFkB, COL1A1, COMP, DCN, TGF-β1, MMP3, MMP13, and GAPDH were designed and validated by us, using PrimerBlast and duplicated whit primer3 (**table II**). The relative change in gene expression

was determined via the comparative $2^{-\Delta\Delta CT}$ method. GAPDH was used as the internal control (housekeeping gene), and LEs samples from all horses that were not incubated with any treatment were used as reference samples.

Table II. Genes and sequence of primers evaluated in the study.

Targeted genes	Primer sequences (5' --> 3')	Product size (bps)
GAPDH, glyceraldehyde-3-phosphate dehydrogenase	Forward TCCCGCCAACATCAAATGGGG Reverse TTCAAGTGAGCCCCAGCCTTCTC	105
NFKB, nuclear factor of kappa light polypeptide gene enhancer in B-cells-like	Forward AAGCTGGCTGGTGGGGACTTG Reverse TCCTTCGCCTCTGCTGTGCTG	128
COL1A1, collagen, type I, alpha 1	Forward ACGCTGCCCTTTCTGCTCCTTTC Reverse TTTGGTTGCTGGGCAGACAATGC	118
COMP, cartilage oligomeric matrix protein	Forward GGCGTGGGGATGGCCTTTG Reverse ACGGAGTTGGGGACGCAGTTATG	95
TGF-β1, transforming growth factor beta 1	Forward CGTGGGCATCAACGTAGGGTTC Reverse GCCATGAGGAGCAGGAAGGG	90
MMP 13, matrix metalloproteinase 13	Forward AGCGAACTCCGTGGTTGACAGG Reverse ACCCACATCAGGGACCCAC	127
MMP 3, matrix metalloproteinase 3	Forward CGGGTCCGCCTTTCTCAAGATG Reverse GGCTCCACGGGGCTATCAGG	86

STATISTICAL ANALYSIS

The Shapiro–Wilk test was used to assess the fit of the data set to a normal distribution (goodness of fit). All the parameters evaluated, except LE histology, demonstrated a normal distribution ($p > 0.05$). Platelet and WBC counts in whole blood and both PRP preparations were compared with a one-way analysis of variance (ANOVA), followed by a *post hoc* Tukey test. PDGF-BB and TGF- β 1 concentrations from both PRG supernatants were compared using a t-non-paired test.

A generalized lineal model (GLM), followed (when necessary) by a Tukey test, was used for comparing HA concentrations in culture media (at 1 h and 72 h) and gene expression at 72 h. Semi-quantitative histology analysis was evaluated by a Kruskal–Wallis test, followed (when necessary) by a Dunn test. A p -value < 0.05 was accepted as statistically significant for all tests. A $p < 0.05$ was accepted as statistically significant for all tests.

RESULTS

Cell concentration in whole blood, Lc-PRP, and Lr-PRP

Platelet counts were significantly ($p < 0.05$) different between whole blood and both PRPs. Lc-PRP presented

the highest platelet concentration followed by whole blood and Lr-PRP (**figure 2 A**). Leukocyte 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 (**figure 2 B**).

Concentration of growth factors PRGS

PDGF-BB and TGF- β 1 concentrations were significantly ($p < 0.01$) higher in Lc-PRGS when compared to Lr-PRGS (**figure 3 A, 3 B**).

Concentration of HA in culture media of LEs at 1 h and 72 h

At 1 h, HA concentrations were significantly ($p < 0.01$) higher in SLE groups cultured with both PRGS at 50% when compared with the same hemoderivatives at 25%. To note, HA was not detected at this time in SLEs from both control groups (**figure 4**). At 72 h, HA was significantly ($p < 0.01$) higher in culture medium of all LE groups when compared to the same experimental group at 1 h. On the other hand, at 72 h LEs of the control group without LPS showed a significant lower HA release in comparison to the LE control LPS-challenged group and those LEs cultured with 50% Lr-PRGS. To highlight, LEs cultured with 25%

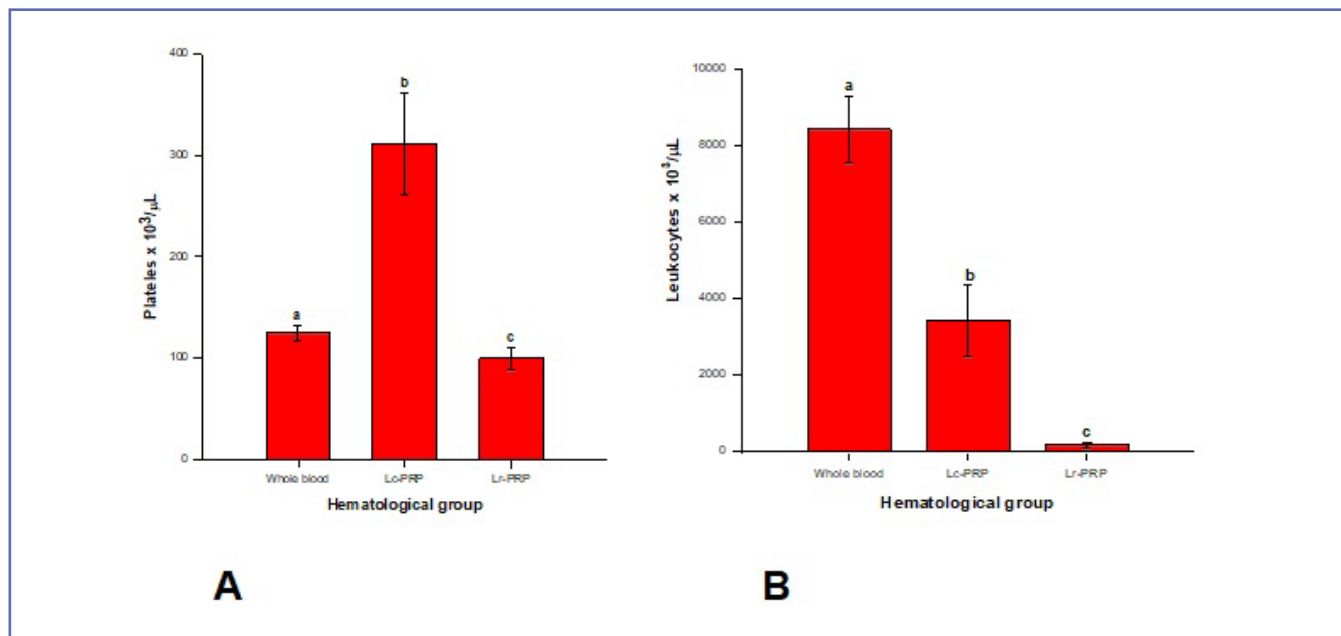


Figure 2. A) Mean (standard deviation (s.d)) platelet counts ($\times 10^3/\mu\text{L}$) in whole blood, Lc-PRP and Lr-PRP. **B)** Mean (s.d) leukocyte counts (μL) in whole blood, Lc-PRP and Lr-PRP. ^{a-c} = Different lower case letters denote significant differences ($p < 0.05$) between hemoderivatives by the Tukey test. Acronyms meaning as in figure 1 legend.

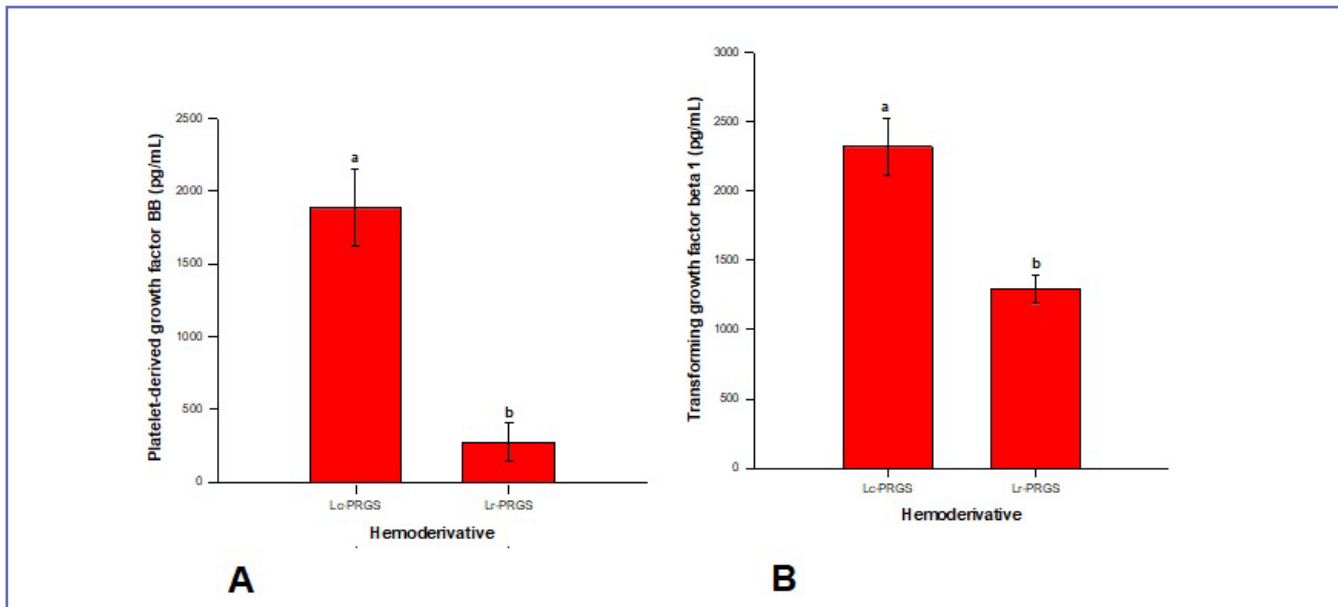


Figure 3. A) Mean (s.d.) PDGF-BB concentration (pg/mL) in Lc-PRGS and Lr-PRGS. **B)** Mean (s.d.) TGF-β1 concentration (pg/mL) in Lc-PRGS and Lr-PRGS. ^{a-b} = different lowercase letters denote significant differences ($p < 0.05$) by the t-test. Acronyms meaning as in figure 1 legend.

Lr-PRGS significantly ($p < 0.01$) released lesser HA when compared to both control groups and those SLEs cultured with 25% Lc-PRGS and 50% Lr-PRGS (figure 4).

Relative gene expression

NFκB was significantly ($p < 0.05$) up-regulated in LEs of the control group when compared to the LE groups cultured with both PRGS at two concentrations. There were no significant differences for the expression of this gene between both control groups; however, LEs of the control group showed a lower NFκB relative expression in comparison to LEs of the control group plus LPS (figure 5 A).

COL1A1 was significantly ($p < 0.05$) up-regulated in LEs of the control group and those cultured with 25% Lr-PRGS in comparison to the rest of the LE groups (figure 5 B). On the other side, COMP was significantly up-regulated in LEs from the control group when compared with the rest of LE groups. LEs cultured with 50% Lc-PRGS exhibited a significant COMP up-regulation when compared to LEs of the control group plus LPS and those cultured with 25% Lc-PRGS and Lr-PRGS at two concentrations (figure 6 A). DCN was significantly ($p < 0.05$) up-regulated in LEs of the control group and those cultured with 25% Lr-PRGS when compared to LEs of the control group plus LPS and those cultured with 50% Lc-PRGS. The expression for this gene was not different between LEs cultured with 25%

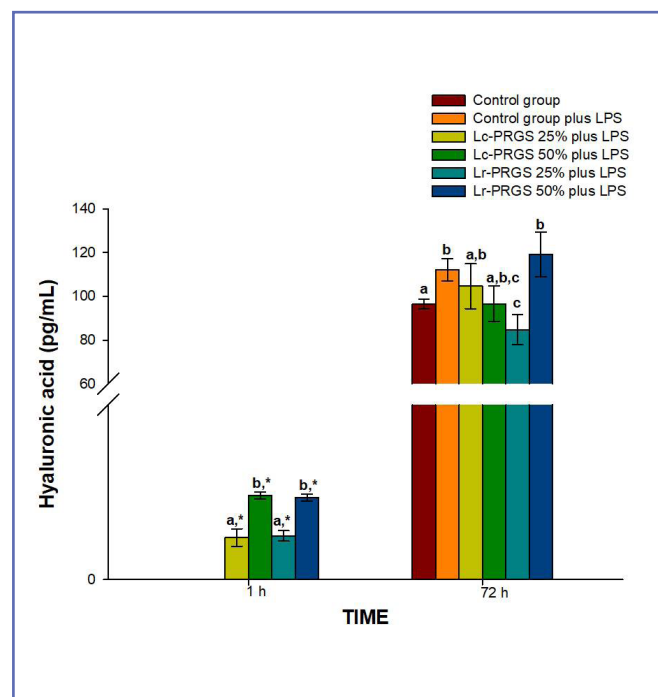


Figure 4. Mean (s.d.) HA concentrations (pg/mL) in culture media of suspensory ligament explant (SLE) experimental groups at 1 h and 72 h. ^{a-c} = different lowercase letters denote significant ($p < 0.01$) differences in SLE experimental groups by the Tukey test. Acronyms meaning as in figure 1 legend.

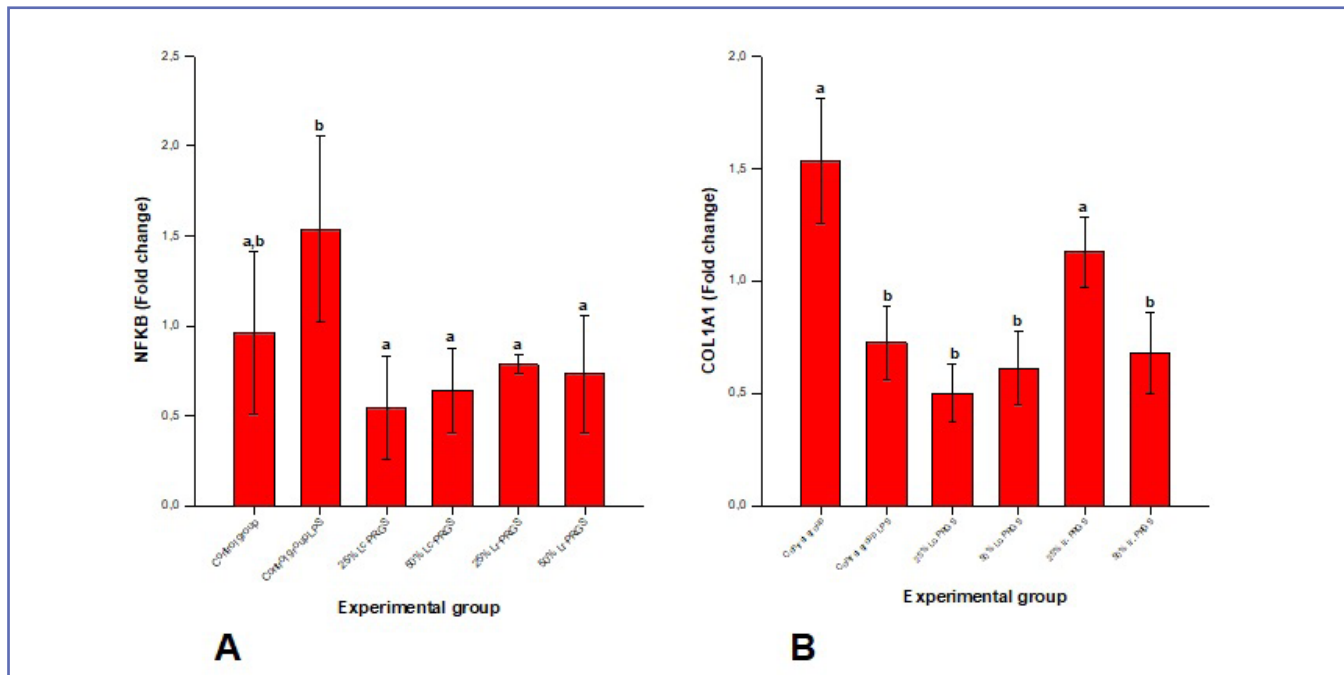


Figure 5. A) Mean (s.d) NFKB relative gene expression (fold change) in SLE experimental groups at 72 h. **B)** Mean (s.d) COL1A1 relative gene expression (fold change) in SLE experimental groups at 72 h. ^{a-c} = different lowercase letters denote significant ($p < 0.01$) differences in SLE experimental groups by the Tukey test. Acronyms meaning as in figure 1 legend.

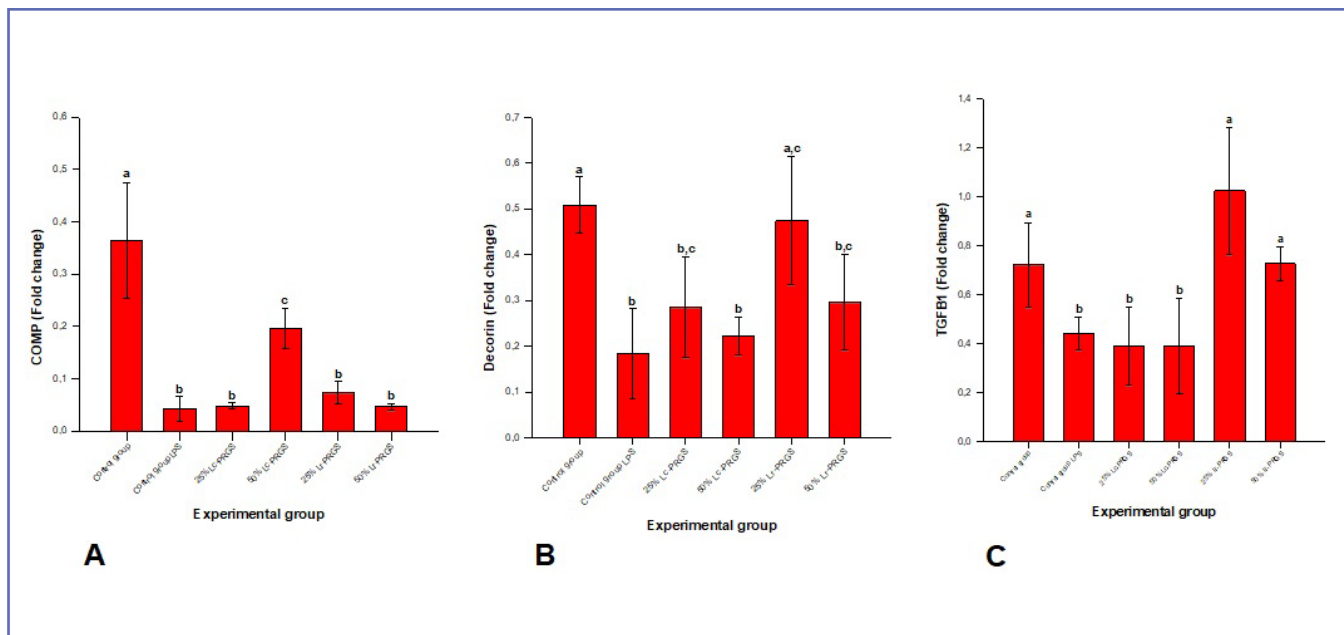


Figure 6. A) Mean (s.d) COMP relative gene expression (fold change) in SLE experimental groups at 72 h. **B)** Mean (s.d) DCN relative gene expression (fold change) in SLE experimental groups at 72 h. **C)** Mean (s.d) TGF- β 1 relative gene expression (fold change) in SLE experimental groups at 72 h. ^{a-c} = different lowercase letters denote significant ($p < 0.01$) differences in SLE experimental groups by the Tukey test. Acronyms meaning as in figure 1 legend.

Lc-PRGS, 25% Lr-PRGS and 50% Lr-PRGS (**figure 6 B**). TGF- β 1 relative expression was not different between LEs of the control group and those cultured with Lr-PRGS at both concentrations. However, the expression for this gene in these three experimental groups was significantly ($p < 0.05$) higher when compared to LEs of the control group plus LPS and those cultured with Lc-PRGS at two concentrations (**figure 6 C**).

MMP3 was significantly ($p < 0.01$) up-regulated in LEs cultured with 50% Lc-PRGS and Lr-PRGS at two concentrations when compared to LEs cultured 25% Lc-PRGS and LEs of both control groups (**figure 7 A**). Moreover, 25% Lr-PRGS induced a significantly ($p < 0.01$) highest MMP13 relative expression in LEs when compared to the rest of LE groups. The relative expression of this gene was not different between LEs cultured with both PRGS at 50%. However, these last two LE groups exhibited a significantly (**figure 7 A**) MMP13 up-regulation in comparison to LEs of both control groups and those cultured with 25% Lc-PRGS (**figure 7 B**).

Histology evaluation

PRGS at two concentrations did not affect the score for ligament fibroblasts cell density, and vascularity (**table III**). However, LEs of the control group exhibited significantly ($p < 0.05$) better (lesser) score for ground substance deposition and collagen pattern orientation. Nevertheless, the total histology score evaluation was similar for LEs of the control group and those cultured with both PRGS at two concentrations. In contrast, LEs of the control group plus LPS showed a significantly ($p < 0.05$) worst total histology score in comparison with the rest of LE groups (**figure 8** and **table III**).

DISCUSSION

All PRGS at two concentrations evaluated in this study produced NF κ B down-regulation, which could indicate that they are anti-inflammatory hemoderivatives. Lr-PRGS, particularly, 25% Lr-PRGS produced an intense anabolic effect in LPS-challenged LEs manifested by up-regulation of COL1A1, DCN and TGF- β 1. Lc-PRGS, particularly,

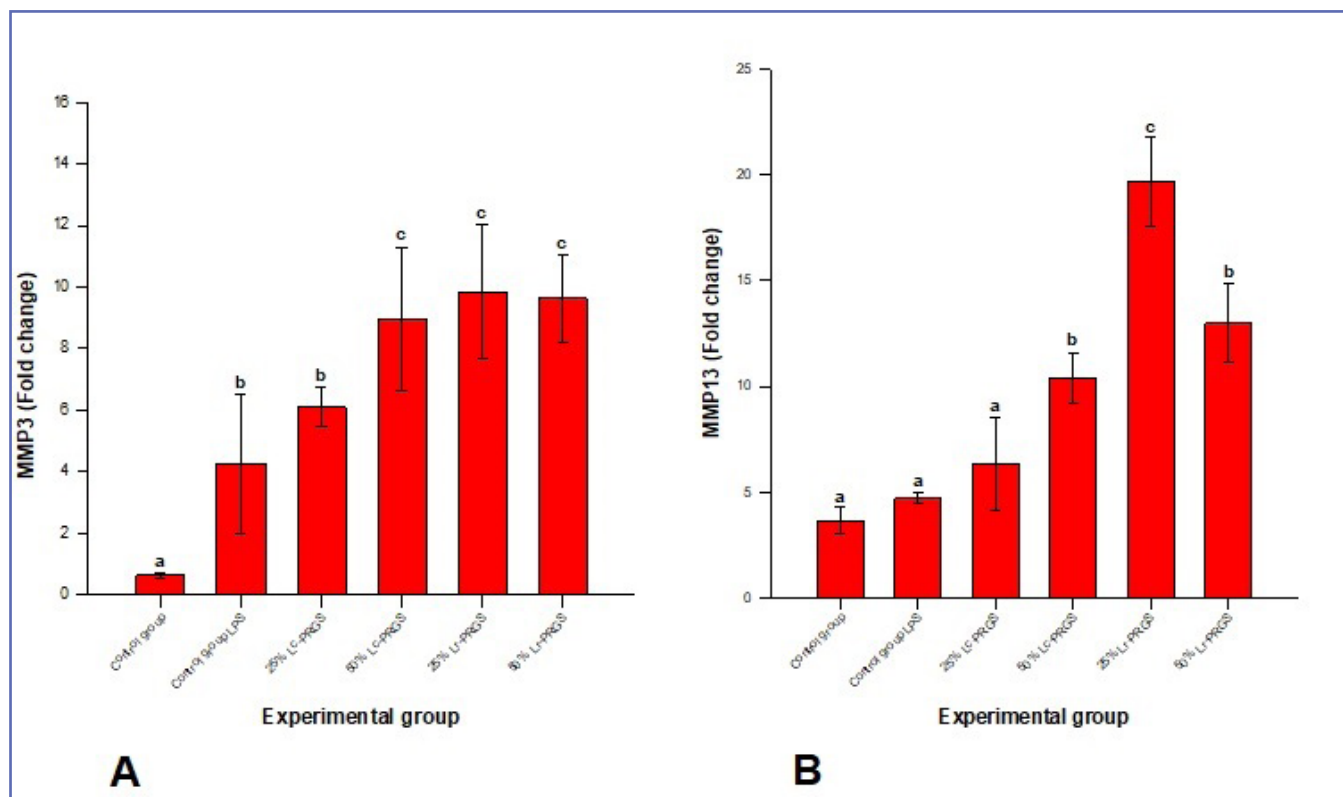


Figure 7. A) Mean (s.d) MMP3 relative gene expression (fold change) in SLE experimental groups at 72 h. **B)** Mean (s.d) MMP13 relative gene expression (fold change) in SLE experimental groups at 72 h. ^{a-c} = different lowercase letters denote significant ($p < 0.01$) differences in SLE experimental groups by the Tukey test. Acronyms meaning as in figure 1 legend.

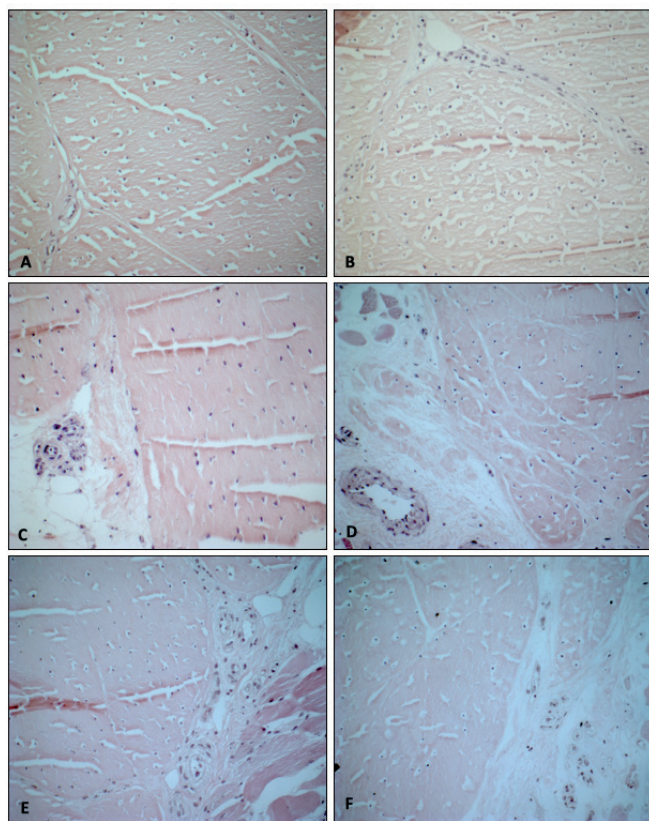


Figure 8. Suspensory ligament explant (SLE) histology slides (H&E 40X). **A)** Transversal section of a SLE of the control group showing normal orientation of the collagen fibers and presence of fibroblasts with elongated nuclei. **B)** Transversal section of a SLE of the control group plus LPS showing increase in the fibroblast number with roundness of their nuclei and loss of the organization of collagen fibers. **C)** Transversal section of a LPS-challenge SLE of the group cultured with 25% Lc-PRGS showing increase in the fibroblast number with roundness of their nuclei. **D)** Transversal section of a LPS-challenge SLE of the group cultured with 50% Lc-PRGS showing increase in the fibroblast number with roundness of their nuclei and loss of the organization of collagen fibers, although in lesser extension than the SLEs of the control group plus LPS. **E)** Transversal section of a LPS-challenge SLE of the group cultured with 25% Lr-PRGS showing fibroblasts with slight changes in the roundness of their nuclei. **F)** Transversal section of a LPS-challenge SLE of the group cultured with 50% Lr-PRGS showing fibroblasts with slight changes in the roundness of their nuclei and slight loss of collagen pattern, although in lesser extension than the SLEs of the control group plus LPS.

Table III. Histology score results for suspensory ligament treated with Lc-PRGS and Lr-PRGS in presence of LPS. * = Data are presented as medians (interquartile range).

Experimental group	Histology item					
	Ligament fibroblasts	Cell density	Ground substance (Alcian blue stain)	Collagen (with and without polarized light)	Vascularity	Total histology score
Control group	1.0 (0.0)	1.0 (0.0)	0.0 (0.0) ^a	0.0 (0.0) ^a	0.50 (1.0)	3.0 (2.0) ^a
Control group LPS	1.0 (0.0)	0.50 (1.0)	1.0 (0.0) ^b	1.0 (0.0) ^b	1.0 (1.0)	10 (5.0) ^b
25% Lc-PRGS	1.0 (0.0)	0.0 (1.0)	0.0 (1.0) ^b	0.0 (0.0) ^b	1.0 (1.0)	6.0 (2.0) ^a
50% Lc-PRGS	1.0 (0.0)	0.0 (0.0)	0.0 (1.0) ^b	0.0 (0.0) ^b	1.0 (1.75)	4.0 (2.0) ^a
25% Lr-PRGS	1.0 (0.0)	0.0 (1.0)	0.0 (0.0) ^b	0.0 (1.0) ^b	0.0 (1.0)	4.0 (2.0) ^a
50% Lr-PRGS	1.0 (0.0)	0.0 (1.0)	0.0 (0.0) ^b	0.0 (1.0) ^b	1.0 (1.0)	4.0 (2.0) ^a

^{a,b} = different lowercase letters in the same column represent significant differences ($P > 0.05$).

25% Lc-PRGS induced MMP3 and MMP13 down-regulation in comparison to the rest of hemoderivatives. However, the general histology score was apparently better in LEs cultured with 25% Lr-PRGS.

This study presents new and complementary information about the anti-inflammatory, anabolic, and anti-catabol-

ic gene expression mechanisms of action of PRGS on an *in vitro* system of equine SL desmitis/desmopathy. Additionally, it describes the protective effects of PRGS on the histology of LEs challenged with LPS. The analytes (PDGF-BB, TGF- β 1 and HA) and genes evaluated in the present study were selected because these are the main polypeptides

(PDGF-BB and TGF- β 1) contained in equine PRP (18) or because of their role as pro-inflammatory (NF κ B), anabolic (COL1A1, COMP, DCN, TGF- β 1) and catabolic (MMP3 and MMP13) genes implicated in the pathophysiological mechanisms of musculoskeletal soft tissue in degenerative/inflammatory conditions, such as equine SL desmitis/desmopathy (1, 16).

PDGF-BB is an anti-inflammatory and angiogenic polypeptide that induces fibroblast proliferation and adhesion (19, 20). This GF has been useful for experimental ligament healing (21). TGF- β 1 enhances ligament repair by stimulating matrix proteins deposition as well as the proliferation and migration of cells (22). This polypeptide produces up-regulation of lysyl oxidases and MMPs that respectively facilitate the extracellular matrix (ECM) ligament repair and degradation (23).

HA is a high-molecular-weight polymer of disaccharide units that enhances the cellular activities of fibroblasts, including their adhesivity, proliferation, and ECM synthesis (24). It could constitute around 20% or more of the total glycosaminoglycans in tendons and ligaments (25). There are several genes encoding protein synthesis in ligament ECM, such as COL1A1, COMP, DCN, TGF- β 1 that maintain the healthiness of this tissue by an equilibrated counterbalance of catabolic genes that encode MMPs, such as MMP3 and MMP13. However, in pathologic conditions (inflammation/degeneration) resident cells in ligament ECM could over-express pro-inflammatory genes, for instance NF κ B that up-regulates inflammatory and catabolic genes like MMP3 and MMP13 with subsequent damage of structural components of ECM (26).

The *in vitro* system used in this study can only evaluate some components involved in desmopathy. Therefore, only two components were evaluated here: LEs and PRGS. The latter is only fibrin-platelet-leukocyte free plasma with higher amounts of GFs. Thus, these *in vitro* experiments are useful for the evaluation of LEs molecular responses when challenged with LPS solutions containing PRGS, and consequently will give more information to design and test treatments that can be used later in animal models of desmopathy or patients with the disease.

We used LPS in this *in vitro* study to induce ligament inflammation/degeneration because the cellular receptors for LPS and catabolic cytokines (mainly IL-1 β) implicated in the genesis and perpetuation of degenerative soft tissue musculoskeletal disease (26) are closely interlinked and described as the TLR-IL-1 receptor (IL-1R) superfamily of receptors (27). When activated they induce a series of intercellular signaling pathways that converge on the activation of the transcription factor NF κ B resulting in pro-inflammatory cytokine and MMP expression (28). Thus, other limita-

tion of our study was that the expression of TLRs was not evaluated.

PRGS used in this study come from two different PRP compositions, one of them (Lc-PRP) with a platelet concentration 3-fold and a leukocyte concentration 0.5-fold in comparison to basal values of these components or cells in whole blood. The other hemoderivative (Lr-PRP) exhibited a platelet concentration 0.9-fold and a negligible leukocyte (0.1-fold) concentration when compared to basal values of these cells in whole blood. Therefore, we also obtained PRGS with different concentrations of GFs, particularly, PDGF-BB and TGF- β 1. Indeed, Lc-PRGS presented the highest concentrations of both polypeptides when compared to Lr-PRGS. However, both types of PRGS exhibited different anti-inflammatory, anabolic and catabolic gene expression responses and protective effect of the histology architecture in LEs challenged with LPS.

Both PRGS exhibited an anti-inflammatory effect in LPS-challenged LEs because they down-regulated NF κ B gene expression when compared to LEs of the control group plus LPS. This anti-inflammatory effect has also been observed previously in other musculoskeletal tissues and cells (29, 30) and it has been associated with an inhibitory effect of some GFs, such as hepatocyte growth factor or PDGF-BB contained in PRP (31).

In general, LPS induced a strong depression in the anabolic gene expression profile of the LE evaluated in this study, affecting markedly the release of HA, which was lower in 25% Lr-PRGS. At this point, the inclusion of a control ("healthy") group without LPS addition was useful to determine the extension of PRGS control of catabolic effect after this harmful biologic stimulus. In line with this, COL1A1 relative expression only was up-regulated in 25% Lr-PRGS. This finding has been previously observed in an *in vitro* study in tendons in which the increase in the platelet concentration resulted in a significant reduction in COL1A1 gene expression in tendons (32). Additionally, there are other similar reports where they used PRP methods that minimized the amounts of leukocytes (PRP releasate) *in vitro* and *in vivo*. The *in vitro* results showed that treating tendon stem/progenitor cells with PRP releasate induced stem/progenitor cells differentiation into active tenocytes, which proliferated quickly and produced abundant collagen (33). We also observed in an animal model of tendinopathy that Lr-PRP produces COL1A1 up-regulation (34).

Interestingly, 25% Lr-PRGS also produced the most significant DCN and TGF- β 1 up-regulation in SLEs challenged with LPS. To note, the most significant COMP up-regulation in LPS-challenged LEs was observed for 50% Lc-PRGS. It is possible that the increased concentrations of GFs (especially PDGF-BB) in this hemoderivative (16)

induced up-regulation of this ECM anabolic gene, similar to what was observed in equine cartilage (35).

Our catabolic gene expression profile results contrast with other studies. In this sense, we observed that 25% Lc-PRGS induced lesser MMP3 and MMP13 expression when compared with 50% Lc-PRGS and Lr-PRGS (both concentrations). However, the MMP3 gene expression in LPS-challenged SLEs cultured with 25% Lc-PRGS were up-regulated when compared to the LEs of the control group, whereas the level of expression for MMP13 was not different between LEs cultured with 25% Lc-PRGS and those of both control groups. In contrast, two *in vitro* studies reported MMP13 down-regulation in equine tendon explants independently of the concentration of platelets and leukocytes in PRP (14, 32). Moreover, our results on MMP3 gene expression were similar to those described by Boswell et al., who found that several PRP preparation with different platelet and leukocyte concentration ratio were able to induce up-regulation of this ECM enzyme (32).

The histology results from our study demonstrated that LEs challenged with LPS were structurally damaged and that both PRGS (25 and 50%) avoided tissue damage when compared to the control groups (healthy and LPS-stimulated SLEs). Although, there was no significant difference between both PRGS (25 and 50%), we can appreciate that 25% Lr-PRGS exhibited the better ligament histology architecture and interestingly, this same concentration showed the lowest release of HA in both moments measured. In addition, SLEs cultured with 25% Lc-PRGS followed by 50% Lc-PRGS presented a worst histology appearance. As previously mentioned, the information about the histology effects of PRP on equine SL is limited. Our histology results could be similar to those described for rabbits with Achilles tendinopathy that were treated with Lr-PRP (34).

These findings suggest that 25% Lr-PRGS is more suitable for SL desmopathy and that possibly the up-regulation of ECM ligament anabolic genes is more important to induce SL healing than the down-regulation of ECM

catabolic genes. Additionally, the exact leukocyte formula is an important parameter since lymphocytes populations are very diverse and do not have the same impact than the monocytes and granulocytes. Then, it is still unclear how to improve significantly the current classification and terminology but this is something interesting for future studies (36). Previously we described a possible anti-inflammatory action of Lr-PRGS that could be associated with a high release of IL-4, which reduces TNF- α release. This could be the case for this situation (37).

PRP is likely a promising treatment to enhance the healing of injured tendons but the factors mentioned above should be considered in clinical practice and research (33). Consequently, to determine the significance of the findings from this study, further studies using *in vivo* models (animal or human) and Lc-PRP and Lr-PRP are needed.

There are many studies and techniques that demonstrate the usefulness in terms of safety and ease of PRP application in musculoskeletal injuries (38) but there is still controversy and variability regarding the amount and types of PRP (33). In the meantime, it is important that all authors in the field accurately describe the products they are testing, in order to make a real and meaningful contribution to the literature (36) and improve the efficiency and results of PRP use in desmopathies (37).

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests (39).

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