Evaluation of a filter-prepared platelet concentrate for the treatment of suspensory branch injuries in horses.

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Keywords

Platelet, horse, suspensory, filtered, concentrate

Summary

Objectives: Platelet preparations have become a treatment for soft tissue injuries in horses. This study evaluated a novel filter-based system to concentrate platelets and assesses its value in the treatment of suspensory ligament branch desmitis.

Methods: Filtered platelet concentrate was prepared from 55 ml of venous blood obtained from 21 normal horses. Platelets and white blood cells in whole blood and filtered platelet concentrate were measured, as was platelet activating factor (PAF)–induced platelet-derived growth factor-BB (PDGF-BB) release. Eleven horses with 18 focal acute suspensory ligament branch injuries were treated intra-lesionally with autologous filtered platelet concentrate and evaluated clinically and ultrasonographically for one to three years.

Results: The increase in concentration of platelets in the filtered concentrate in comparison with whole blood (6.9 ± 1.9-fold) was significantly greater than the increase in white blood cells (3.8 ± 0.8-fold) ($p < 0.0001$). There was no effect of sex or breed on platelet concentration. Platelets were responsive to PAF with >100-fold higher levels of PDGF release over basal levels. All hypoechoic lesions re-evaluated within three months had resolved. Five of the 11 horses returned to their previous level of work, one was exercising at a lower level, three were retired, one died for unrelated reasons, and one was still convalescing.

Clinical significance: Filtered platelet concentrate was easily and reliably prepared and injected into suspensory ligament branch injuries without short-term complications. This treatment was associated with rapid resolution of ultrasonographic lesions and lameness. Filtered platelet concentrate represents a convenient alternative for the treatment of suspensory ligament branch injuries.

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Vet Comp Orthop Traumatol 2011; 24: 363–369
doi:10.3415/VCOT-11-01-0001
Received: January 3, 2011
Accepted: May 9, 2011
Pre-published online: September 2, 2011

Financial support:
The study was supported, in part, by VetCell Ltd. and the Pall Corporation.

Introduction

Equine suspensory ligament injuries are a common cause of persistent lameness and wastage within the equine industry, even with current treatment strategies, thus making an effective, convenient, and economical treatment attractive to veterinarians and horse owners alike.

The two branches of the suspensory ligament arise from the body of the suspensory ligament in the distal half of the metacarpal and metatarsal regions. Injuries to the branches are common in most equine disciplines (1). In a study in non-racehorses, forelimb injuries occurred in both medial and lateral branches with similar frequency, whereas in the hindlimb most injuries affected the lateral branch (2). Clinical onset is usually sudden and associated with over-extension of the metacarpo- or metatarso-phalangeal joint, and it is believed that the clinical injury is preceded by a phase of cumulative degeneration (3). In support of this hypothesis, a study of racehorses in Japan showed that suspensory ligament injuries occurred five-fold more frequently after five years of age compared to two-year-old racehorses (4).

Diagnosis of suspensory ligament injuries is often straight-forward as there is usually lameness with swelling over the affected ligament. Diagnosis can be confirmed ultrasonographically where the injury is manifested as focal or generalised hypoechogenicity and enlargement (1). Diagnostic analgesia requires peripheral nerve blocks of both the palmar or plantar and palmar metacarpal or plantar metatarsal nerves, proximal to and on the same side as

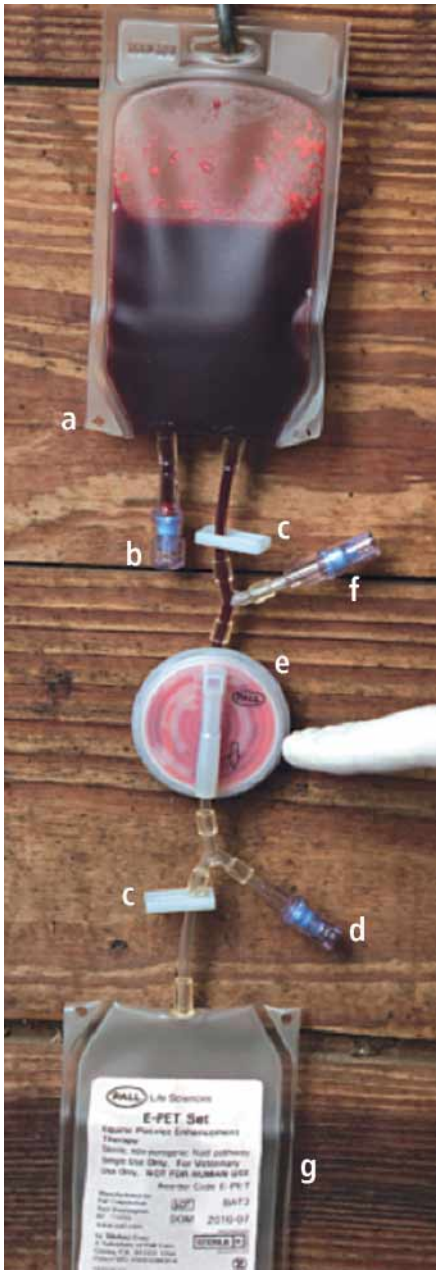

Fig. 1 The filtration system for preparing platelet concentrate: (a) Upper bag containing venous blood, anti-coagulant, and sterile water for injection – all added through (b) Port 1. (c) Clamp. (d) Port 2– used for injection of harvest solution. (e) Filter. (f) Port 3– used for sterile collection of platelet concentrate. (g) Collecting bag.

the lesion in the suspensory ligament branch (5, 6). More chronic cases are associated with peri-ligamentar fibrosis and, occasionally, peripheral lesions can communicate with the adjacent metacarpo- or metatarso-phalangeal joint (7, 8).

There is little objective outcome data available regarding the management of suspensory branch injuries and hence it is difficult to determine the effectiveness of one treatment over another. Van den Belt reported that 58% of forelimb injuries, but only 22% of hindlimb injuries, return to the same level of exercise although the position of the lesion within the suspensory ligament was not recorded and proximal suspensory desmitis carries very different prognoses between fore- and hindlimbs (9–11). However, suspensory ligament injuries are traditionally thought to be problematical, either because the ligament heals with fibrosis (both intra- and peri-ligamentous) which compromises the mechanical function of the ligament and increases the risk of re-injury, or healing is incomplete or defective and results in persistent pain (and lameness) and ultrasonographically evident lesions (1, 12). Suspensory ligament lesions in eventing horses have been reported to carry a poorer prognosis (13).

In an attempt to minimise these complications, a number of different treatments have been proposed, including intralesional administration of hyaluronic acid and beta-aminopropionitrile fumarate, as well as extracorporeal shockwave therapy and surgical splitting (1). More recently, the intra-lesional use of 'biological' preparations such as lyophilised swine bladder submucosa, stem cells, and platelet-rich plasma (PRP) have been reported in small case series (14-16).

Platelet-rich plasma is a preparation made from autologous whole blood with a composition of cellular elements that vary with the centrifuge used and the protocol employed. Common to most products is that the final preparation contains enriched numbers of platelets and may arguably be more correctly termed platelet concentrate (17). Some devices produce a product with low white blood cell (WBC) or red blood cell content, whereas others have these cells present. Preparations of platelet concentrate typically contain in excess of four times the concentration of platelets in comparison to that of whole blood although this is not always the case for preparations used clinically (15, 18).

The rationale for the use of PRP is that it is capable of delivering high concentrations of a number of anabolic growth factors present in platelets, most notably transforming growth factor beta 1 (TGF-β1) and platelet-derived growth factor BB (PDGF-BB). Significant anabolic effects of PRP on equine superficial digital flexor tendon explants as well as suspensory ligament explants and desmocytes have been demonstrated *in vitro* (19–21). Clinically, the use of PRP has become popular in human orthopaedics, including for the treatment of chronic tendinopathy (22, 23). In the horse, recent publications have suggested beneficial effects of PRP when used in an experimental surgically created lesion in the superficial digital flexor tendon (24, 25). There are no published controlled studies of naturally-occurring injuries in horses that demonstrate beneficial effects, although recent publications of small case series have suggested positive effects on suspensory ligament and superficial digital flexor tendon (15, 16).

Platelet-rich plasma can be prepared in a number of ways, most commonly involving two-stage centrifugation or apheresis methods (26, 27). The aim of this study was to evaluate a novel system that utilises filtration rather than centrifugation to prepare a platelet concentrate from equine venous blood. This method is worth evaluating because it is convenient by virtue of it being self-contained, easily transported and fast with only 15 minutes required for the preparation of platelets. Furthermore, it is a functionally closed system, thereby minimizing the risk of infection. This study characterizes the cell product, and evaluates its short-term effect in the treatment of focal suspensory ligament branch injuries in horses.

Materials and methods

Evaluation of recovery and quality of platelet concentrate

Platelet concentrate was prepared from 55 ml venous blood collected in 5 ml acid citrate dextrose anticoagulant from 21 normal horses utilising a closed filtration system[a] (▶ Fig. 1). Briefly, the 60 mL of antico-

agulated venous blood was added to the blood bag to which 9 ml of sterile water for injection (capture solution) had been previously added. This ratio of water to blood results in a slight lowering of the osmolarity which causes the platelets to swell and assists in the filtration process, before the solution is passed through a filter where the platelets were captured. Back-flushing 8 ml of a proprietary harvest solution through the filter allowed for recovery of the platelets into a syringe in approximately 6 ml of the plasma and harvest solution mixture with restored (normal) osmolarity.

For each horse, the breed, age, and sex were recorded. The platelet and WBC concentrations in the venous blood and in the platelet concentrate were measured, and the platelet and WBC concentration factors calculated. Cell counts were performed using an automated haematology analyser with a veterinary pack validated for horse blood[b].

To determine whether the recovered platelets were intact and functional, agonist-induced growth factor release was measured. A working solution of platelet activating factor (PAF; β-Acetyl-γ-O-alkyl-L-α-phosphatidylcholine[c]) was prepared by dissolving 1 mg/mL PAF in 0.15M sodium chloride containing 2.5 mg/mL bovine serum albumin (BSA). Five hundred µL whole blood or filter harvested samples were added to 1.5 mL microfuge tubes to which 5 µL of PAF working solution were added (1:100 dilution), incubated at room temperature for 30 minutes, centrifuged at 3,768 x g for 7 min to sediment cells, platelets and cellular debris before an aliquot of the supernatant was transferred to a separate microfuge tube and kept frozen at –80 C prior to assay for PDGF-BB. Platelet-derived growth factor-BB was assayed using an enzyme-linked immunosorbent assay[d] in accordance with the manufacturer's instructions for use. This assay had previously shown cross-reactivity to horse PDGF-BB (19).

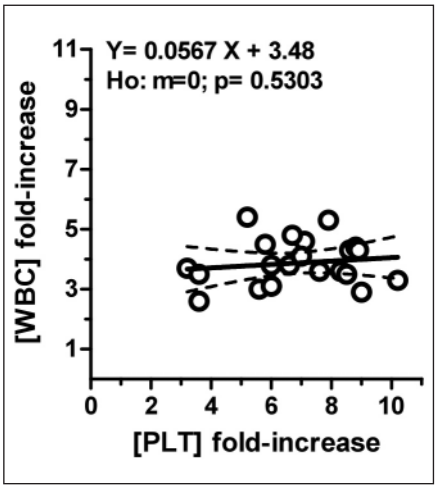

Fig. 2 Relationship between concentration factors for platelets and white blood cells using the filtration system in normal horses. The concentration of platelets (average = seven-times) appears to be independent of the white blood cells which average approximately four-times that of venous blood.

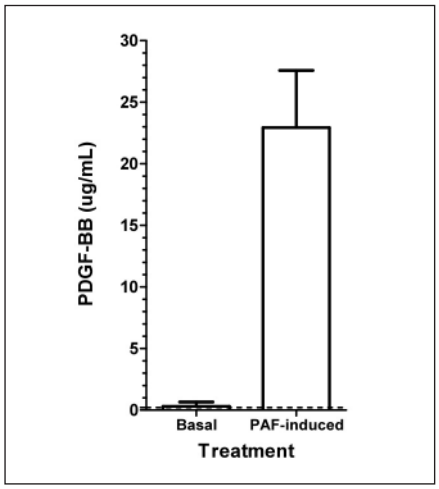

Fig. 3 Platelet-derived growth factor (PDGF) concentrations before and after activation with platelet activating factor (PAF) in normal horses, demonstrating the presence of functional platelets.

Clinical cases

Eighteen focal suspensory branch injuries in 11 horses were treated with the autologous platelet concentrate. All injuries, apart from in one horse (horse 8), had occurred within the previous six weeks. In seven of the treated cases, platelet numbers were calculated from both the venous blood and platelet concentrate. Under standing sedation and after regional analgesia of the palmar and palmar metacarpal, or plantar and plantar metatarsal nerves, immediately proximal to, and on the same side as the branch injury, 2.5 ml of platelet concentrate was injected aseptically under ultrasonographic guidance into the lesion.

Lameness was graded on a scale of 0–10 before treatment and up to three months afterwards (28). Ultrasonographic examinations were performed prior to, and up to three months after treatment. Resolution of lameness, and the ultrasonographic improvement in hypoechogenicity and cross-sectional area of the lesion were assessed. Follow-up of cases was made by telephone contact with the owners up to three years after treatment.

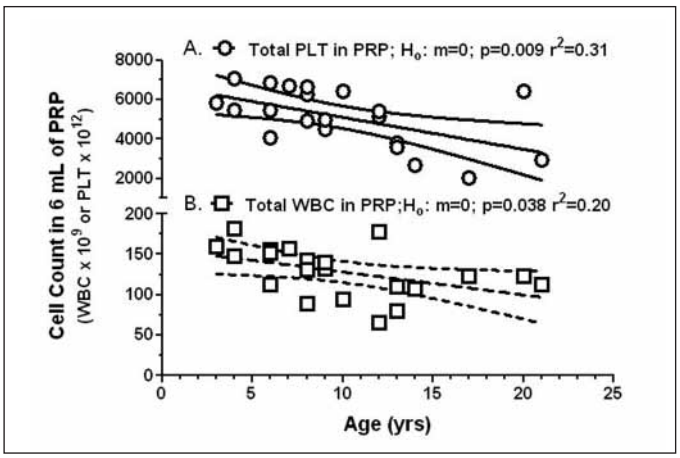

Fig. 4 The variation of total recovered (A) platelet and (B) white blood cell numbers (in 6 ml of capture solution) with age in normal horses.

[a] Equine Platelet Enhancement Therapy (product code, E-PET): Pall Corporation, Port Washington, NY, USA
[b] CellDyn 3700: Abbott Labs, Abbott Park, IL, USA
[c] Cat No. P7568: Sigma-Aldrich, St. Louis, MO, USA
[d] DuoSet® ELISA Development System DY220: R&D Systems, Inc.; Minneapolis, MN, USA

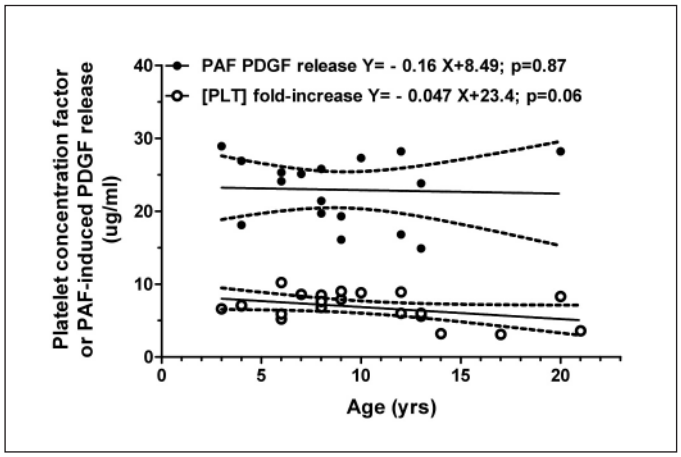

Fig. 5
The variation of platelet concentration factor and platelet-derived growth factor concentrations in the platelet concentrate with age in normal horses.

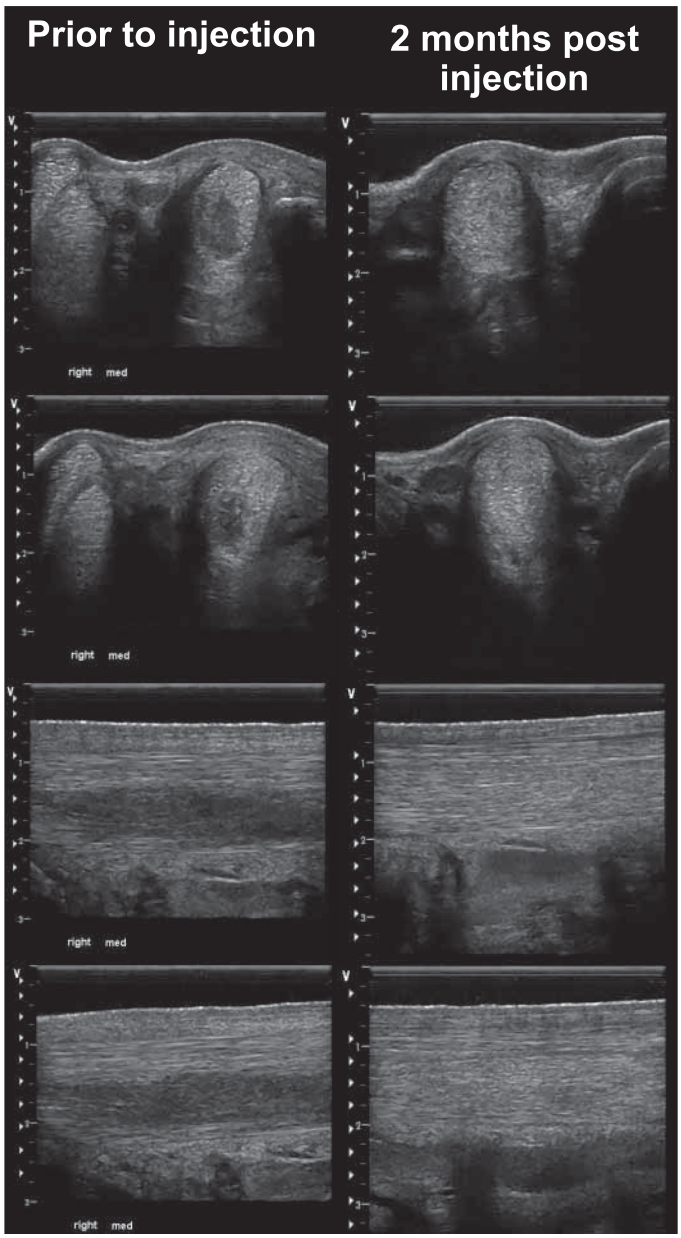

Fig. 6
The resolution of a focal lesion within the medial suspensory ligament branch of the right forelimb in a five-year-old Thoroughbred racehorse. Note the filling in the lesion and the good longitudinal pattern two months after injection with platelet concentrate.

Statistics

Data are presented as means and standard deviations. A Wilcoxon signed rank test was used to assess significance of changes in PDGF before and after activation with PAF. Linear regression analysis was used to determine any significant association between platelet concentration and PDGF levels with age. In order to test the hypothesis that the slope of the regression line was different from zero, the F test was was used. Values of $p < 0.05$ were taken as statistically significant. Data analyses were performed using either Excel[e] or Prism[f].

Results

Evaluation of recovery and quality of platelet concentrate

The age range of the 21 donor normal horses was three to 21 years (mean 11 ± 5 years). There were 10 mares and 11 geldings. There were seven Thoroughbreds, four Quarter horses, five Warmbloods, three ponies, one Standardbred, and one Cleveland Bay. There were not any significant differences in platelet recovery, nor platelet concentration between the sexes and breeds, and thus values from all the normal horses were pooled.

The average platelet concentration of the venous blood was 125 x 10^9/l (± 21) while that of the concentrate was 850 x 10^9/l (± 244). The platelet concentration factor averaged almost seven-times that of venous blood (6.9 ± 1.9). In contrast, the average WBC count of the venous blood was 5.66 x 10^9/l (± 1.34) compared to 21.4 x 10^9/l (± 5.2) for the concentrate, which was an average concentration factor of only four-times (3.8 ± 0.8). The fold increase in platelet concentration did not correlate with that of WBC (▶ Fig. 2; $p = 0.53$ for the slope being significantly different from zero).

Platelet-derived growth factor concentrations before activation (n = 17) were negligible (0.04 µg/ml ± 0.15). Following activation, PDGF concentrations were 22.9

[e] Microsoft Corp.; Redmond, WA, USA
[f] Prism, Version 5: Graph Pad, Inc., San Diego, CA, USA

Table 1 Affected branch, initial lameness score and outcome for 11 horses with desmitis of one or more suspensory ligament branches.

Horse	Limb affected	Branch affected	Lameness score at treatment	Lameness score at 3 months	Resolution of hypoechoic lesion on ultrasound at 3 months	Long-term outcome	Long-term follow-up details
1	Right forelimb	Lateral	3	0	Yes	Died (unrelated reason)	Died during convalescence.
2	Left hindlimb	Medial	2	0	Yes	Retired	Retired due to suspensory origin breakdown after convalescence.
		Lateral			Yes		
3	Left forelimb	Medial	2	0	Yes	Returned to previous level	Sound and resumed full work after 9 months.
		Lateral			Yes		
	Right forelimb	Medial	3	0	Yes		
4	Right forelimb	Lateral	1	0	Yes	Retired	Retired to stud.
	Left forelimb	Lateral	1	0	Yes		
5	Left hindlimb	Medial	2	0	Yes	Returned to previous level	Raced successfully 9 months post-injury and 14 times since.
6	Left hindlimb	Lateral	2	0	Yes	Returned to lower level	Sound and in work at a lower level.
7	Right forelimb	Medial	3	0	Yes	Returned to previous level	Rested for 8 months, raced successfully 8 times since.
8	Right hindlimb	Lateral	2	1	ND	Still convalescing	Became sound 10 months after treatment. Re-examined 12 months after treatment when sound but not returned to work. Hypoechoic lesions on ultrasound resolved.
		Medial			ND		
	Left hindlimb	Lateral	2	1	ND		
		Medial			ND		
9	Right forelimb	Lateral	4	0	Yes	Returned to previous level	Sound and in full work for 3 years post-injury.
10	Right forelimb	Lateral	1	0	Yes	Retired	Retired to breeding after 1 year.
11	Left forelimb	Lateral	3	0	Yes	Returned to previous level	Sound and in full work for 3 years post-injury.

Key: ND = No data available.

(± 4.5) µg/ml of platelet concentrate indicating the presence of functional platelets (▶Fig. 3).

The variation in the total numbers of platelets and WBC recovered with age is shown in ▶Figure 4. There was an age-related decrease in both of these parameters ($p = 0.009$ and $p = 0.038$, respectively), but when the platelet concentration factor and concentrations of PDGF were compared with age, there was not any statistically significant reduction in PDGF release, and only an age-related trend (▶Fig. 5; $p = 0.06$) toward a decline in platelet concentration factor was evident.

Clinical cases

The venous sample averaged 92.8 (± 3.5) x 10^9 platelets/l, while the concentrate averaged 648.0 (± 312.6) x 10^9 platelets/l, indicating an average 6.9 (± 3.1) concentration factor, which was not significantly different from the concentration factor calculated in the normal horses.

Lameness scores reduced from an average of 2 out of 10 (± 0.79) at the time of treatment to 0 for 10 of the 11 horses within three months (▶Table 1). In all suspensory ligament lesions which underwent an ultrasonographic examination at three months (10/11 horses), the platelet concentrate resulted in resolution of the focal lesion (▶Fig. 6, ▶Table 1). Five of the 11 of the horses returned to their previous level of work, three out of 11 were retired, one was exercising at a lower level, one died for unrelated reasons during convalescence, and one was still convalescing.

Discussion

The preparation of platelet concentrate using the filtration system appears to yield platelet counts averaging seven-times the counts in venous blood, which is higher than that in preparations used in previously published case series (15, 16). The added advantage of the filtered system is

that it avoids the need for a centrifuge so that it can be used 'horse-side', and it is a closed system which minimises the risk of iatrogenic sepsis.

Although there appeared to be an age-related decline in the total number of platelets recovered, this was not evident in either the whole blood (data not shown), nor in the platelet concentration factor and, most importantly, the total PDGF released, suggesting that platelet responsiveness to agonist-induced growth factor release is retained even in older animals.

The presumed mechanism of therapeutic action associated with administering high concentrations of platelets is not clear, although the anabolic effects of the growth factors contained within the platelets provide one possible rationale for their use. Hence it was important to demonstrate that there were high concentrations of the PAF-induced platelet-derived growth factor in the filter-derived platelet preparation (on average 22.9 µg/ml. The functionality of the platelets was demonstrated by the absence of significant PDGF activity before activation. While activation can be considered necessary for the use of platelet preparations topically, it has been suggested that endogenous activators are sufficient to negate the need of activation of the platelet concentrate prior to injection into damaged ligaments (20).

Recent experimental data has indicated a beneficial effect of PRP administered to surgically created lesions in the superficial digital flexor tendon (24, 25). However, the reported data of increased tissue stiffness, higher glycosaminoglycan content, and increased neovascularisation are also signs of increased fibrosis, and so the exact benefit of PRP still needs to be further clarified. Increased fibrosis of the suspensory ligament may impart greater strength to the tissue to resist re-rupture, but it may also have an adverse effect on increasing ligament stiffness. The suspensory ligament appears to stretch more than the other palmar and plantar soft tissue structures at the walk and trot, but it is not known to what extent the suspensory ligament contributes to the energetic efficiency of the equine distal limb, and hence the consequences of increasing its structural stiffness (29, 30).

The use of PRP has also gained recent popularity in human medicine for the treatment of tendon injuries although there is, as yet, no current convincing evidence of efficacy in clinical cases because many of these studies are inadequately powered (31–33).

As the cases treated were naturally-occurring lesions, the size of the lesion was not controlled and thus varied between cases. However, the location, type and size of the lesion has been reported to not be correlated with outcome (7). It was not possible to determine if this treatment is more effective than other treatments as this study was not controlled, and to the best of our knowledge, there were no published reports for outcome of suspensory branch desmitis treated conventionally. It was interesting to note that the lameness was slower to respond in the horse that presented for treatment with more chronic pathology (horse 8).

The cases treated in this series demonstrated that the platelet concentrate was well tolerated, showed no short-terms complications, and was associated with resolution of suspensory branch ultrasonographic lesions and lameness. It was not possible to compare this treatment with spontaneous healing to determine if the platelet concentrate accelerates the rate of resolution of the lesion. Longer-term follow-up is needed to provide evidence that the effects of platelet concentrate are to improve the functional outcome of suspensory branch injuries.

Acknowledgements

The authors acknowledge the assistance of Mr. Greg McGarrell and VetCell Ltd. in the running of this study. The study was supported, in part, by VetCell Ltd and the Pall Corporation.

Conflict of interest

RKWS is a Technical Adviser of VetCell Ltd. Both GAO and JS work for Pall Corporation, which is the manufacturer of the filtration device used in study.

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