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Ultrastructure and growth factor content of equine platelet-rich fibrin gels

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Objective—To compare fiber diameter, pore area, compressive stiffness, gelation properties, and selected growth factor content of platelet-rich fibrin gels (PRFGs) and conventional fibrin gels (FGs).

Sample—PRFGs and conventional FGs prepared from the blood of 10 healthy horses.

Procedures—Autologous fibrinogen was used to form conventional FGs. The PRFGs were formed from autologous platelet-rich plasma of various platelet concentrations (100×10^3 platelets/μL, 250×10^3 platelets/μL, 500×10^3 platelets/μL, and $1,000 \times 10^3$ platelets/μL). All gels contained an identical fibrinogen concentration (20 mg/mL). Fiber diameter and pore area were evaluated with scanning electron microscopy. Maximum gelation rate was assessed with spectrophotometry, and gel stiffness was determined by measuring the compressive modulus. Gel weights were measured serially over 14 days as an index of contraction (volume loss). Platelet-derived growth factor-BB and transforming growth factor-β_1 concentrations were quantified with ELISAs.

Results—Fiber diameters were significantly larger and mean pore areas were significantly smaller in PRFGs than in conventional FGs. Gel weight decreased significantly over time, differed significantly between PRFGs and conventional FGs, and was significantly correlated with platelet concentration. Platelet-derived growth factor-BB and transforming growth factor-β_1 concentrations were highest in gels and releasates derived from $1,000 \times 10^3$ platelets/μL.

Conclusions and Clinical Relevance—The inclusion of platelets in FGs altered the architecture and increased the growth factor content of the resulting scaffold. Platelets may represent a useful means of modifying these gels for applications in veterinary and human regenerative medicine. (*Am J Vet Res* 2014;75:392–401)

Combined cell-matrix grafts have promise as a means of regenerative treatment for tissue defects of critical size or poor healing capacity. Fibrin has been used as a scaffold for cardiovascular,[1] musculoskeletal,[2,3] respiratory,[4] neural,[5] and cutaneous[6] tissues. Cells are typically injected into the lesion site in a solution of fibrinogen, which is then polymerized with thrombin to create a custom-fit graft. Because it is a natural vertebrate protein that is autologously available and completely degradable, fibrin has advantages over nonbiological synthetic hydrogels. It contains native peptide sequences that engage cellular integrins,[7] which provide sites for adhesion, physical support to grafted cells, and instructive mechanical cues. Fibrin gels have predictable material properties that can be adjusted to create specific graft characteristics by changing factors such as fibrinogen concentration, pH, and ionic strength.[8–11] These features make fibrin a useful substrate for bioengineering purposes and one of the most popular hydrogels in the field of tissue engineering and regenerative medicine.

Abbreviations

FG	Fibrin gel
PDGF	Platelet-derived growth factor
PRFG	Platelet-rich fibrin gel
PRP	Platelet-rich plasma
TGF	Transforming growth factor

Grafted cells require specific signals from the extracellular matrix to survive. Anoikis is a term used to describe the premature death of cells that do not receive these mechanical and chemical cues from the matrix, and this phenomenon is considered to be a major cause of cellular graft failure.[3,12,13] Matrix stiffness is a major determinant of cellular phenotype and behavior,[5,10,14] and cells that are exposed to mechanical cues and growth factor gradients are more likely to proliferate, differentiate, migrate, and orient correctly within a nascent tissue.[14–17] Fibrin creates a provisional matrix in a graft bed, but its fibers lack directionality and tension, and fibrin has little associated growth factor content. In

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some musculoskeletal and cardiovascular applications, FGs may be too soft to perform as desired.[18–20] Soft gels may be adequate for the restoration of certain tissues such as nerves,[5,21] but stiffer hydrogel substrates are required to support processes such as osteogenesis,[22] cardiac fibroblast orientation,[23] and cardiomyocyte contractility[24] in constituent cells intended for grafting.

Platelet-rich plasma is an autologous, therapeutic biomaterial favored for use because of its high growth-factor content and scaffold properties.[25] When activated by thrombin, PRP forms a PRFG that can provide a provisional matrix for ingress and support of migrating cells. After anchoring to fibrinogen through the integrin receptor $\alpha_{IIb}\beta_{IIIa}$,[26–28] platelets contract gels by virtue of their actomyosin motors. In this way, platelets generate tension in a matrix and provide preliminary orientation to the scaffold as they maintain attachment to the lesion walls. Whereas conventional FGs are isotropic and must be remodeled by resident cells,[15] PRFGs are anisotropic because of the ability of platelets to generate tension[8,29] against the tissue margins to which they are anchored. Platelet-rich fibrin gels are in use clinically in people and experimentally in other animals,[30–32] but the authors are not aware of any reports on direct comparisons of PRFGs and conventional FGs in any species.

The purpose of the study reported here was to compare biomechanical and structural characteristics of PRFGs with characteristics for the more traditionally used tissue engineering substrate, conventional FGs. We hypothesized that the inclusion of platelets would increase gel stiffness by virtue of an increase in fibrin fiber diameter and decrease in pore area (on the basis of known effects of platelets, thrombin, and the rate of polymerization on FG structure[33–35] and clot stiffness[29] as well as the inherent contractility of platelets[29]). We further hypothesized that PRFGs would contain more PDGF-BB and TGF-β_1 (on the basis of the known growth factor content of platelets[36,37]) than would conventional FGs.

Materials and Methods

Sample—Blood samples were obtained from each of 10 horses of various breeds (4 Quarter Horses, 4 Thoroughbreds, 1 Hanoverian, and 1 Paint) and ages (mean ± SD, 10 ± 4.1 years; range, 4 to 17 years). Five of the horses were mares and 5 were geldings. Animal use was conducted in accordance with institutional animal care and use requirements of the University of California-Davis. Blood samples from each horse were collected into evacuated tubes containing acid citrate dextrose (ratio of blood to acid citrate dextrose solution A, 9:1) for preparation of PRP (14 tubes) and fibrinogen precipitate (4 tubes). One blood sample was also collected from each horse into a sodium citrate–containing evacuated tube for determination of the whole blood fibrinogen concentration.

Fibrinogen preparation—Fibrinogen was prepared by use of ethanol precipitation.[38] Whole blood in glass tubes was centrifuged (200 × *g* for 15 minutes), and plasma was harvested and transferred to new polypropylene tubes. Then 95% ethanol was added to the plasma in each polypropylene tube (0.88 mL/5 mL of plasma), and tubes were inverted several times to ensure proper mixing. Tubes were incubated on ice for 30 minutes and then centrifuged at 1,500 × *g* for 15 minutes. The fibrinogen precipitate was apparent at the bottom of each tube. Supernatant plasma was discarded, and precipitates were warmed in a 37°C water bath before being resuspended in PBS solution. The resuspended samples and the sodium citrate–containing whole blood samples were submitted for fibrinogen quantification.[a]

PRP preparation—Whole blood was centrifuged at 200 × *g* for 15 minutes. Plasma was harvested and placed in new polypropylene tubes, and prostaglandin E_1[39,40] was added (final concentration, 10 µg/mL) to prevent premature aggregation and activation of platelets during processing. The tubes were again centrifuged at 400 × *g* for 15 minutes, which created a platelet pellet and a supernatant of platelet-poor plasma. Platelet-poor plasma was removed, and each platelet pellet was resuspended in the smallest possible volume of platelet-poor plasma to create PRP. Platelet concentrations were determined,[b] and dilution was performed with platelet-poor plasma to achieve concentrations of 100 × 10^3 platelets/µL, 250 × 10^3 platelets/µL, 500 × 10^3 platelets/µL, and 1,000 × 10^3 platelets/µL. The concentrations spanned a 10-fold dilution that ranged from the approximate systemic concentration of platelets in horses to the concentration often cited as desirable for clinical PRP use in humans.[25]

Gel preparation—For each horse, 25 gels (5 sets, with each set consisting of 5 types of gels) were generated as described elsewhere.[10] Four gel types were PRFGs with each of the various platelet concentrations (100 × 10^3 platelets/µL, 250 × 10^3 platelets/µL, 500 × 10^3 platelets/µL, and 1,000 × 10^3 platelets/µL), and the fifth gel type was a conventional FG with no platelets. For each gel type, 1 set was created for compression testing, 2 sets (10 gels; 2 replicates/horse) were created for measuring gelation time and subsequently used for growth factor analysis, 1 set was created for ultrastructure determination, and 1 set was created for monitoring gel weight over a 14-day incubation period and growth factor analysis in spent medium.

Fibrinogen concentrate was diluted with PBS solution to achieve a final fibrinogen concentration of 20 mg/mL. The PRP was diluted with PBS solution or supplemented with fibrinogen concentrate, as required, to achieve a fibrinogen concentration of 20 mg/mL. One-milliliter aliquots of PRP (containing 1 of the 4 platelet concentrations) or fibrinogen solution were placed into separate wells of a 48-well plate. Aprotinin[c] (50 µg) was added to each well to reduce proteolytic degradation of the gels.[5,10] Gel formation was induced by the addition of bovine thrombin[d] (final concentration, 2.5 U/mL) and calcium chloride[d] (final concentration, 20mM), which was followed by incubation at 37°C for 1 hour.

Gelation over time—Solutions of PRP or fibrinogen were added to separate wells of a 96-well plate and measured with a microplate reader-spectrophotometer.[e] Thrombin-calcium solution (30°C) was added, and gelation time was determined by measuring the rate of

change of absorbance (optical density) at 550 nm, on the basis that gels become more turbid as they polymerize.[10,41] Measurements were obtained every 51 seconds (the minimum interval possible for this microplate reader) for 20 minutes (total time period for complete gel polymerization[10]). Control wells, without the addition of thrombin-calcium solution, were included for each gel type. The optical density of the corresponding control well was subtracted from that of the test wells at each time point to correct for differences in the baseline turbidity of PRP and fibrinogen solutions as well as differences among 96-well plates. The maximum slope of the curve generated for each set of optical densities over time was recorded as the maximum gel-ation rate for a given sample.

Gel ultrastructure—Gels were placed in PBS solution for 1 hour, incubated overnight in 3% glutaraldehyde for fixation, and then returned to PBS solution until dehydration prior to scanning electron microscopy. Samples were dehydrated with a graded series of alcohol, critical point dried, and sputter-coated with gold.[f] Fibrin fiber diameter and percentage porosity were measured on scanning electron microscopic images[g] with image analysis software.[42,43,h] Briefly, analysis (3 transverse images/gel) was performed with the default thresholding algorithm, which highlighted areas of space (no fibers). The analysis was also performed with the cross-entropy thresholding algorithm described in another study.[44] The black (space) portion of the image was quantified with the area fraction option to determine the 2-D percentage porosity. Pore area was determined by the particle analysis function in the image analysis software.[h] Fiber diameter was measured on 3 images of each gel's horizontal surface; diameters of each of 3 randomly selected fibers/image were measured with the tool, which yielded 9 data points/gel.

Compressive moduli—Gel stiffness was quantified with a compressive testing system.[i] Gels were allowed to polymerize for 24 hours. Gels then were incubated in PBS solution for 1 hour to allow swelling before quantification of gel stiffness. Compression (unconfined) was performed at a rate of 1 mm/min for 1 minute. The linear region of the stress-strain curve was determined within strains that ranged from 0% to 5%, and the slope was determined as Young's modulus (compressive stiffness).[10,45]

Gel weight over 14 days—After polymerization, gels were removed from the plates, blotted, and weighed. Gels were placed in wells containing Dulbecco modified Eagle medium (high-glucose medium, with 4.5 g of D-glucose/L, 584 mg of L-glutamine, and 110 mg of sodium pyruvate/L)[j] with penicillin-streptomycin (100 U/mL) and gentamicin (0.1 mg/mL). Plates were incubated at 37°C; they were serially weighed on days 1, 3, 5, 7, 9, 11, and 14 (day 0 was the day the gels were created and the first day of incubation).

Growth factor analysis—One hour after polymerization was completed, 1 set of gels was liquefied by sonication (ten 1-second pulses at 60 W), placed briefly on ice, and centrifuged at 21,000 × *g* for 10 minutes. After gels were released from the wells 1 hour after polymerization was completed, extruded fluid was collected as the gel releasate sample for day 0. Gels were placed in wells containing Dulbecco modified Eagle medium with penicillin-streptomycin (100 U/mL) and gentamicin (0.1 mg/mL); plates then were incubated at 37°C. Medium was collected and completely replaced on days 1, 3, 5, 7, 9, 11, and 14 (day 0 was the day the gels were created and the first day of incubation). All samples were collected and frozen at –20°C until analysis. Quantification of PDGF-BB and TGF-β_1 was performed on sonicated gels, initial releasates, and spent medium samples with ELISAs[k,l] previously validated for use in horses.[46]

Statistical analysis—Prior to the study, a power analysis[m] was performed with data on FGs previously obtained by our laboratory group. The analysis revealed that for detection of a 25% difference in compressive stiffness among gel types, 10 subjects would provide a power of 99.7%.

All analyses were performed with statistical software.[n] Logarithmic transformation was performed prior to testing whenever data were not normally distributed. A repeated-measures 1-way ANOVA with post hoc Tukey testing was used to compare compressive moduli, maximum gelation rate, maximum optical density, and growth factor concentrations of gels and gel releasates among gel types. A 2-way ANOVA (factors were gel type and time) was used to analyze growth factor concentrations in samples of medium obtained on days 1, 5, 9, and 13 and also gel weight loss over time. Post hoc Tukey multiple comparisons among time points and gel types were performed. A Spearman correlation test was performed to determine whether a significant linear relationship existed between mean gel weight and platelet concentration. A 2-way ANOVA (factors were gel type and horse) with a post hoc Tukey multiple comparison was used to compare pore area and fiber diameter among gel types. Significance for all analyses was set at $P < 0.05$.

Results

Ultrastructural features—The appearance of PRFGs was qualitatively different from that of conventional FGs. In general, conventional FGs had a regular, lattice-like arrangement of pores, which were demarcated by clumps of short, fine fibers (**Figure 1**). In contrast, PRFGs consisted of discrete, separate, larger fibers arranged in a more random and often extremely dense meshwork. Conventional FGs were not entirely acellular; a few platelets and leukocytes were observed on scanning electron micrographs.

Quantitatively, fiber diameter was largest for PRFGs containing 100×10^3 platelets/μL (mean ± SD, 117.7 ± 10.53 nm) and smallest for conventional FGs (56.8 ± 5.11 nm; **Figure 2**). The fiber diameters of all PRFGs were significantly ($P < 0.001$) greater than those of conventional FGs. Percentage porosity ranged from 40.8% to 51.7% for all gels; there were no significant differences in percentage porosity on the basis of the thresholding algorithm used for analysis, despite the subjective appearance of a larger pore diameter in conventional FGs. Pore area then was assessed by specify-

ing a range for pore size (0.16 to 36 μm^2), which was selected on the basis of the diameters of the smallest and largest identifiable pores in representative images of each gel type. Image analysis software automatically identified pores within this range and determined their area. For all gel types, there was a mean ± SD of 977 ± 503.2 pores evaluated/gel by use of this method. Mean pore areas of all PRFGs were significantly ($P < 0.01$) smaller (range, 1.203 to 1.292 μm^2) than the mean pore area of conventional FGs (1.592 μm^2; **Figure 3**). There was also a significant ($P < 0.001$) effect of horse for both fiber diameter and pore area.

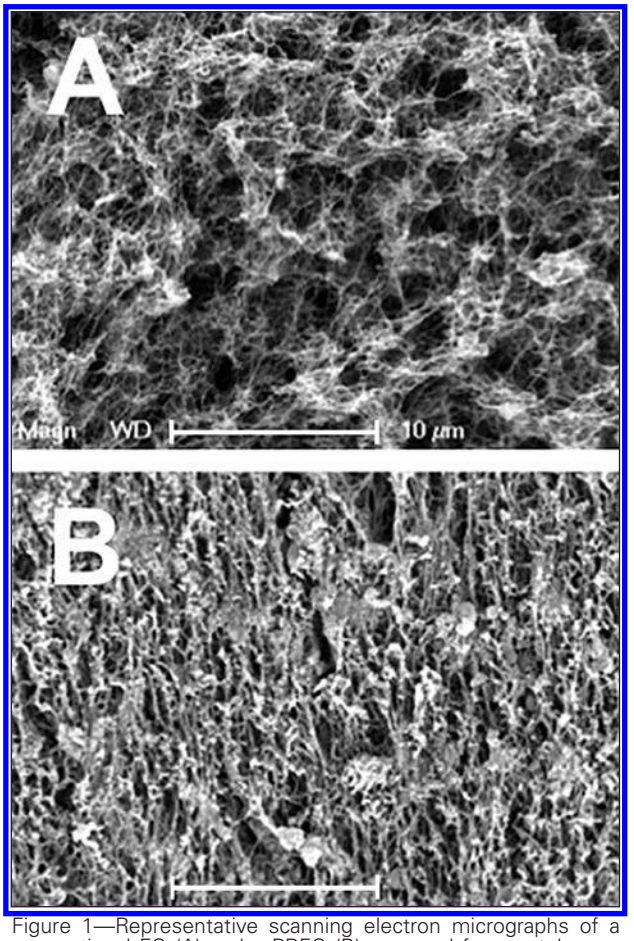


Figure 1—Representative scanning electron micrographs of a conventional FG (A) and a PRFG (B) prepared from autologous fibrinogen obtained from a healthy horse. Notice that the conventional FG has larger pores and fibers with a smaller diameter, compared with those of the PRFG. Sputter coated with gold. Bar = 10 µm.

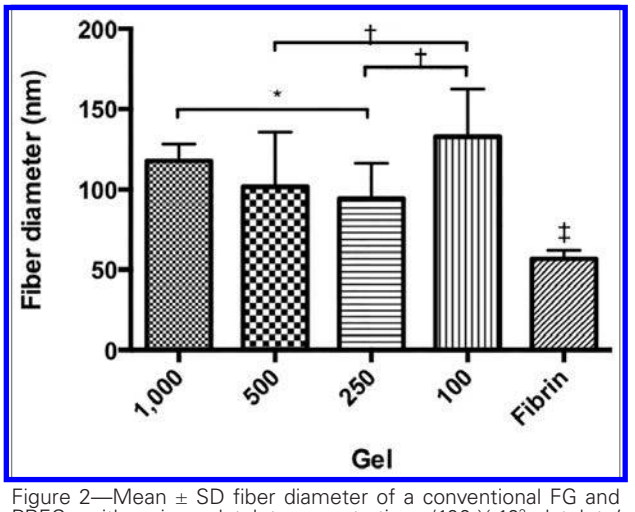


Figure 2—Mean ± SD fiber diameter of a conventional FG and PRFGs with various platelet concentrations (100 × 10^3 platelets/µL, 250 × 10^3 platelets/µL, 500 × 10^3 platelets/µL, and 1,000 × 10^3 platelets/µL) formed from autologous PRP obtained from 10 healthy horses. *,†Values differ significantly (*$P < 0.01$; †$P < 0.001$) between the indicated gels. ‡Value differs significantly ($P < 0.001$) from the value for each of the PRFGs.

Compression testing—Results of the ANOVA indicated that compressive modulus was significantly affected by horse ($P < 0.001$) and gel type ($P = 0.047$). However, post hoc multiple comparisons among gels with various platelet concentrations did not identify significant differences (**Figure 4**).

Maximum gelation rate—Gelation occurred rapidly, despite a subphysiologic test temperature that was intended to slow the enzymatic cleavage of fibrinogen

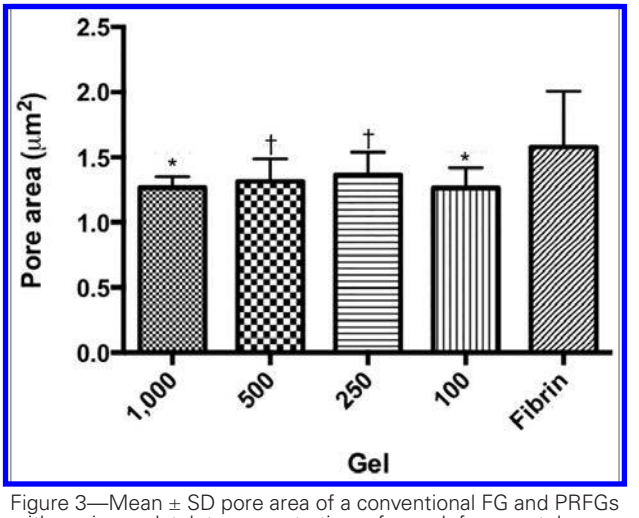


Figure 3—Mean ± SD pore area of a conventional FG and PRFGs with various platelet concentrations formed from autologous PRP obtained from 10 healthy horses. Values did not differ significantly ($P \geq 0.05$) among PRFGs. *,†Value differs significantly (*$P < 0.001$; †$P < 0.01$) from the value for the conventional FG.

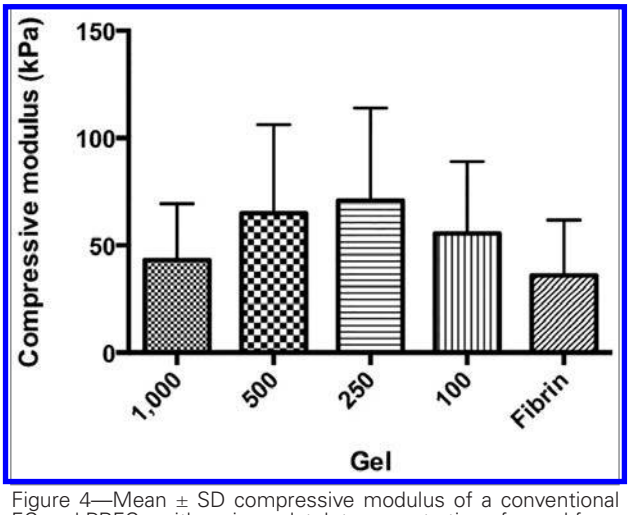
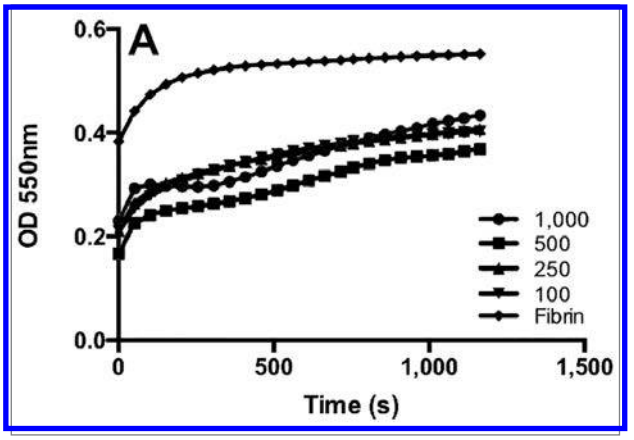


Figure 4—Mean ± SD compressive modulus of a conventional FG and PRFGs with various platelet concentrations formed from autologous PRP obtained from 10 healthy horses. Values did not differ significantly ($P \geq 0.05$) among gels.



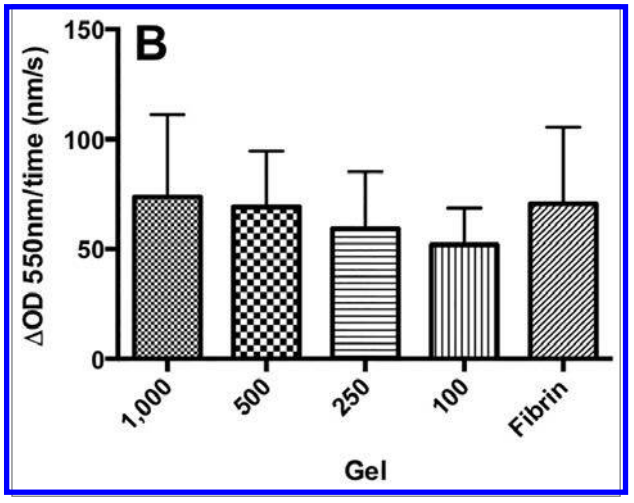


Figure 5—Optical density measured over time at 550 nm (OD 550 nm) for polymerization of a conventional FG and PRFGs formed from authologous PRP obtained from 10 horses (A) and mean ± SD maximum gelation rate for the conventional FG and PRFGs (B). In panel B, values did not differ significantly ($P \geq 0.05$) among gels. ΔOD 550 nm = Change in OD 550 nm.

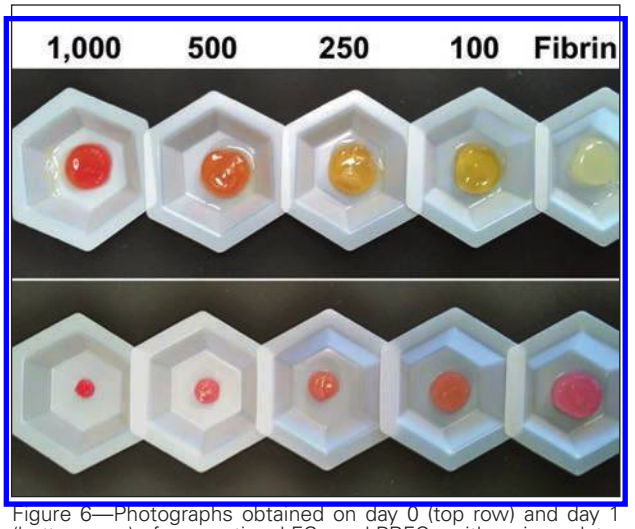
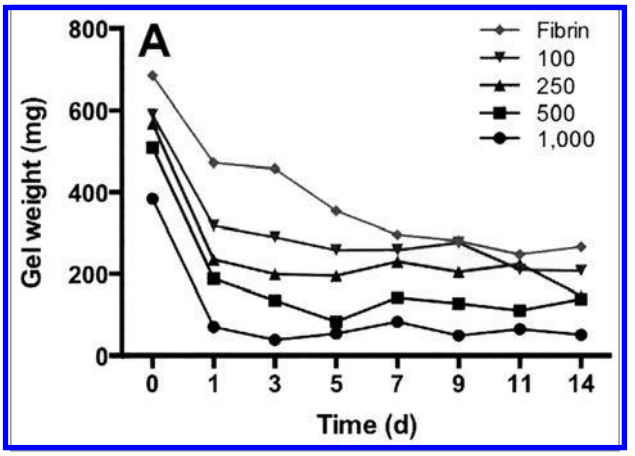
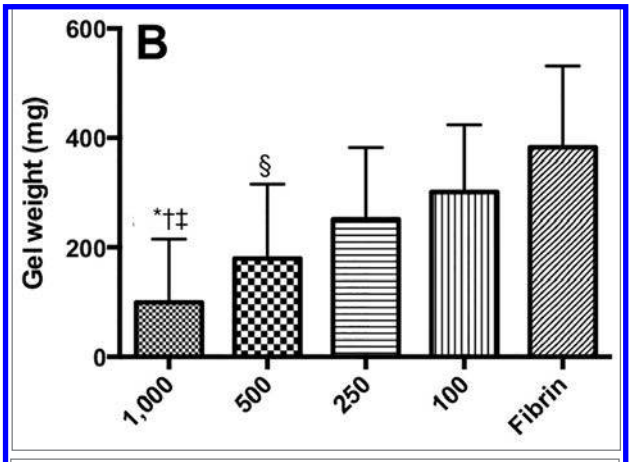


Figure 6—Photographs obtained on day 0 (top row) and day 1 (bottom row) of conventional FGs and PRFGs with various platelet concentrations formed from autologous PRP obtained from 10 healthy horses. Day 0 was the day the gels were created and the first day of incubation. Notice that the PRFGs contracted substantially within the first 24 hours after polymerization. The pink color in day 1 gels is the result of incubation in culture medium containing phenol red.

by thrombin. A significant ($P < 0.001$) effect of horse was identified for maximum gelation rate, but there was not a significant ($P = 0.161$) difference in maximum gelation rate among gel types (**Figure 5**).





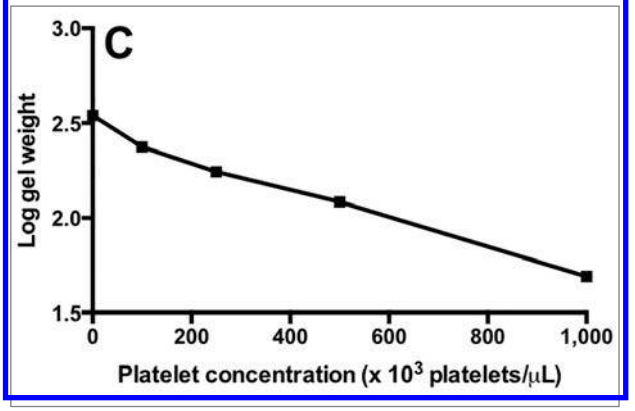


Figure 7—Weight of a conventional FG and PRFGs with various platelet concentrations, measured over 14 days (A), mean ± SD weights for the conventional FG and PRFGs over a 14-day period (B), and the correlation between gel weight and platelet concentration (C). Gels were formed from autologous PRP obtained from 10 healthy horses. In panel C, gel weight was significantly correlated ($r^2 = 0.986$; $P < 0.001$) with platelet concentration in the gels. *Value differs significantly ($P < 0.001$) from the value for the PRFG with 100 × 10^3 platelets/μL and the conventional FG. †Value differs significantly ($P < 0.01$) from the value for the PRFG with 250 × 10^3 platelets/μL. ‡Value differs significantly ($P < 0.05$) from the value for the PRFG with 500 × 10^3 platelets/μL. §Value differs significantly ($P < 0.01$) from the value for the conventional FG.

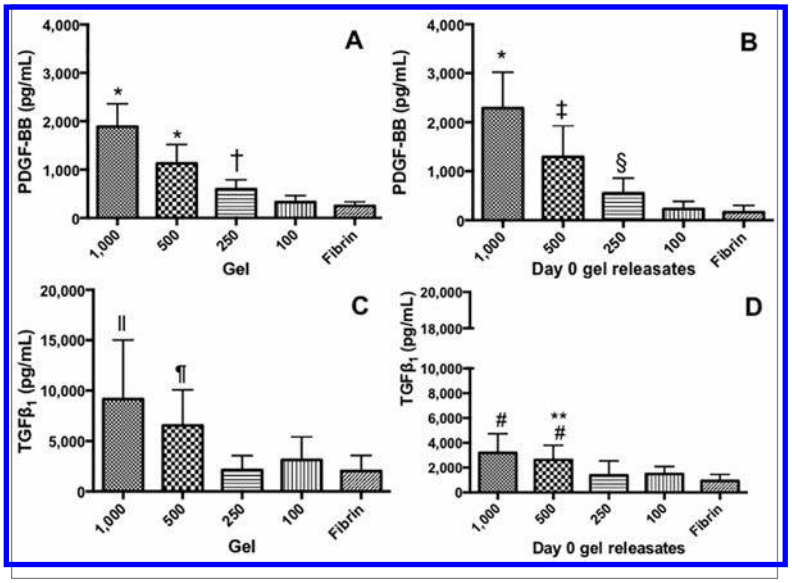


Figure 8—Mean ± SD concentrations of growth factors (PDGF-BB and TGF-β_1) contained in a conventional FG and PRFGs with various platelet concentrations (A and C) and in fluid released from the gels on day 0 (B and D). *Value differs significantly ($P < 0.001$) from the value for each of the other gels. †Value differs significantly ($P < 0.01$) from the value for the PRFG with 100 × 10^3 platelets/μL and the conventional FG. ‡Value differs significantly ($P < 0.01$) from the value for the PRFGs with 250 × 10^3 platelets/μL and 100 × 10^3 platelets/μL and the conventional FG. §Value differs significantly ($P < 0.05$) from the value for the PRFG with 100 × 10^3 platelets/μL and the conventional FG. ‖Value differs significantly ($P < 0.05$) from the value for the PRFGs with 250 × 10^3 platelets/μL and 100 × 10^3 platelets/μL and the conventional FG. ¶Value differs significantly ($P < 0.01$) from the value for the conventional FG. #Value differs significantly ($P < 0.05$) from the value for the conventional FG. **Value differs significantly ($P < 0.01$) from the value for the PRFG with 250 × 10^3 platelets/μL.

Changes in gel weight—Most gel weight loss was within the first 24 hours after polymerization (**Figures 6 and 7**). Gel weight differed significantly ($P < 0.001$) on the basis of time. Baseline gel weights were not significantly different among gel types, but over the 14-day incubation period, mean gel weights differed significantly ($P < 0.001$) among gel types and were significantly ($P < 0.001$) correlated with platelet concentration.

Growth factor concentration of gels and releasates—There was no detectable PDGF-BB or TGF-β_1 in control samples of the medium used for gel incubation. Growth factor concentrations of the gels and the initial releasates differed in proportion to platelet concentration for both PDGF-BB ($P < 0.001$) and TGF-β_1 ($P = 0.007$; **Figure 8**). Most growth factor release from gels occurred within the first 24 hours after polymerization and diminished thereafter. There was a significant effect of gel type on release of PDGF-BB ($P = 0.008$) and TGF-β_1 ($P = 0.005$). Similarly, there was a significant ($P < 0.001$) effect of time on release of both PDGF-BB and TGF-β_1.

Discussion

The structural (and resulting functional) properties of fibrin can be modified by altering polymerization factors, such as fibrinogen concentration, pH, salt concentration, or temperature.[10,47] The incorporation of platelets into FGs results in structural alterations and increases in growth factor concentrations and may ultimately improve the performance of cell-scaffold grafts after transplantation. However, in the present study, the inclusion of platelets did not confer an increase in gel stiffness over that for gels prepared from an ethanol precipitate of fibrinogen.

Fibrin gels are desirable as tissue-engineering scaffolds for several reasons. The most important reason is fibrin's inherent compatibility with cell viability, which is in contrast to that for many of the components and processes involved in fabrication of synthetic scaffolds.[10,20] Fibrin is a natural substance, is completely biodegradable, and facilitates the transition to a new extracellular matrix.[10] Graft cells can be incorporated into a gel at the time of polymerization, which ensures a uniform distribution, rather than having to be seeded into the scaffold after the fabrication process (as for some synthetic scaffolds).[1,20] The main disadvantage of FGs is their relative softness as a substrate, which, unless there is some modification, makes them unsuitable for certain applications.[18,20]

The process of fibrin polymerization consists of several steps, beginning with thrombin cleavage of fibrinopeptide A from a fibrinogen molecule to form a fibrin monomer. This cleavage results in exposure of a molecular knob, which engages a hole in the γ-nodule of another fibrinogen molecule and results in a half-staggered arrangement of fibrin monomers. This early form of fibrin is referred to as an oligomer or protofibril, and these then assemble laterally in a helical manner to create a fibrin fiber.[48] Fibers may also aggregate laterally to produce thicker fiber bundles, depending on the polymerization conditions.[47]

At the culmination of fibrin clot formation in vivo, platelets bind fibrin via β_3 integrins[28,35,49,50] and contract the clot. In so doing, they also contract against the margins of the wound bed, which generates tension and orients the new, provisional matrix.[29] By virtue of their growth factor content, platelets directly contribute to the growth, development, and restoration of tissue.[51–54] Platelet-derived growth factor and TGF-β_1 are the most plentiful growth factors contained within the α granules of platelets[36,37] and are released to the extracellular space after platelet activation. These factors direct cell proliferation, cell differentiation, matrix production, angiogenesis, and wound contraction.[55–60] Growth factor supplementation improves the survival and differentiation of transplanted cells in a number of scaffold materials and tissue environments.[61–64] Data for the present study confirmed that PRFGs contain substantial amounts of growth factors and, on this basis, may be better able to support grafted cells than are conventional FGs.

The mechanical properties of FGs reflect a highly complex interaction of individual structural characteristics of each fibrin network, including fiber diameter and pore area. In the study reported here, the addi-

tion of platelets to FGs resulted in increased fiber diameter and decreased pore area. Large fiber diameter[47] and small pore area[5,20,65] have historically been considered the major determinants of FG stiffness, but in the present study, significant differences in compressive stiffness were not detected. Other variables such as fibrin assembly rate, fiber density, and branch point density are also key contributors to ultimate gel stiffness,[66] but the definitive determinant of clot stiffness is unknown.[67] Fibrin clots with intermediate fiber diameter and branch point density are stiffer than clots with large fibers and low branch point density or small fibers and high branch point density, which indicates that the combined effect of ultrastructural features is greater than the sum of the parts.[66] In the present study, the range of measured fiber diameter was small (50 to 66 nm) in conventional FGs but varied considerably (range, 55 to 187 nm) in PRFGs. As measured in this study, fiber diameter for PRFGs likely reflected the degree of lateral aggregation of individual fibers because the diameter of individual fibrin fibers in humans is surprisingly consistent (85 nm), whereas larger fibers represent lateral aggregation of some number of individual fibers.[47] Interestingly, the largest fiber diameter measurements were associated with the lowest platelet concentration (100×10^3 platelets/μL), which approximated the systemic platelet concentration of clinically normal horses. Although we cannot offer a definitive explanation for this observation, it may be that less tension was applied to these fibers by the lower number of platelets, such that the fiber diameter was effectively larger than the fiber diameter for those stretched more tautly by a higher number of platelets. In addition, gels with lower platelet concentrations likely contained less thrombin, which is produced on the surface of activated platelets.[68] Fibrin fiber diameter increases as thrombin concentration decreases.[69] Finally, PRFGs in the present study contained a full complement of plasma proteins, whereas the conventional FGs contained predominantly fibrinogen. The impact of various plasma proteins on gel mechanics is not completely defined,[70,71] but gels prepared from plasma have larger fiber diameters than the fiber diameters of gels prepared from purified fibrinogen alone.[72] Interestingly, all gels in the study reported here were approximately 5 times as stiff (approx 50 kPa) as FGs formed from purified human fibrinogen (approx 10 kPa) and tested on the same equipment,[10] which may have been a result of their greater plasma protein content.[70,71]

Pore area and percentage porosity are important structural indices of any biological scaffold. In general, larger pores favor cellular ingrowth and proliferation, whereas smaller pores favor cell attachment (especially early cell attachment), owing to the greater overall surface area they provide.[73] More porous scaffolds represent softer constructs overall.[74] Compared with scaffolds with other pore sizes and percentage porosities reported in the literature,[74–77] the gels in the present study (both PRFGs and conventional FGs) were of intermediate porosity and had extremely small pore areas. Although the mean pore areas differed significantly between PRFGs and conventional FGs, this difference was small relative to the range of reported pore sizes and may therefore be inconsequential in terms of clinical use.

Concentrations of thrombin, calcium, and sodium chloride can also dramatically affect the rate of gelation and ultimate structure of FGs.[9,10,70] We expected that platelets would accelerate the polymerization process, compared with the process for conventional FGs. Therefore, we were surprised that no significant differences in gelation time were detected between gels with and without platelets. We suspect this failure to detect a difference was most likely attributable to technical limitations: namely, the minimum interval between optical density measurements was 51 seconds. The fibrin polymerization process is quite rapid even in the absence of platelets; the steepest portion of the optical density–time curves (the slope of which is the maximum gelation rate) lasts only approximately 100 seconds (Figure 5). During that time, we would have collected only 1 or 2 data points, and this likely explains our failure to detect a difference in maximum gelation rate between conventional FGs and PRFGs. Unfortunately, we did not realize this technical limitation at the outset of the study. In addition, the use of a lower concentration of thrombin (ie, 0.1 to 1 U/mL) than that used in the present study may slow the polymerization process enough to allow the detection of differences in gelation rate. To maintain consistency in methods and facilitate the comparison of results, we chose thrombin, calcium chloride, and fibrinogen concentrations reported in a previous study[10] conducted by the laboratory group of one of the authors.

The desired concentration of leukocytes in PRP and PRFGs is a contentious topic.[78,79] Neutrophils affect fibrinolysis when present in a thrombus[80,81]; however, to the authors' knowledge, the specific effect of leukocyte concentration on clot formation, FG formation, or clot stiffness has not been reported. We did not control for and did not examine the effect of leukocyte concentration in the present study. The PRP used for PRFG formation was of intermediate WBC concentration (mean, 9.34×10^3 WBCs/μL; range, 3.2×10^3 WBCs/μL to 16.0×10^3 WBCs/μL). Investigators in a recent study[82] concluded that the inclusion of DNA and histones (as found in leukocytes but not platelets) increased clot stiffness and resulted in fibrin fibers with a larger diameter, so it is conceivable that leukocytes affected gel ultrastructure and compressive modulus in the present study.

There is significant variation in PRP composition between and within individuals, even when the same preparation method is used.[83] In the present study, we used samples obtained from 10 horses and prepared 25 gels/horse, but because we tested several variables, we were able to test only 1 replicate gel/horse for compressive modulus, ultrastructure, and weight loss over time. The inclusion of multiple replicates for each horse and each test would have resulted in a more robust data set that is less subject to the effects of intraindividual variability. However, given that the key compositional variables investigated (ie, fibrinogen concentration and platelet concentration) were controlled as fixed values in these experiments, we suspect that the effect of intraindividual variability was much less than that typically encountered in a clinical setting.

This study illustrated the substantial effects of platelets on gel contraction, once gels were physically removed from culture wells. During contraction, fluid is extruded from the gels, similar to the process for a clot in vivo, and weight is lost because of this dehydration.[84] Conventional FGs lost some weight during the first 24 hours after polymerization, but PRFGs lost 50% to 60% of their weight in that interval. The clinical use of PRFGs requires that gels be formed in situ (ie, in the lesion), where they will remain attached to lesion walls and thus prevent graft shrinkage. If a gel is instead produced in a laboratory prior to clinical application, substantial contraction must be anticipated, and thus the gel should be larger than the lesion size when fabricated.

Platelet-rich fibrin gels have been widely used in human mandibular and periodontal reconstructions since the mid 1990s[31,85,86] as well as in orthopedic surgical applications such as repair of avascular necrosis of the hip,[87] rotator cuff,[30] and Achilles tendon.[32] The main use of PRP products in regenerative medicine has been to deliver high concentrations of growth factors, as indicated by the results of the present study. In addition, results of this study confirmed that platelets altered the structural properties of FGs, although these alterations did not affect overall gel stiffness. Nonetheless, these findings suggest that the particular growth factor and structural requirements of transplanted cells or recipient tissues may be more specifically addressed by changing the platelet concentration in PRFGs. Further studies are required to determine the platelet concentrations best suited for a specific tissue or lesion type, whether the porosity of PRFGs can be manipulated to enhance cellular support, and whether PRFGs will improve the survival and site-specific differentiation of grafted cells, compared with results for conventional FGs.

a. STA Compact, Stago-Diagnostica, Parsippany, NJ.
b. Coulter Ac•T diff analyzer, Beckman Coulter, Miami, Fla.
c. Santa Cruz Biotechnology, Santa Cruz, Calif.
d. Sigma-Aldrich, St Louis, Mo.
e. Synergy HTTR, BIO-TEK, Wisnooski, Vt.
f. Auto sputter coater SC-7, Pelco, Redding, Calif.
g. XL30 TMP, Philips, Eindhoven, Netherlands.
h. Image J, version 1.39d, National Institutes of Health, Bethesda, Md.
i. Instron 3345 compressive testing system, Instron, Norwood, Mass.
j. Catalogue No. 11995-065, Gibco, Invitrogen/Life Technologies, Carlsbad, Calif.
k. Quantikine human PDGF-BB ELISA, R&D Systems Inc, Minneapolis, Minn.
l. Quantikine human TGF-β1 ELISA, R&D Systems Inc, Minneapolis, Minn.
m. DSS Research, Fort Worth, Tex.
n. Prism, version 6, GraphPad Software Inc, La Jolla, Calif.

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