



Autologous conditioned serum: The comparative cytokine profiles of two commercial methods (IRAP and IRAP II) using equine blood

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Summary

Reasons for performing study: Osteoarthritis (OA) is one of the most prevalent and debilitating conditions affecting the horse. Autologous conditioned serum (ACS), commercially available as IRAP and IRAP II, is a recently developed treatment for OA in which plasma is prepared from venous blood by incubation with glass beads for 24 h. This product has been shown to increase anti-inflammatory cytokines and growth factors in human blood. However, data for equine ACS preparations are lacking.

Objectives: To characterise the protein profiles produced by commercially available ACS systems in equine blood.

Methods: Blood was drawn from 5 horses into 6 groups: red top vacutainer (control), IRAP and IRAP II, with and without heparin. Samples were collected 1 or 24 h post draw and analysed for IL-1ra, IL-10, IGF-1, TGF- β , TNF- α and IL-1 β using ELISAs.

Results: Twenty-four hour IRAP and IRAP II samples contained significantly higher levels of all cytokines relative to 1 h serum controls. At 24 h, IRAP II contained significantly higher levels of IL-1ra and IRAP contained significantly higher levels of TNF- α , compared to 24 h controls. **In addition, TGF- β , IL-10 and IL-1 β in IRAP and IRAP II sera were similar to 24 h serum controls.** The addition of heparin significantly reduced levels of IGF-1, TNF- α and TGF- β , and significantly elevated levels of IL-1ra.

Conclusions: The cytokine profile that IRAP II produced is modestly better than IRAP. Incubation of whole blood in glass tubes stimulated cytokine synthesis, although not as efficiently as IRAP II.

Potential relevance: Although high levels of IL-1ra were found in ACS, elevation of other factors suggests these cytokines play a previously understated role in clinical improvements. Because ACS has been shown to alleviate clinical symptoms of OA, the present study suggests that factors other than IL-1ra alone might be involved in its clinical efficacy. Species-dependent elevations of cytokines warrant further investigation and optimisation of the systems appears to be necessary based on the differences between human and equine blood.

Introduction

Osteoarthritis (OA) is a significant problem in horses due to both the prevalence and economic impact of the disease. Recently, novel biological therapies have emerged as treatment options for both human and equine OA. Autologous conditioned serum (ACS) is one option with published support in both the human and equine fields (Frisbie *et al.* 2007; Moser *et al.* 2007). The method of preparing ACS revolves around culturing whole blood with borosilicate glass beads and harvesting the serum as the therapeutic agent. Presumably the stimulation of the white blood cells is responsible for the cytokine production during the culture period. The initial description of ACS using human blood reported a significant increase in interleukin-1 receptor antagonist (IL-1Ra) of 140-fold post culture (Meijer *et al.* 2003). This study also reported a significant 2-fold increase in interleukin-4 and 10 (IL-4 and IL-10) with no significant increase in the proinflammatory cytokines interleukin-1 β (IL-1 β) and tumor necrosis factor α (TNF- α). Clinical benefits have been realised in man in a variety of musculoskeletal applications including OA (Moser *et al.* 2007). The first controlled study assessing the symptom and disease modifying properties of IL-1ra (generated by another technique) was completed in an equine OA model (Frisbie *et al.* 2002). That study was also able to show both symptom and disease modifying effects. Furthermore, a study by the same authors using a similar model of equine OA showed an increase in endogenous IL-1ra was systemically induced following ACS treatment (Frisbie *et al.* 2007). Since this period another commercial technique has been developed using borosilicate beads, named IRAP II², and is marketed by a different company compared to the original IRAP¹ commercial product.

The manufacturer of IRAP II has also published cytokine levels using human blood with this refined method (Bare 2008). In this study IRAP II was compared to uncultured blood and IRAP ACS, showing superior results to both. Specifically, this study showed IRAP II had a 10 x and 7 x significant increases in IL-1Ra compared to baseline and IRAP, respectively. An increase in proinflammatory cytokines was also seen with both ACS methods, more consistent with what would be expected. Serum from IRAP II also had a 4 x level of IL-1ra/IL-1 β ratio compared to IRAP.

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While the *in vitro* results of this new method appear promising, clinical results are not yet available. Furthermore, it is apparent that species differences exist with many of the biological therapies and *in vitro* equine-specific information on either ACS system is still lacking.

The objective of this *in vitro* study was to determine levels of inflammatory cytokines (TNF- α), anti-inflammatory cytokines (IL-10 and IL-1ra) as well as a growth factors (IGF-1 and TGF- β) in equine whole blood incubated with the IRAP and IRAP II systems compared to baseline serum and whole blood (clot tube) cultured in a glass tube. Protein levels were quantified by enzyme-linked immunosorbent assay (ELISA) and real-time PCR for mRNA levels.

Materials and methods

Sample collection

All protocols and procedures were approved by Colorado State University Animal Care and Use Committee. Blood was collected from 5 healthy horses, 2–5 years of age. Specifically, using a single venipuncture, blood from each horse was collected into two 10 ml glass red-top vacutainer tubes³ as a control, four 60 ml IRAP¹ and four 50 ml IRAP II² syringe systems (Fig 1). One set of IRAP and IRAP II syringes, which were processed in parallel, had 1 μ l/ml heparin⁴ added to ensure no clot formation.

Sample preparation

All blood samples were incubated at 37°C for 1 h. Samples (one red top, one heparinised IRAP as well as one heparinised IRAP II) were then centrifuged and serum collected and stored at -80°C until analysis. These samples were designated as the baseline controls. A second sample for each condition was cultured for

24 h at 37°C before centrifugation. All samples were spun in a Hermle Z300 centrifuge⁵ at 2500 $\times g$ for 10 min. After centrifugation, the serum was removed using an 18 gauge needle and a 20 ml polypropylene syringe. All serum samples were filtered through a 0.22 μ m filter⁶, aliquoted into 2 ml cryovials⁷ and stored at -80°C until analysis.

Serum protein analysis

Commercially-available ELISA kits were used to determine serum concentrations of TNF- α ⁸, IL-1 β ⁹, IL-10¹⁰, IL-1ra¹⁰, IGF-1¹¹ and TGF- β ¹². All assays were run according to the manufacturer's instructions, with the exception of minor protocol modifications for the equine-specific IL-1ra, TNF- α and IL-10 assays. The cross-reactivity of the TGF- β and IGF-1 ELISAs in equine serum have been previously validated (De Kock *et al.* 2001; Dalhgren *et al.* 2005). For the IL-1ra ELISA, a 2-fold capture and 1.5-fold detection antibody concentration over the recommended levels were used for all serum samples. A plate shaker was utilised to increase antibody-antigen interactions.

Serum (from one red top, IRAP and IRAP II at 1 and 24 h with and without heparin) was thawed to room temperature and tested according to each kit's protocol. Plates were washed using a Bio-Tek ELx405 plate washer¹³ and serum samples from each horse assayed in triplicate. Absorbance was determined using a Spectramax 384 Plus microplate absorbance reader¹⁴ and each serum concentration determined based on a standard curve of optical density vs. concentration.

Gene expression analysis

Extraction of RNA was performed at either one (baseline) or 24 h post collection utilising a buffy coat method with modifications for

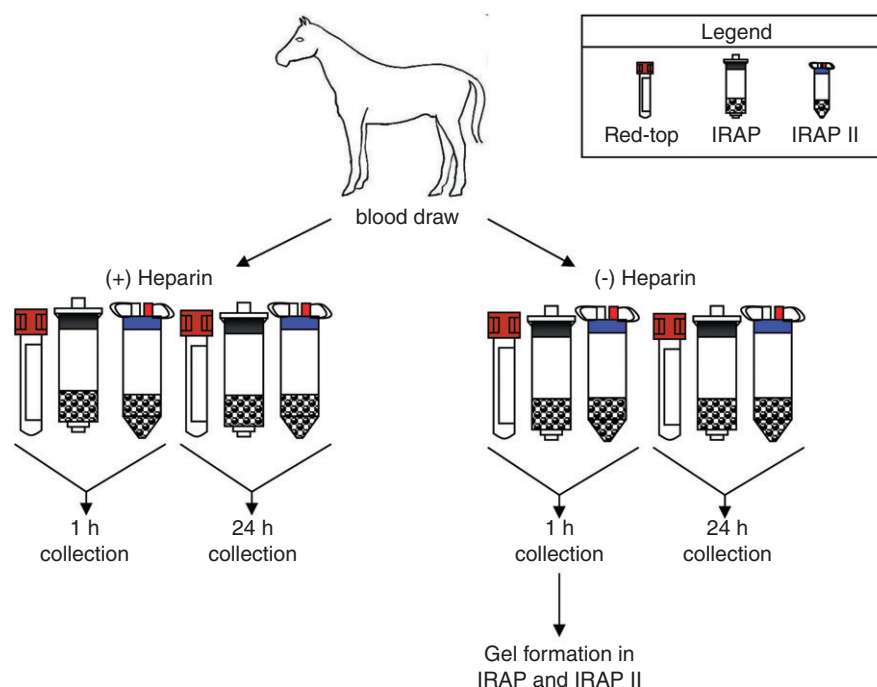


Fig 1: A flow diagram of the blood draw, treatment and incubation. Blood was drawn into 4 sets of red top, IRAP or IRAP II tubes. Heparin was added to 2 sets and 2 sets did not receive heparin. Of these, half the samples were incubated for 1 h and half for 24 h before collection. A gel-like clot formed in the 1 h IRAP and IRAP II.

TABLE 1: Forward and reverse primer sequences for quantitative real-time PCR

Gene		Sequences	References
IL-1 β	F	5'-GCAGTACCCGACACCAGTGA	Leutenegger <i>et al.</i> (1999)
	R	5'-TTTGGGCCATCCTCCTCA	
IL-1Ra	F	5'-GAGAAGATAGATGTGGTGCCATTG	
	R	5'-GTCAGTGATGTTAACTGCCTCCAAT	
IL-4	F	5'-TCGTGCATGGAGCTGACTGTA	
	R	5'-GCCCTGCAGATTTCTTTCC	
IL-10	F	5'-TTCAGCAGGGTGAAGACTTTTC	Riihimaki <i>et al.</i> (2008)
	R	5'-CTTGGCAACCCAGGTAACCCTTA	
COMP	F	5'-ACCCAGACCAGAGGGACGTG	
	R	5'-CCCATCCTTGCTTGGTCGCT	
Decorin	F	5'-GATTTGGGTCTGGACAAAGT	
	R	5'-GTTGACAAGAATCAACGCATGAAG	
FGF	F	5'-ACCCGACGGCCGA	
	R	5'-CTCTTCTGCTTGAAGTTGTAGTTTGA	
IGF	F	5'-TGTA CTGCGCACCCCTCAA	Dalhgren <i>et al.</i> (2005)
	R	5'-TGC GTTCTTCAAATGACTTTCCTTCT	
TGF- β	F	5'-TCCTGGCGCTACCTCAGTAAC	Dalhgren <i>et al.</i> (2005)
	R	5'-GGTGACATCAAAGGACAGCCATTC	
TNF- α	F	5'-GCCCAGACACTCAGATCATCTTC	Riihimaki <i>et al.</i> (2008)
	R	5'-CATTGACAGCCCACTCA	
18s	F	5'-CGGCTTTGGTGACTCTAGATAACC	Dalhgren <i>et al.</i> (2005)
	R	5'-CCATGGTAGGCACAGCGACTA	

coagulated blood (Feezor *et al.* 2004). Briefly, following removal of the serum layer for protein analysis, the remaining fraction was homogenised by vortexing at high speed for 2 min. Samples were re-separated by centrifuging at $500 \times g$ for 5 min and the buffy coat removed using a pipette tip. This fraction contains the leucocyte population as well as contaminating erythrocytes and thrombocytes. Residual erythrocytes were lysed in 3 consecutive washes of 0.01 mol Tris-HCl in a ratio of 20:1 (lysis buffer:cell pellet) and incubated at 4°C for 15 min. The leucocyte fraction was collected by centrifugation at $200 \times g$ for 10 min followed by subsequent decanting of the lysis buffer. Following the third wash, the supernatant was completely aspirated and the cell pellet resuspended in 350 μ l of RLT buffer¹⁵ in order to lyse the cells. RNA was then extracted using a commercial RNA purification kit¹⁵. The RNA quantity and quality was verified using a Nanodrop ND-1000 spectrophotometer¹⁶ and 1 μ g of RNA was reverse transcribed to cDNA using a SuperscriptIII Reverse Transcription Kit¹⁷.

Quantification of gene expression was carried out by means of real-time polymerase chain reaction using an ABI Prism 7000 Sequence Detection System¹⁸ in 25 μ l reaction volumes containing 12.5 μ l of Sybr Green Master Mix¹⁵, 1.25 μ l of forward and reverse primers (at 20 μ M), 11.25 μ l of nuclease-free water and 10 ng cDNA. Primers were either previously published or newly designed for equine-specific sequences (Table 1). Newly designed primers were designed utilising Vector NTI software¹⁸ to span exon-exon boundaries and validated for application efficiencies. Results were normalised to 18S expression and analysed using the $\Delta\Delta$ Ct method (Livak and Schmittgen 2001).

Statistical analysis

A mixed model analysis of variance was utilised to assess the data. Fixed variables were the collection period (1 or 24 h post collection) or treatment (red top, IRAP or IRAP II). Both main and interaction effects were assessed, where the horse was considered a random effect. When individual comparisons were made, a least square means was used and for all comparisons a P value <0.05 was

considered significant. Log transformations were used when inadequate variance was indicated by residual plots.

Results

Sample collection

Baseline samples were collected for each treatment group and initially given 1 h to clot. However, the serum layers for both IRAP and IRAPII tubes without heparin formed a gel-like clot upon centrifugation. In the 24 h incubation the clot retracts and the serum can be collected. To investigate this phenomenon further blood was collected and centrifuged 0.25, 0.5, 1 and 2 h after collection. All additional time points produced a clot and only the 24 h period was not associated with clot formation. The addition of heparin was found to prevent clot formation in all cases. Because of this clotting, the 1 h IRAP and IRAP II samples did not produce serum and were, therefore, unusable as controls. For this reason the 1 h serum data is presented as a control.

Growth factor and cytokine quantification

The 24 h serum control was found to significantly increase levels of IL-1ra when compared to the 1 h control (Fig 2). Concentrations of IL-1ra in IRAP were not found to be significantly different from the 24 h control. IRAP II significantly increased IL-1ra protein concentration compared to IRAP as well as the 1 and 24 h controls. When levels of IL-1ra are normalised to IL-1 β , the only significant difference was IRAP II when compared to the 1 h control (Fig 3). Likewise, IL-10 levels were significantly higher in IRAP and IRAP II samples compared to the 1 h control (Fig 2). There were no significant differences in the levels of IL-10 found in IRAP, IRAP II and 24 h control samples.

No significant change in TNF- α concentration was seen in the 24 h control when compared to the 1 h control (Fig 4). The TNF- α concentrations in IRAP were found to be significantly higher compared to both 1 and 24 h controls, but not significantly higher than IRAP II. Concentrations of TNF- α in IRAP II showed no

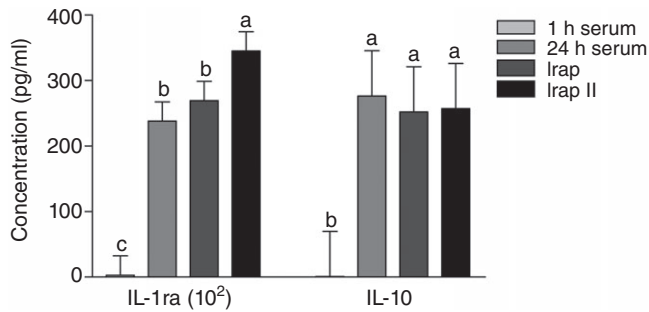


Fig 2: Serum and ACS concentrations of anti-inflammatory cytokines IL-1ra (10^2) (pg/ml) and IL-10 (pg/ml). Bars represent the mean of $n = 5 \pm$ s.e. Different letters indicate significant differences between groups.

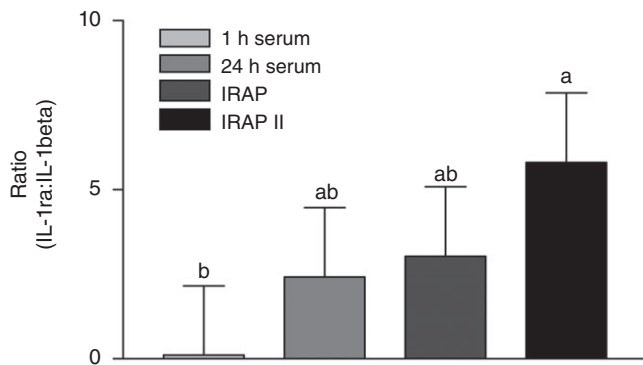


Fig 3: Ratio of IL-1ra:IL-1 β in serum and ACS. Bars represent the mean of $n = 5 \pm$ s.e. Different letters indicate significant differences between groups.

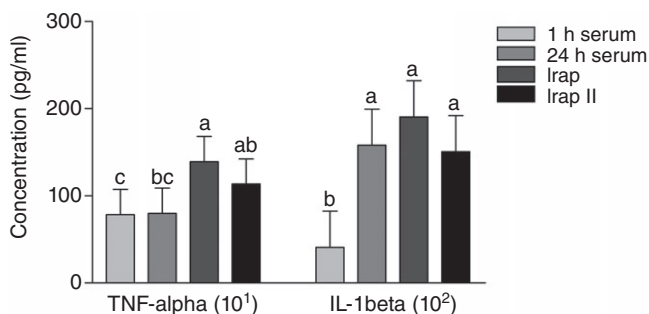


Fig 4: Serum and ACS concentrations of inflammatory cytokines TNF- α (10^1) (pg/ml) and IL-1 β (10^2) (pg/ml). Bars represent the mean of $n = 5 \pm$ s.e. Different letters indicate significant differences between groups.

significant increase compared to the 24 h control and were significantly higher than the 1 h control. Levels of IL-1 β were significantly higher in IRAP, IRAP II and 24 h samples compared to the 1 h controls (Fig 4). There were no significant differences in the levels of IL-1 β found in IRAP, IRAP II and 24 h control samples.

The IGF-1 concentrations were not significantly different when the 1 and 24 h controls were compared (Fig 5). ACS from IRAP and IRAP II demonstrated significant increases in IGF-1 compared to both the 1 and 24 h controls. There was no significant difference in IGF-1 between IRAP and IRAP II. The TGF- β concentrations were significantly higher in the 24 h compared to the 1 h control (Fig 5). A significant increase in TGF- β was also seen with IRAP and IRAP II when compared to the 1 h, but not the 24 h control.

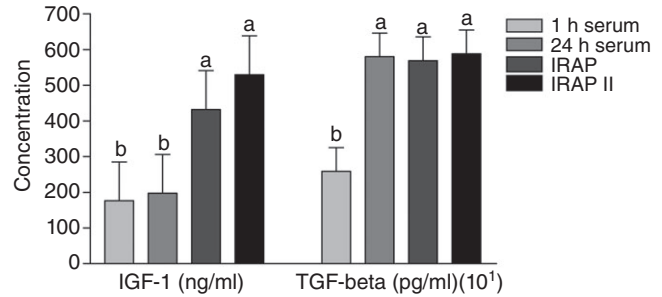


Fig 5: Serum and ACS concentrations of growth factors IGF-1 (ng/ml) and TGF- β (10^1) (pg/ml). Bars represent the mean of $n = 5 \pm$ s.e. Different letters indicate significant differences between groups.

To test the effects of preventing the clotting cascade heparin was added to a duplicate set of IRAP, IRAP II and serum samples. A similar amount of heparin was added in samples that were not treated *in situ* with heparin and no effect on assay performance was noted. The prevention of the clot formation by the addition of heparin resulted in a significant reduction in estimated levels of IGF-1, TNF- α and TGF- β . Conversely, heparin was found to significantly increase IL-1ra concentrations by 1.5-fold.

mRNA quantification

The IL-1ra mRNA levels were significantly higher in the 24 h control and IRAP samples when compared to the 1 h control. No other significant differences were noted in IL-1ra mRNA levels. Both the 24 h control and IRAP II showed a significant increase in IGF-1 mRNA levels when compared to the 1 h control. None of the other target mRNA sequences demonstrated significant differences for any comparison.

Discussion

Interleukin-1 receptor antagonist, commonly thought to be a main contributor to the therapeutic efficacy of ACS, was found to be significantly increased in the IRAP and IRAP II systems. Though levels of IL-1ra were found to be elevated in both ACS systems compared to the 1 h control, the difference was not found to be statistically different when IRAP I was compared to the 24 h control. Previous work using human blood has shown a range between a 3.2- and a 140-fold increase in ACS after a 24 h culture period when compared to baseline levels. Treatment of equine blood with IRAP and IRAP II was found to increase IL-1ra levels 93- and 119-fold over the 1 h control, respectively. Surprisingly, an 82-fold increase in IL-1ra was also seen in the 24 control serum. Meijer *et al.* (2003) previously found consumer-grade glass to increase IL-1ra synthesis in incubated serum; however, the magnitude of the increase depended on the type of glass used (Meijer *et al.* 2003). Here, we demonstrate that incubation of equine whole blood with a medical-grade borosilicate blood tube has the ability to increase IL-1ra levels, but at a lower level compared to IRAP II and without a concomitant increase in IGF-1.

Incubation of whole blood was also found to increase IL-1 β independent of treatment or processing system. Concentrations in IRAP, IRAP II and the 24 h control were all found to be significantly increased when compared to the 1 h control. Compared to the 1 h control, IRAP, IRAP II and the 24 h control

were found to be increased 3.95-, 4.75- and 3.75-fold, respectively. Due to its role as a mediator of osteoarthritis in addition to other orthopaedic diseases, IL-1 β production in ACS is of great importance when considering the therapeutic value of this system. It is also interesting that no increase in IL-1 β or TNF- α were reported by Meijer *et al.* (2003). Therefore, it is considered appropriate to use the IL-1ra/IL-1 β ratio as an indicator of therapeutic efficacy. Only IRAP II was found to have a ratio of IL-1ra/IL-1 β that was significantly better than the 1 h control. Interestingly, this ratio of IL-1ra to IL-1 β is more than 100 times less favourable with equine blood compared to levels recently found with human blood (Bare 2008).

Culture of whole blood for 24 h was found to upregulate the anti-inflammatory cytokine IL-10 independent of the treatment. Concentrations of IL-10 in IRAP, IRAP II and the 24 h control treatment groups were all found to be increased greater than 250-fold compared to the 1 h control. Previous work (Meijer *et al.* 2003; Moser *et al.* 2007; Bare 2008) has shown IL-10 to be significantly increased in ACS as a result of culture with the IRAP syringe; however, these studies used a 1 h baseline as a comparison and did not include a 24 h control. Therefore, this data suggests that IL-10 upregulation may be a consequence of whole blood culture rather than treatment with the IRAP or IRAP II ACS systems.

Likewise, concentrations of the growth factor TGF- β were found to be elevated in the 24 h control as well as the IRAP and IRAP II systems compared to the 1 h control. Independent of treatment condition, incubation elicited a greater than 2-fold increase in TGF- β over the 24 h period. TGF- β concentrations have historically been found to be elevated in ACS (Wright-Carpenter *et al.* 2004a; Moser *et al.* 2007); however, these studies did not include a 24 h control sample. Therefore, our findings indicate that increases in TGF- β , like IL-10, may be a result of whole blood culture, independent of the processing system.

Significant upregulation of the inflammatory cytokine TNF- α was found with IRAP when compared to both the 1 and 24 h control samples. While the TNF- α level was higher than both control samples with IRAP II, the difference was not found to be significant. Because of the potency of TNF- α as a proinflammatory cytokine this difference between the 2 systems is noteworthy.

The IGF-1 protein levels were increased 2.2- and 2.7-fold for IRAP and IRAP II compared to the 1 h control, respectively, with no simultaneous increase in the 1 or 24 h controls. There was a significant upregulation of IGF-1 mRNA in the IRAP, IRAP II and 24 h control serum samples. However, since no increase in IGF protein was measured in the 24 h control, we suspect that transcriptional regulation prevented protein production. We do not have an explanation for this particular finding. A previous study by Wright-Carpenter also found a small, but not significant, elevation in IGF-1 levels in human ACS (Wright-Carpenter *et al.* 2004a), making this significant increase unique to the horse. Because IGF-1 has been found to be a major factor in tissue healing and regeneration, it may play a previously understated role in the clinical improvements seen with equine ACS.

A significant reduction in estimated levels of IGF-1, TNF- α and TGF- β was caused by the addition of heparin to ACS samples before incubation. Conversely, the addition of heparin was found to significantly increase IL-1ra concentrations by 1.5-fold. This indicates that increases in TNF- α and TGF- β may be dependent on the clotting cascade, suggesting the involvement of platelet

degranulation as a cause for this increase in protein levels since it was seen in all other nonheparin samples. Because IGF-1 was not increased in the 24 h nonheparin controls and mRNA levels were increased in both ACS systems, the data suggests that IGF-1 protein levels are dependent on production from the nucleated cell fraction. Also, IL-1ra levels increase independent of the addition of heparin, indicating that this increase may be due to adherence to glass beads, as previously stated (Meijer *et al.* 2003; Wehling *et al.* 2007).

The finding of increased levels of IL-1 β , IL-10, IL-1ra and TGF- β in serum isolated from incubated red top tubes is surprising. However, the stimulatory action of medical-grade glass on whole blood during incubation has been previously reported by Meijer *et al.* (2003), though the stimulatory action depended on the quality of the glass. Therefore, it can be postulated that stimulation of whole blood by the glass of the red top control tubes was responsible for these increases.

To the authors' knowledge, previous *in vitro* published work has utilised human blood. Specifically, one of the first studies published by Meijer *et al.* (2003) reported a 140-fold and 2.2-fold increase for IL-1ra and IL-10, respectively, with no significant increase in the inflammatory cytokines TNF- α and IL-1 β (Meijer *et al.* 2003). Two nonpeer reviewed, commercial reports from Rice University and Arthrex found an increase in both inflammatory and anti-inflammatory cytokines again using human blood. Our findings using equine blood are in accordance with previous reports from Arthrex and Rice University, showing an increase in anti-inflammatory cytokines as well as an increase in TNF- α protein levels in ACS from the IRAP syringe. Our results also indicate that the increases seen in IL-10 levels are largely due to the culture of whole blood rather than treatment with either of the ACS systems. Also of note is the significant increase in IGF-1 found in both IRAP and IRAP II. Although studies in human blood have shown small increases in IGF-1, they were not found to be statistically significant.

The list of cytokines present in ACS is likely to be more extensive than what has been previously reported until now. Also, both within and across studies, mean concentrations of the cytokines detected show a high standard deviation, indicating that cytokine production varies greatly between individuals (Wright-Carpenter *et al.* 2004a,b; Frisbie *et al.* 2007; Moser *et al.* 2007; Bare 2008). Our high standard error is in accordance with the previous data (Wehling *et al.* 2007; Bare 2008), suggesting there is high variability in how each patient's blood reacts to treatment with the ACS systems. In tandem with our findings of elevated growth factor levels in ACS, it stands to reason that other cytokines may play a previously understated role in the clinical improvements seen with ACS in the equine industry. Also, the finding of species-dependent elevations of certain cytokines underscores the need for further study into the effects of ACS as well as species dependent optimisation of the systems.

Conflict of interest

There are no conflicts of interest for the authors.

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Manufacturers' addresses

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- ²Arthrex, Bonita Springs, Florida, USA.
- ³Tyco Healthcare, Mansfield, Massachusetts, USA.
- ⁴APP Pharmaceuticals, Scaumburg, Illinois, USA.
- ⁵Labnet, Edison, New Jersey, USA.
- ⁶Whatman, Maidstone, UK.
- ⁷Wheaton, Millville, New Jersey, USA.
- ⁸Thermo-Scientific, Rockford, Illinois, USA.
- ⁹Promega, Madison, Wisconsin, USA.
- ¹⁰R&D Systems, Minneapolis, Minnesota, USA.
- ¹¹Diagnostic Systems Laboratory, Webster, Texas, USA.
- ¹²Kingfisher, St. Paul, Minnesota, USA.
- ¹³Bio-Tek, Winooski, Vermont, USA.
- ¹⁴Molecular Devices, Sunnyvale, California, USA.
- ¹⁵Qiagen, Valencia, California, USA.
- ¹⁶Nanodrop Products, Wilmington, Delaware, USA.
- ¹⁷Advanced Biosystems, Worthington, UK.
- ¹⁸Invitrogen, Carlsbad, California, USA.

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