Effects of platelet-rich plasma on the repair of wounds on the distal aspect of the forelimb in horses

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Objective—To evaluate the effect of platelet-rich plasma on wounds on the distal aspect of the forelimb in horses.

Animals—6 mixed-breed 10- to 15-year-old mares.

Procedures—3 wounds were created on metacarpal regions in each of 6 horses (n = 36 wounds total). Eighteen wounds were treated with platelet-rich plasma and bandaged, whereas 18 control wounds were similarly bandaged with no prior topical treatment. Decrease in wound surface area and the required number of excisions of exuberant granulation tissue were recorded until complete healing. Tissue specimens were taken from wounds at 1 week for histologic examination and measurement of transforming growth factor β1 concentrations and at closure for histologic examination, biomechanical evaluation, and measurement of collagen type I and type III mRNA.

Results—Platelet-rich plasma favored excessive development of granulation tissue and significantly slowed wound healing at 1, 2, and 3 weeks after surgery. Transforming growth factor β1 had a 1.6-fold higher concentration in treated wounds, compared with untreated wounds. Histologic, biomechanical, and gene expression data did not differ significantly between treated and control wounds.

Conclusions and Clinical Relevance—Topical application of autologous platelet-rich plasma did not accelerate or improve the quality of repair of small granulating wounds on limbs of horses. This treatment may better suit wounds with massive tissue loss or, alternatively, chronic wounds that would benefit from a fresh source of mediators to accelerate the healing process. (Am J Vet Res 2009;70:277–282)

Horses are afflicted with chronic nonhealing wounds and, conversely, the development of exuberant granulation tissue (ie, proud flesh) when wounds are located on distal regions of the limbs. Both conditions lead to extensive scarring that may limit an athletic career. Wounds occurring in this location are an important concern to owners and veterinarians.

Several mechanisms have been implicated in problematic wound repair in horses, including poor blood supply,1,2 inefficient inflammatory response to trauma,3,4 persistent upregulation of profibrotic growth factors,5,6 and a disparity between collagen synthesis and lysis7 as well as microvascular occlusion and deficient apoptosis of the cellular components of granulation tissue.8 These irregularities are limited to wounds located on the limb, whereas even extensive wounds of the trunk and head usually heal uneventfully.1,3,5,9 Attempts to ameliorate wound repair in the horse have been disappointing. Indeed, costly treatments are often unsuccessful at preventing or resolving chronic wounds or the development of exuberant granulation tissue.

Bioactive molecule-based treatments constitute an area of tissue engineering that currently commands much attention. Results of studies on a variety of animals with wounds suggest that cytokine treatment might accelerate healing of tissues and, especially, promote the repair of impaired wounds. Unfortunately, clinical use of purified bioactive agents has fallen short of expectations.10,11 This may relate to the fact that cytokines work in concert, both temporally and spatially.12 Because wound repair is a dynamic process, cytokine combination treatment will no doubt be required for efficacy.
In an effort to provide this combination treatment, investigators have turned to PRP. Platelet-rich plasma represents a concentrated form of multiple cytokines released from platelet α-granules at sites of tissue injury. These include, among others, platelet-derived growth factor, TGF-β1, and insulin-like growth factor-1. The principal therapeutic advantage of PRP over isolated purified cytokines is that it represents a physiologically natural mixture of mediators designed to have synergistic biological effects in a wound healing environment. The use of PRP has been reported in the management of acute and chronic wounds.

In a report on 1 horse, a commercially available platelet gel was applied to wounds of the distal region of the limb. The heterologous platelet gel reportedly accelerated epithelial differentiation and favored the production of repair tissue, resulting in organized, interlocking collagen bundles. The objective of the study reported here was to evaluate the effect of topical application of autologous PRP to wounds on the forelimb in a larger population of horses. It was hypothesized that by increasing the concentration of mediators present in the wound bed via the addition of PRP, the acute inflammatory response might be enhanced and consequently accelerate or improve the quality of wound repair and obviate the development of exuberant granulation tissue.

Materials and Methods

Animals—This project was approved and performed according to the guidelines of the Animal Ethics Committee of the École Nationale Vétérinaire de Lyon. Six mixed-breed adult horses, 10 to 15 years of age and weighing between 463 and 570 kg, were used. Horses were free of any clinically detectable medical disorder, received tetanus prophylaxis prior to the study, and were kept under constant conditions throughout. Horses were examined daily for signs of discomfort, lameness, illness, and bandage slippage.

Preparation of autologous PRP—Platelet-rich plasma was prepared by the tube (manual) method in commercially designed platelet sequestration tubes. Blood samples were collected atraumatically through a single jugular venipuncture with an 18-gauge needle into 8-mL citrated anticoagulant platelet sequestration tubes. Blood samples were centrifuged at 1,500 X g for 8 minutes to achieve separation of cell layers. Red blood cells were isolated from the overlying Buffy coat and plasma by the plug within the patented platelet sequestration tubes. Eight milliliters of blood thus yielded approximately 4 to 5 mL of platelet-poor plasma, of which between 0.75 to 1.0 mL of platelet-poor plasma to produce PRP.

Surgical procedure—Horses were sedated by IV administration of detomidine (0.01 mg/kg) and butorphanol tartrate (0.04 mg/kg). A proximal metacarpal ring block was performed by use of a 2% solution of lidocaine hydrochloride. Surgical sites were aseptically prepared, and 3 square 6.25-cm2 full-thickness (epidermal, dermal, and subcutaneous tissue) wounds were created 2 cm apart on the dorsolateral surface of each metacarpus (n = 36 wounds). Both forelimbs were bandaged in a layered fashion to control bleeding; a nonadherent permeable dressing was secured with sterile conforming cotton gauze that was held in place with a cohesive bandage and adhesive tape at the extremities. Wounds were left to heal by second intention under bandage for 1 week in an effort to simulate a typical clinical situation. A scalpel blade was used to level the bed of granulation tissue and retrace the wound periphery. A layered bandage was applied for 24 hours to control bleeding prior to commencing treatment.

Experimental design—In each horse, the 3 wounds on 1 randomly assigned forelimb were treated with PRP (TWs; n = 18), whereas those on the contralateral forelimb received no treatment (CWs; 18). In this manner, each horse served as its own control. One and a half milliliters of PRP was prepared for each TW per horse and was activated immediately prior to its application to the wound surface by adding 50 units of human thrombin reconstituted in 1 mL of CaCl2; this resulted in the production of a gel-like substance. All TWs and CWs were dressed with a paraffin strip dressing followed by application of a secondary and tertiary layer of bandage as described earlier.

Wounds were identified as A, B, and C, from distal to proximal locations on the limb. Bandages were changed 1 week later, at which time, wounds A were biopsied to collect specimens for histologic evaluation and determination of TGF-β1 concentrations. A second dose of PRP was applied to wounds B and C of the treated forelimb, and all wounds were dressed as described. From then on, bandages were changed a minimum of once weekly until complete healing that corresponded to a fully epithelialized surface. Wounds B and C served for macroscopic observation throughout the healing period. Once fully healed, wounds B were biopsied to collect specimens for histologic evaluation and measurement of collagen type I and type III mRNA, whereas wounds C were biopsied to collect specimens for biomechanical evaluation.

Biopsy technique—Sedation and local anesthesia were performed as for the surgical procedure. Specimens were obtained with an 8-mm diameter biopsy punch from wounds A at 1 week after the first PRP treatment and from wounds B upon complete healing. Biopsy specimens for TGF-β1 protein and for collagen type I and type III mRNA measurements were obtained from the center of wounds A and B, respectively. Blood was rinsed off with sterile saline (0.9% NaCl) solution, and biopsy specimens were blotted dry with gauze, placed in an Eppendorf tube and immediately plunged into liquid nitrogen, and transferred to a −80°C freezer. Specimens used for histologic evaluation were taken from the edge of wounds A (1 week old) or B (healed) to encompass a 3- to 4-mm strip of intact skin, the migrating epithelium, and a 3- to 4-mm strip of granulation tissue from the wound center, when present. Specimens were fixed in neutral-buffered formalin, processed in paraffin blocks, sectioned for histologic examination, and stained with H&E by use of standard procedures.
Full-thickness, vertically oriented, 1.5- to 2.0-cm-wide by 6.0- to 8.0-cm-long rectangular tissue strips were taken from healed wounds C for biomechanical evaluation. Tissue strips encompassed the healed wound at their midpoint. Blood was rinsed off with sterile saline solution, and tissue strips were rolled up in humid gauze, stored in a zipper storage bag and immediately plunged into liquid nitrogen, and transferred to a −80°C freezer. All biopsy sites were left to heal by second intention under bandage.

Macroscopic assessment—Wounds B and C were examined weekly. Upon bandage change, they were cleaned with saline solution–soaked gauze and subjectively assessed and photographed with a digital camera alongside a 2-dimensional scale to measure the WSA, which corresponded to the area of denuded granulation tissue, by use of commercial software. From duplicate measurements, the percent decrease in WSA was calculated for wounds B and C. Exuberant granulation tissue was sharply excised with a scalpel when appraised as grade IV (range, grade I to grade IV) as described by Bigbie et al. Granulation tissue elevated above the skin edges and projecting over the advancing epithelial border; moreover, the number of times excision was required was recorded.

Protein extraction and TGF-β1 measurements—Biopsy specimens of wounds A were used to measure tissue concentrations of TGF-β1 at 1 week after topical application of PRP. Tissues were minced with a scalpel blade and mixed with 10 mL of tissue protein extraction reagent containing 1 tablet of protease inhibitor. Samples were centrifuged at 1,500 g for 8 minutes, after which total protein concentration was determined by the Bradford method. A commercial human TGF-β1 ELISA kit was used according to the instructions of the manufacturer to determine TGF-β1 concentrations in duplicate aliquots of each sample following acid-activation with 1 N hydrochloric acid and dilution to 1:10 with the commercial calibrator diluent. The TGF-β1 assay is specific for equine TGF-β1. Data on TGF-β1 were normalized against the total protein concentration for each sample.

Histologic examination—Two observers, blinded to the origin of the wound biopsy specimens, evaluated inflammation, epithelialization, and angiogenesis as well as fibroplasia within the healing tissue by use of the semiquantitative grading scale of Lepault et al. This scale was adjusted for wounds B, biopsied upon complete epithelialization, in which neoepipithelial thickness was graded as 0 = inferior to that of adjacent intact epidermis, 1 = equal to that of adjacent intact epidermis, and 2 = superior to that of adjacent intact epidermis.

Biomechanical evaluation—Biomechanical evaluation was performed by use of a single-column testing system equipped with pneumatic side action grips. Tissue strips were trimmed down in width to exclude any surrounding intact skin such that, once mounted longitudinally, only scar tissue was appraised for the distance between grips. Thickness and width of the tissues loaded in the grips as well as length of the tissue between the grips were measured with electronic calipers. The required force on the grips (80 psi) had previously been determined by testing 3 necropsy specimens. Jaws were moved apart at a rate of 40 mm/s. Chart recordings were obtained by use of commercial software and scanned into a personal computer. Values for stiffness, stress, and strain were calculated for each tissue.

RNA extraction and semiquantitative reverse transcriptase PCR analyses of collagen type I and type III—Total cellular RNA was isolated from tissue specimens taken from healed wounds B by use of a commercial kit. The concentration of total RNA was quantified by measuring optical density at 260 nm. First-strand cDNA template was synthesized from mRNA (50 ng).

Semiquantitative analysis of the expression of collagen types I and III was performed via PCR by use of a programmable thermocycler for estimation of scar-tissue maturity. Immature collagen type III is particularly abundant in the early stages of wound repair but is replaced later by the stronger mature collagen type I. Resulting cDNA (0.5 μL) underwent PCR amplification. Primers were designed by use of commercially available software to read the open frame of equine cDNA sequences for COL1A2, COL3A1, and GAPDH.

The number of cycles was optimized for each gene to fall within the linear range of PCR amplification as follows: GAPDH, 25 cycles; COL1A2, 32 cycles; and COL3A1, 33 cycles. The PCR reactions (5 μL of PCR solution/reaction) were resolved on a 2% agarose gel (40 mM Tris acetate; pH, 8; 1 mM EDTA) with ethidium bromide (0.5 μg/mL). The PCR products were observed by UV light, and the images were digitized. Digitized signals for each gene were analyzed by densitometry with a public domain computer image program. A ratio of collagen type I to collagen type III mRNA was calculated for each scar.

Statistical analysis—All data were compared between TWs and CWs to determine the effects of PRP on the repair of wounds on the distal region of the forelimb in horses. A repeated-measures linear model with treatment and time as repeated factors followed by a priori contrasts to compare means between levels of the independent variables was used to examine differences in the percentage decrease in WSA. The Wilcoxon signed rank test was used to examine differences in the median number of resections for each type of wound separately. The Cochran-Mantel-Haenszel test for repeated measures was used to compare histologic scores obtained for TWs and CWs. A Wilcoxon signed rank test was used to examine differences in biomechanical data obtained for TWs and CWs. The Wilcoxon signed rank test was used to compare the ratio of collagen type I to collagen type III mRNA obtained for TWs and CWs. Values of P < 0.05 were considered significant. Analyses were performed with a commercially available software program.

Results—A pilot study was performed on 4 horses, with blood samples collected at 2 separate times, to verify the efficacy and reproducibility of the method for preparing PRP. Manually prepared PRP had a 3.5-fold increase in platelet count over the counts for whole blood
Macroscopic observations—The repeated-measures linear model with treatment and time as within-subject factors revealed no significant ($P = 0.25$) difference in percentage decrease in WSA among wounds according to treatment but revealed a progressive increase over time (Figure 1). However, a priori contrasts revealed that the percentage decrease in WSA was significantly inferior for TWs, compared with CWs, at location B at 1 week ($P = 0.048$) and 2 weeks ($P < 0.001$) and at location C at 1 week ($P = 0.01$), 2 weeks ($P = 0.04$), and 3 weeks ($P = 0.01$) after treatment (Figure 2). There were no significant ($P = 0.33$) differences in percentage decrease in WSA among wounds in locations B and C when time and treatment were combined. Control wounds took a mean of 62 days to heal, whereas TWs took a mean of 72 days ($P = 0.09$).

Grade IV exuberant granulation tissue$^{22}$ was observed in 7 of 12 TWs and in 2 of 12 CWs. Moreover, the affected TWs required a mean of $3.0 \pm 1.37$ excisions, whereas the affected CWs required a mean of $0.5 \pm 0.84$ excisions. Despite the lack of a significant association between the frequency of resection and treatment for wounds at either location B ($P = 0.19$) or C ($P = 0.19$), the development of more exuberant granulation tissue was observed in wounds treated with PRP.

Histologic observations—No significant effect of treatment was found on the microscopic morphology of the wounds biopsied at either 1 week or once fully healed. A larger inflammatory focus was not detected within 1-week-old healing TWs than within equivalent CWs. In healed wounds, inflammation had completely dissipated, neopithelium was thickened in TWs and CWs, and angiogenesis was more substantial in 2 TWs and 1 CW, whereas fibroblast organization was superior in 2 TWs and 1 CW.

Wound strength—Tissue strips of healed wounds were loaded under tension until the breaking point. Mean stiffness values of CWs ($8.69 \pm 0.87$ N/m) did not differ significantly ($P = 0.63$) from those of TWs ($8.43 \pm 1.76$ N/m), nor did values of stress (CWs, $2.78 \pm 0.43$ mPa; TWs, $2.47 \pm 0.67$ mPa, $P = 0.44$) or strain (CWs, $20.80 \pm 2.14$ N; TWs, $20.74 \pm 3.37$ N, $P = 0.81$) differ significantly ($P = 0.15$) between TWs and CWs (0.502 ± 0.118).

Collagen type I and type III mRNA expression—The mean ratio of collagen type I to collagen type III mRNA expression was higher in 5 CWs, compared with 1 TW, but mean (± SD) ratios of expression did not differ significantly ($P = 0.22$) between TWs (0.502 ± 0.135) and CWs (0.542 ± 0.118).

Discussion

Topical medications, as opposed to systemically administered drugs, are not required to undergo stringent FDA testing and approval. Consequently, a plethora of new products have recently entered the market, often with limited scientific evidence to support the claims of the company. In an effort to improve the quality of wound management in horses, new methods should be critically evaluated to establish their efficacy, prior to commercialization.

Technologies to provide autologous PRP are now being used in a wide variety of clinical applications. Platelet concentrates can be obtained by at least 3 means: the tube (manual), buffy coat (semiautomated), and apheresis (automated) methods. Advantages of the tube method are its low cost and minimal technical requirements. The single and double centrifugation tube method is reported to be reliable for concentrating equine platelets and for obtaining potentially therapeutic TGF-β1 concentrations.

TGF-β1 concentrations—The difference in mean ± SD concentrations of TGF-β1 between TWs ($1,092 \pm 493.28$ ng/g) and CWs ($676 \pm 134.79$ ng/g) was not significant ($P = 0.09$). However, mean TGF-β1 concentration was 1.6-fold higher in 1-week-old wounds treated with PRP than in CWs.

Figure 1—Graph of WSA (mean ± SD) and number of wounds versus time in forelimbs of 6 horses. The WSA measurements from all wounds (treated [white] or control [black]) contributed to the values shown by each bar. Diamonds indicate the number of TWs and triangles indicate the number of CWs that were not fully healed. A larger inflammatory focus was not detected a maximum load at break equivalent to 60% of the breaking force of normal intact skin.

Figure 2—Macroscopic appearance of CW (a) and TW (b) on forelimbs of horses, 14 days following topical application of PRP. Granulation tissue within the TW appears exuberant (grade IV), according to the Bigbie scale, and requires surgical excision.
The centrifugation tube method used in this study was simple, easy to perform, and rapid (20 minutes). It increased platelet and TGF-β1 concentrations 3.5-fold and 2.8-fold over concentrations in blood and serum, respectively, which is in agreement with values attained in a previous study with a similar technique. Althoughuffy coat and aphepsis techniques used to concentrate equine platelets previously have resulted in greater yields (8.9-fold and 5.2-fold, respectively), the increase in TGF-β1 concentrations was comparable with that in the study reported here (2.8-fold and 3.1-fold, respectively). It is suggested that PRP should contain a 3- to 5-fold increase in platelet concentration over baseline, although platelets collected from different horses may require distinct platelet concentrations to attain comparable biological effects.

In this study, the rate of healing was assessed in a relative manner (ie, percentage decrease in WSA over time), which took into account the exact dimensions of the original wounds. Indeed, although every effort was made to standardize wound size at surgery, it was nearly impossible to ensure that each wound possessed identical dimensions at the onset.

Findings in this study do not support the hypothesis that by increasing the presence of mediators in the wound, via the addition of PRP, the quality of repair would be accelerated or improved. The addition of PRP did not obviate the development of exuberant granulation tissue in this particular model of wounding in horses.

In the present study, it was hypothesized that by increasing the concentration of proinflammatory mediators (in particular, TGF-β1) present in the acute wound bed via the topical application of autologous PRP, the chemotaxis of WBCs would be improved, which would ameliorate dermbridegment and decrease the stimulus for a prolonged inflammatory response, thereby limiting attending fibrosis. Moreover, higher concentrations of TGF-β1 should accelerate the differentiation of fibroblasts toward a myofibroblast phenotype, thereby enhancing wound contraction and simultaneously limiting the number of remaining synthetic fibroblasts; consequently, the amount of extracellular matrix deposited in the wound would be decreased.

In contrast to the hypothesis, topical application of autologous PRP to small granulating wounds on the distal aspect of the forelimb favored the excessive development of granulation tissue and significantly slowed wound healing at weeks 1, 2, and 3, though the final rate of healing was not influenced by the treatment. Protrusion of granulation tissue above and beyond the wound margins may have physically impeded epithelial migration and may have inhibited epithelial cell mitosis, thus contributing to the observed delay in healing. Nonetheless, when exuberant granulation tissue developing under bandages is excised in a timely fashion, rates of epithelialization and wound contraction return to normal. Autologous platelet gel accelerates epithelialization of full-thickness wounds in humans. Specifically, when the platelet count in the gel is > 6 times the baseline count, neoepithelium appears 3 days earlier than when gel is not applied. In the study reported here, topical application of autologous PRP did not enhance wound epithelialization, although platelet counts in the PRP were < 6 times baseline values.

Transforming growth factor-β1 plays a major role in wound healing through recruitment of macrophages and fibroblasts as well as stimulation of collagen production and inhibition of its degradation via down-regulation of matrix metalloproteinases. Transforming growth factor-β1 concentrations in TWs were 1.6-fold higher at 1 week following PRP application than in CWs. It is possible that the increase in TGF-β1 concentration was of insufficient magnitude to bolster a more robust inflammatory response or to promote the differentiation of fibroblasts toward a contractile phenotype. Alternatively, prolonged expression of TGF-β1 would favor collagen buildup, leading to the development of exuberant granulation tissue. To verify this, it would be interesting to measure TGF-β1 concentrations at a later time point in wound specimens in an effort to evaluate whether a 1-time topical application of PRP induces long-term increases in the expression of this protein. Although release of secretary proteins by platelets begins within 10 minutes of clotting, with more than 95% of the presynthesized mediators secreted within 1 hour, platelets continue to produce and secrete additional proteins for the balance of their lifespan (5 to 10 days). Moreover, a unique feature of TGF-β1 is that it can regulate its own production by monocytes and activated macrophages in an autocrine manner, resulting in sustained expression at the wound site following a single exogenous application.

Despite the considerable amount of granulation tissue within TWs, an increase in biomechanical strength was not detected in healed scars in the study reported here. This contrasts the findings of Kimura et al, who reported that treatment with platelet releasate enhanced the strength of wounds allowed to heal by second intention in guinea pigs. It also contrasts the findings of Ksander et al who reported increased breaking strength of incisional wounds of rats, 14 days following creation and treatment with PRP. Biomechanical data from the study reported here indicate that PRP did not alter, in a positive or negative fashion, the maturity of the wound during the remodeling phase of repair, which is corroborated by the lack of significant increase in the ratio of collagen type I to collagen type III mRNA found in TWs.

In conclusion, 2-time topical application of autologous PRP to small, granulating full-thickness wounds healing on the limbs of horses did not accelerate or improve the quality of repair; nor did it obviate the development of exuberant granulation tissue. These findings indicate that this treatment might hold more promise for the management of avulsion-type wounds with massive tissue loss or, alternatively, of chronic wounds that might benefit from a fresh source of mediators to accelerate the healing process.

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