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Surgical stress influences cytokine content in autologous conditioned serum

C. T. FJORDBAKK*, G. M. JOHANSEN†, A. C. LØVÅS, K. L. OPPEGÅRD and A. K. STORSET†

Department of Companion Animal Clinical Sciences, Norwegian School of Veterinary Science, Oslo, Norway
†Department of Food Safety & Infection Biology, Norwegian School of Veterinary Science, Oslo, Norway.

*Correspondence email: cathrine.fjordbakk@nmbu.no; Received: 29.09.13; Accepted: 05.04.14

Summary

Reasons for performing study: No recommendations have been made regarding the relative timing of blood collection for autologous conditioned serum (ACS) preparation and surgical procedures.
Objectives: 1) To identify effects of surgical stress on cytokine levels in ACS, 2) identify haematological markers for prediction of cytokine production in ACS and 3) investigate the necessity for specialised ACS containers when preparing a cytokine-rich serum.
Study design: Experimental *in vitro* study.
Methods: Blood was drawn from 15 stallions admitted for elective castration preoperatively and 22–24 h post operatively and incubated in ACS containers and plastic vacutainer tubes containing Z Serum Clot Activator. Concentrations of interleukin (IL)-1 receptor agonist (IL-1Ra), IL-10, IL-1β, tumour necrosis factor (TNF)-α, insulin-like growth factor (IGF)-1 and transforming growth factor (TGF)-β were determined in all serum samples and compared between preparation methods and sampling time by ANOVA. Changes in cytokine levels induced by incubation, defined as delta cytokine, were calculated by subtracting the baseline levels from the levels in incubated samples. Based on post operative serum amyloid A (SAA), horses were grouped into 'mild', moderate' and 'marked' surgical stress; delta cytokine levels in post operative samples were compared between these groups by ANOVA.
Results: Delta IGF-1 was significantly lower in post operative samples compared with preoperative. Horses in the 'marked' surgical stress group had significantly lower delta IL-1Ra and delta TGF-β than the 'moderate' group and significantly lower delta IGF-1 than the 'mild' group. No association between cytokine levels and haematology variables were identified. Cytokine levels were comparable between serum prepared in blood tubes and in specialised ACS containers.
Conclusions: Surgical stress influences the cytokine content in ACS. Useful predictors of cytokine production in ACS were not identified. Specialised ACS containers may not be necessary for preparation of a cytokine-rich serum.

Keywords: horse; regenerative medicine; autologous conditioned serum

Introduction

In recent years, 'biological therapy' for joint disease has gained considerable interest in both human and veterinary medicine [1]. This term usually implies any form of treatment that exploits the body's natural abilities to fight disease; for example by utilising inflammatory cytokine antagonists. As interleukin 1 (IL-1) is considered one of the pivotal inflammatory cytokines in osteoarthritis [2], the endogenous interleukin 1 receptor antagonist (IL-1Ra) has been a focal point of research in biological therapy for joint disease in horses [1]. The potential beneficial effects of this protein in diseased joints were demonstrated in a model of experimentally induced osteoarthritis in horses, where intra-articular treatment with IL-1Ra gene transfer reduced clinical signs and improved histological appearance of the articular cartilage [3].

Autologous conditioned serum (ACS) represents a practical alternative for biological treatment of joint disease. Autologous conditioned serum refers to the cytokine-rich serum harvested after exposing circulating leucocytes to activating surfaces, thereby stimulating *de novo* synthesis of several anti-inflammatory cytokines and growth factors [4,5]. Compared with unmanipulated horse blood, ACS contains increased levels of anti-inflammatory cytokines such as IL-1Ra and IL-10, as well as the growth factors transforming growth factor (TGF)-β and insulin-like growth factor (IGF)-1 [6]. However, the exact composition of cytokines and growth factors in ACS is undetermined [1] and considerable inter-individual differences in cytokine levels have been documented [6]. Also, as incubation of equine whole blood in plastic vacutainer tubes containing Z Serum Clot Activator[a] results in comparable levels of IL-1Ra and IL-10, the need for expensive specialised ACS harvesting systems may be questioned [6]. Intra-articular treatment with ACS resulted in improved clinical signs such as reduced lameness and joint effusion in an experimental model of carpal osteoarthritis in horses [7]. However, histological appearance of the articular cartilage was not different from the untreated placebo group. A recent study investigating the effects of ACS on equine chondrocytes *in vitro*, found only minimal beneficial effect of ACS vs. unconditioned serum on chondrocyte metabolism [8]. Despite obvious paucities in clinical evidence, ACS is popular among equine practitioners and is commonly used in joints unresponsive to other intra-articular remedies or as first-line treatment when cost is of no concern [9]. Prophylactic and post arthroscopic use have also been reported [1].

No recommendations have been made regarding the timing of blood collection for ACS preparation in relation to surgical procedures such as arthroscopy. Elective surgical procedures induce an acute-phase response in the horse quantifiable as an increase in the acute-phase protein serum amyloid A (SAA) peaking approximately 24 h post operatively [10]. As the acute-phase response interferes with leucocyte function [11], ACS prepared from blood drawn post operatively may be inferior in content of cytokines and growth factors compared with ACS prepared from blood drawn preoperatively. Therefore, the objectives of the study were 1) to identify potential effects of surgical stress on the content of cytokines and growth factors in ACS, 2) identify potential haematological or inflammatory markers for prediction of cytokine production in ACS and 3) investigate the necessity of specialised ACS containers when preparing a cytokine-rich serum. Our hypotheses were that 1) cytokine and growth factor content of ACS prepared 22–24 h post operatively would be lower compared with ACS prepared preoperatively, 2) cytokine levels in ACS are associated with leucocyte levels and 3) blood incubation in plastic vacutainer tubes containing Z Serum Clot Activator[a] results in comparable cytokine and growth factor levels as incubation in specialised ACS systems containing medical-grade glass beads.

Materials and methods

Horses

Fifteen stallions (age 1–8 years, mean 3.1 years) admitted to the hospital for elective castration or cryptorchidectomy were recruited to the study. These horses comprised 7 Standardbreds, 4 Norwegian/Swedish Coldblooded Trotters, 2 Icelandic horses, one Pura Raza Española and one American Quarter Horse. All stallions were considered to be healthy based

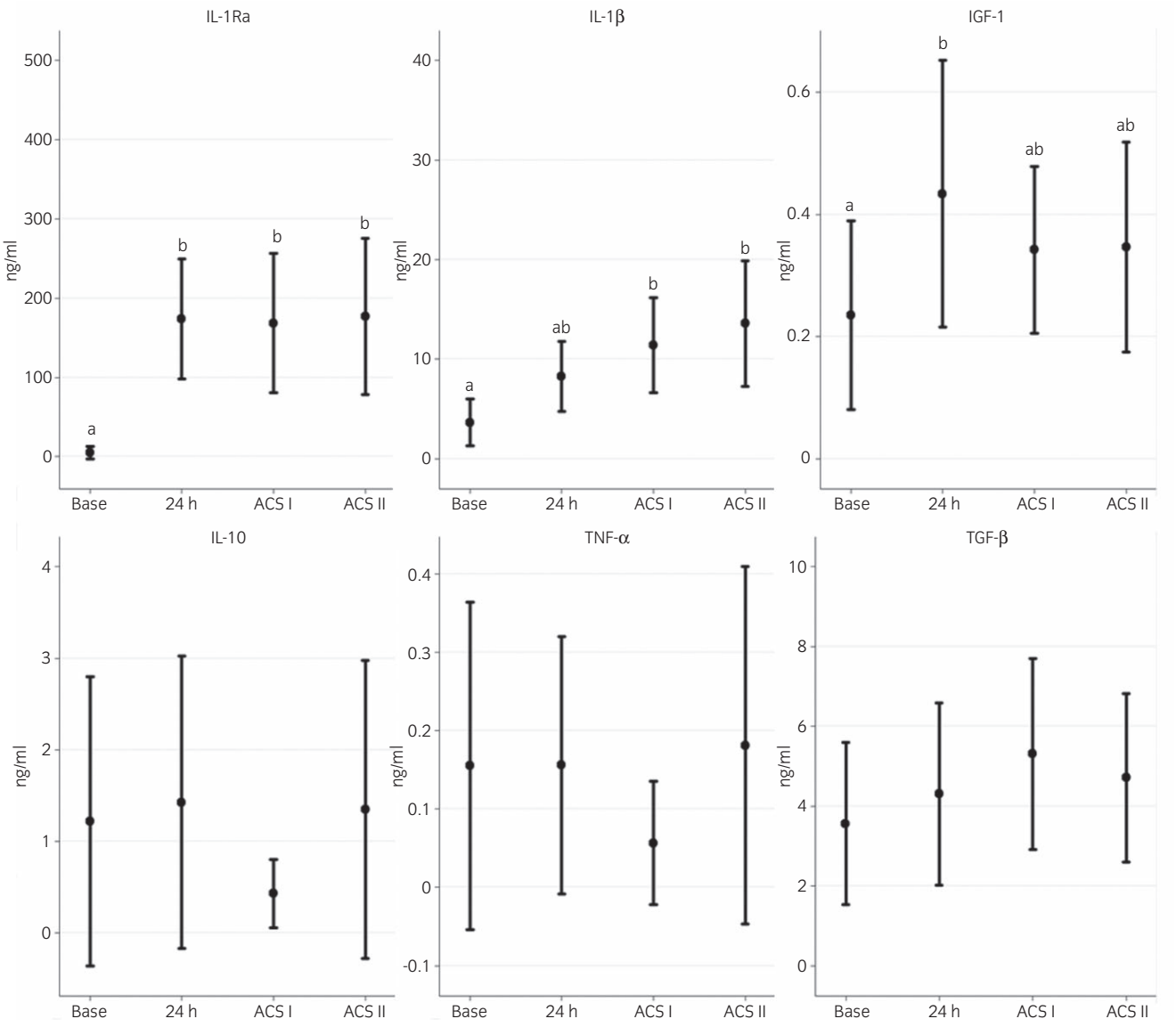

Fig 1: Levels of cytokines and growth factors (mean and 95% confidence interval) determined by commercially available ELISA tests in preoperative serum samples of 15 healthy stallions admitted for elective castration/cryptorchidectomy. Different letters indicate significant differences (P<0.05) between the preparation methods. ACS = autologous conditioned serum; IGF = insulin-like growth factor; IL = interleukin; IL-1Ra = IL-1 receptor agonist; TGF = transforming growth factor; TNF = tumour necrosis factor.

on physical examination and preoperative haematology and biochemistry analyses including SAA (reference range 0–20 mg/l).

Surgical procedures

Surgical procedures were performed aseptically under general anaesthesia; normally descended testes were removed through scrotal incisions whereas inguinally or abdominally retained testes were removed using an inguinal approach as previously described [12]. Spermatic cords were ligated prior to emasculation and primary closure of all surgical incisions was performed. Penicillin[b] (22,000 iu/kg bwt i.v.) and flunixin meglumine (Finadyne[c], 1.1 mg/kg bwt i.v.) were administered perioperatively.

Sample collection and preparation

Blood was collected aseptically preoperatively and 22–24 h post operatively from an indwelling jugular vein catheter (BD Secalon 14 gauge catheter)[d] placed using standard hospital routines. Preoperatively, one plastic EDTA vacutainer tube[a] was submitted for routine haematology analysis within 2 h of sample collection. At both sampling times, the remainder of the blood was distributed into different containers. One plastic vacutainer tube containing Z Serum Clot Activator[a] was allowed to coagulate at room temperature for 1 h prior to centrifugation (2500 ***g***, 10 min) and serum collection (baseline serum). One identical plastic vacutainer tube containing Z Serum Clot Activator[a] and 2 ACS containers (ACS I: 60 ml polypropylene syringe; ACS II: 30 ml polypropylene tube)[e,f] both containing medical-grade glass spheres were incubated at 37°C for 24 h, prior to centrifugation (2500 ***g***, 10 min for the vacutainer tube and 3700 ***g***, 10 min for the ACS containers, respectively) and serum collection (24 h serum, ACS I and ACS II, respectively). Serum from the ACS containers was filtered through a 0.2 μm filter (Sterifix 0.2 μm Luer Lock)[g]; all serum samples were stored in 5 ml cryovials at -20°C until analysis.

Sample analyses

Serum amyloid A analysis was performed in pre- and post operative baseline serum samples using an anti-SAA coated latex agglutination

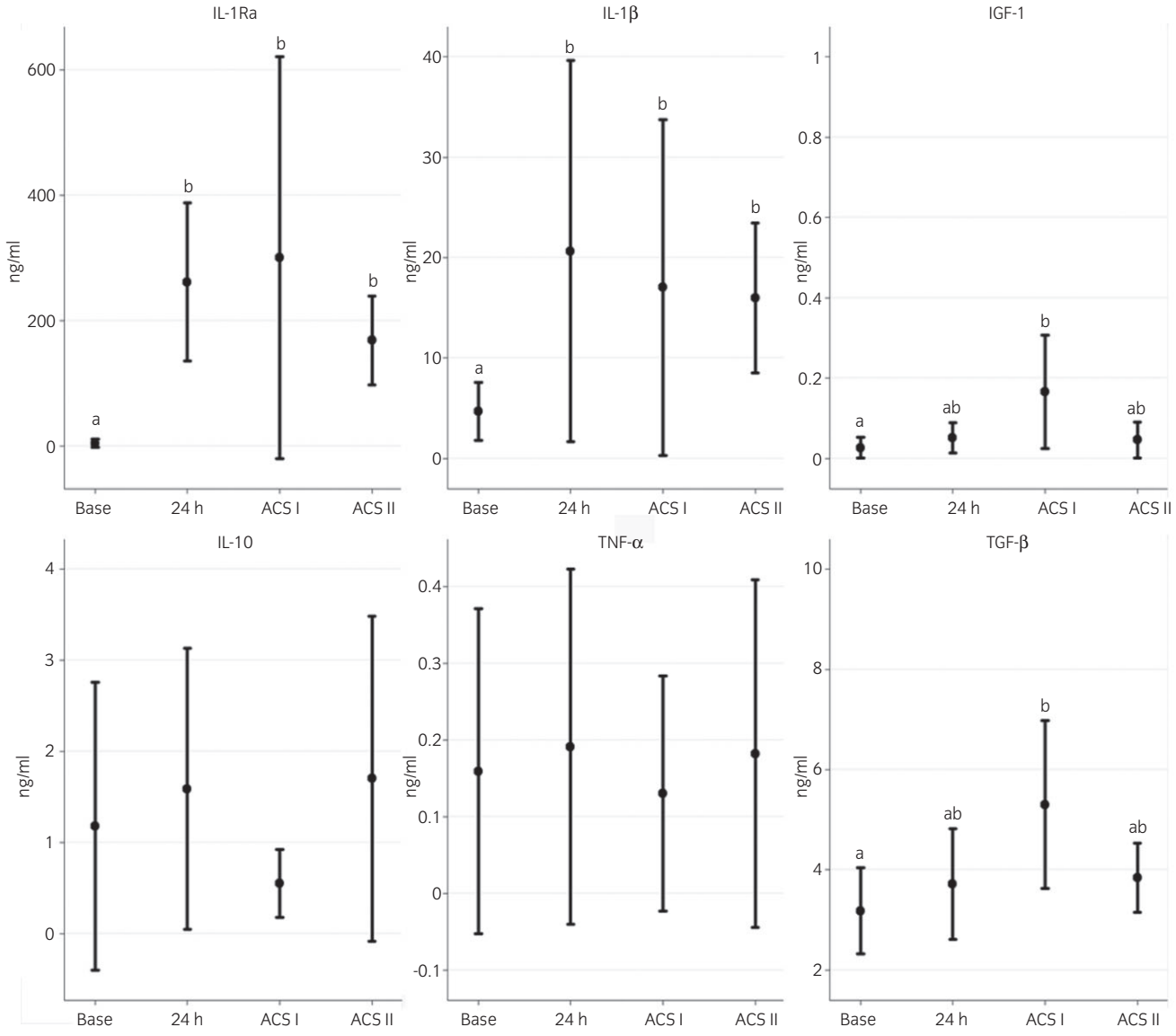

Fig 2: Levels of cytokines and growth factors (mean and 95% confidence interval) determined by commercially available ELISA tests in post operative serum samples of 15 healthy stallions admitted for elective castration/cryptorchidectomy. Different letters indicate significant differences (P<0.05) between the preparation methods. ACS = autologous conditioned serum; IGF = insulin-like growth factor; IL = interleukin; IL-1Ra = IL-1 receptor agonist; TGF = transforming growth factor; TNF = tumour necrosis factor.

photometric immunoassay. Commercially available ELISA kits were used to determine serum concentration of cytokines and growth factors in baseline serum, 24 h serum, ACS I and ACS II. Equine-specific kits for IL-1Ra[h], IL-10[h], IL-1β[i] and tumour necrosis factor (TNF)-α[h] and human-specific kits for IGF-1[h] and TGF-β1[i] were used; the latter 2 kits have previously been validated for cross-reactivity in equine serum [13,14]. All assays were run according to the manufacturer's instructions.

Data analysis

The arithmetic mean and 95% confidence interval (CI) was calculated for all variables. Due to positive skewness, variables were transformed using either log transformation (preoperative SAA) or square root transformation (post operative SAA, cytokines and growth factors) until normal distribution was confirmed visually and by the Shapiro–Wilk test prior to further analyses. All variables including the IL-1Ra:IL-1β ratio were compared between the 2 time points (pre- and post operatively) and serum preparations (baseline serum, 24 h serum, ACS I and ACS II) by repeated measures ANOVA with *post hoc* Tukey's HSD tests. The change in cytokine and growth factor levels induced by incubation was defined as 'delta cytokine' and calculated as follows: delta cytokine = incubated level - baseline level; these variables were compared pre- and post operatively using ANOVA analyses. Based on exploratory data analysis, the variable post operative SAA was grouped into 3 categories and defined as 'mild surgical stress' (<21 mg/l); 'moderate surgical stress' (21–200 mg/l) and 'marked surgical stress' (>200 mg/l); delta cytokine in post operative samples were subsequently compared between these groups using ANOVA analyses with *post hoc* Tukey's HSD tests.

Associations between the haematology variables plus SAA and each of the cytokines and growth factors in all incubated preoperative samples were determined by Pearson correlation coefficients with 95% CIs and by linear regression modelling. Variables associated with each of the cytokines/growth factors in a univariate regression analysis with P≤0.20 were included in multivariate analysis; both purposeful forward/backward selection was performed. For all analyses, P<0.05 was considered significant; analyses were performed using commercial statistical software[j].

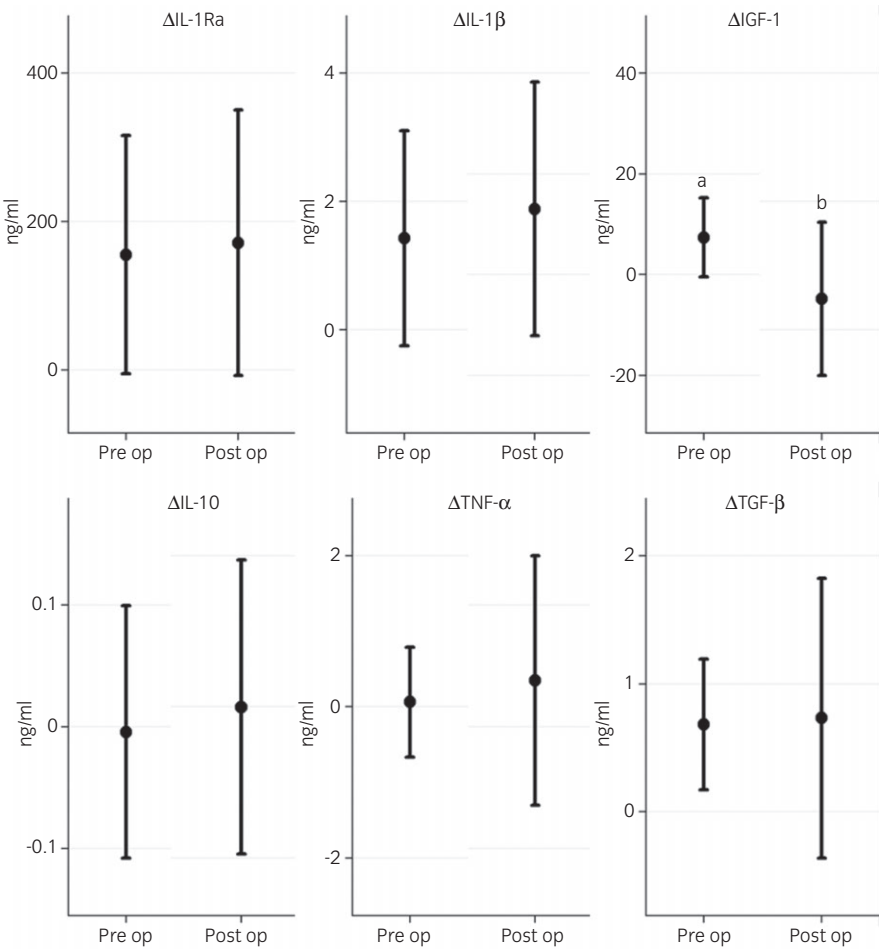

Fig 3: Change in cytokine and growth factor content induced by incubation (delta cytokine = incubated level - baseline level) in pre- and post operative serum samples (mean and 95% confidence interval). Different letters indicate significant difference (P<0.05) between the timepoints. IGF = insulin-like growth factor; IL = interleukin; IL-1Ra = IL-1 receptor agonist; TGF = transforming growth factor; TNF = tumour necrosis factor.

Results

Routine castration was performed in 11 stallions. Unilateral inguinal cryptorchidectomy was performed in 2 stallions and unilateral abdominal cryptorchidectomy performed in 2 stallions. In the cryptorchid horses, the descended testis was routinely removed through a scrotal incision. All surgical procedures went without complications and all horses were discharged from the hospital on the first post operative day.

Serum amyloid A

A significant increase in SAA was seen post operatively in all horses (preoperative 0.31 mg/l, 95% CI 0.2–0.4 mg/l; vs. post operative 164.6 mg/l, 95% CI 54.1–275.1 mg/l, respectively, P<0.001). Five horses (normal stallions only) had post operative SAA levels <21 mg/l (mean 5 mg/l, 95% CI -5.8 to 15.9 mg/l) and were subsequently categorised in the 'mild surgical stress' group. Four horses (normal stallions only) had post operative SAA levels 21–200 mg/l and were categorised in the 'moderate surgical stress' group (mean 111 mg/l, 95% CI 86.4–134.7 mg/l), whereas 6 horses (4 cryptorchid and 2 normal stallions) had post operative SAA >200 mg/l and were categorised in the 'marked surgical stress' group (mean 333.3 mg/l, 95% CI 100.1–566.5 mg/l).

Preoperative serum analyses

Due to kit availability, ACS I was used in 8 horses only. Levels of cytokines and growth factors determined in preoperative serum samples are displayed in Figure 1. Compared with baseline serum incubation resulted in significantly increased levels of IL-1Ra in all serum preparations (P<0.001) and significantly increased levels of IL-1β in ACS I and ACS II (P = 0.02 and P = 0.004, respectively). Twenty-four h serum contained significantly more IGF-1 than baseline serum (P = 0.03). There were no significant differences between the preparation methods (24 h serum, ACS I and ACS II) in cytokine or growth factor content or in IL-1Ra:IL-1 β ratio.

Post operative serum analyses

Due to kit availability, ACS I was used in 8 horses only. Levels of cytokines and growth factors determined in post operative serum samples are displayed in Figure 2. Baseline cytokine levels were comparable with preoperative levels except for IGF-1 which was significantly lower (26.5 pg/ml, 95% CI -1.4 to 54.5 pg/ml post operatively vs. 241.2 pg/ml, 95% CI 65.4–404.2 pg/ml preoperatively, P = 0.007). Compared with baseline serum, incubation of post operative samples resulted in significantly increased levels of IL-1Ra and IL-1β in all serum preparations (P<0.001 and P<0.05, respectively). Autologous conditioned serum I contained significantly more IGF-1 and TGF-β than baseline serum (P = 0.01 for both growth factors, respectively). There were no significant differences between the serum preparation methods (24 h serum, ACS I and ACS II) in cytokine or growth factor content or in IL-1Ra:IL-1 β ratio.

Effect of surgical stress

Post operatively, incubation resulted in significantly lower delta IGF-1 compared with preoperative samples (P<0.001; Fig 3). Horses categorised in the 'marked surgical stress' group had significantly lower delta IL-1Ra and delta TGF-β vs. horses categorised in the 'moderate surgical stress' group (P = 0.02 and P = 0.049, respectively, Fig 4) and significantly lower delta IGF-1 vs. horses categorised in the 'mild surgical stress' group (P = 0.049).

Considerable interindividual differences in cytokine levels were found in all serum preparations. No association between cytokine levels and haematology variables or post operative SAA were identified. A strong

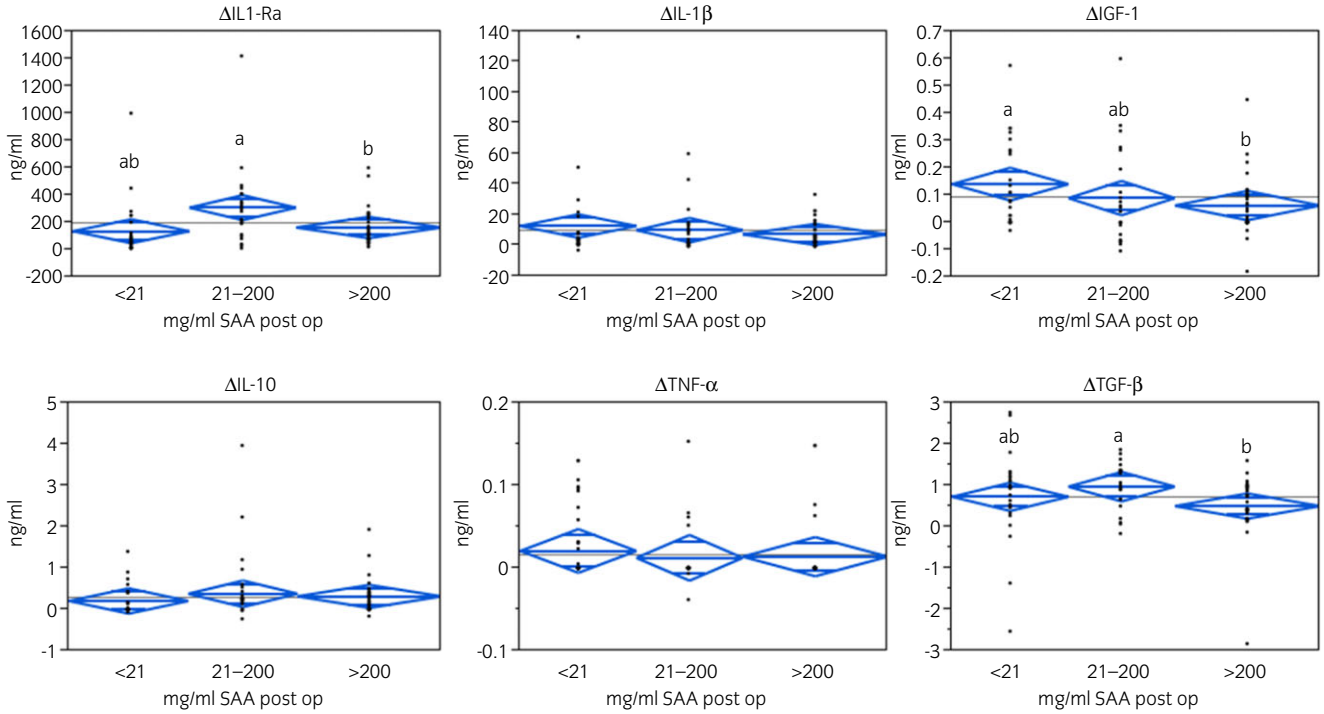

Fig 4: Diamond plot (mean with 95% confidence interval) of delta cytokine determined in post operative serum samples after grouping the horses into 3 categories based on post operative serum amyloid A (SAA) levels; mild surgical stress = post operative SAA <21 mg/l; moderate surgical stress = post operative SAA 21–200 mg/l; marked surgical stress = post operative SAA >200 mg/l. Different letters indicate significant differences (P<0.05) between groups. IGF = insulin-like growth factor; IL = interleukin; IL-1Ra = IL-1 receptor agonist; TGF = transforming growth factor; TNF = tumour necrosis factor.

positive correlation was found between thrombocyte count and the level of TGF-β in all preoperative serum samples (r = 0.79; 95% CI 0.66–0.88). This relationship was confirmed using linear regression modeling, where 64% of the variance in TGF-β level was accounted for by thrombocyte count. The level of TGF-β was found to increase with 37 pg/ml for every 1×10^9/l increase in thrombocyte count and the model showed significant fit (P<0.001).

Discussion

Elective surgery resulted in significant lower baseline serum content of IGF-1 and significantly reduced *de novo* synthesis of IGF-1 in incubated serum samples. The finding of reduced circulating level of IGF-1 post operatively was not surprising, as similar findings have been reported during acute-phase responses in man [15]. However, the reduced *de novo* synthesis of IGF-1 observed in incubated serum samples post operatively has, to the authors' knowledge, not previously been described and demonstrates that surgical stress may influence constituents of ACS. This was also evidenced by the significant decrease in delta IL-1Ra, delta IGF-1 and delta TGF-β seen in horses with post operative SAA >200 mg/ml vs. horses with lower post operative SAA. A proposed mechanism of SAA is alterations in leucocyte recruitment and function [11] that might explain these findings. However, the exact physiological effects of SAA are poorly understood as contradicting effects regarding leucocyte function have been described [11].

Acute-phase protein levels increase with increasing tissue damage [16], which formed the basis for categorising the horses in the current study into groups of 'mild', 'moderate' and 'marked' surgical stress. Surgical stress level corresponded with the castration method performed; not surprisingly, cryptorchidectomy elicited higher SAA levels than regular castrations. However, high SAA levels were seen in 2 stallions routinely castrated also; this might reflect differences in tissue handling and surgical technique. Surgeon effect was not evaluated as all routine castrations were performed by final year veterinary students under supervision. Although considered minimally invasive, elective arthroscopy also induces significantly elevated post operative SAA levels evident from the first to the fifth post operative day [16]. The results from the current study are therefore relevant to clinical practice where ACS treatment is recommended after joint surgery. In instances of high post operative SAA, postponing blood collection for ACS preparation until SAA is within normal range may optimise ACS quality.

The considerable interindividual differences in cytokine levels in ACS identified in the current study and as reported by others [6] probably contributed to our failure in predicting cytokine levels in incubated samples. Assuming that the clinical effects of ACS are due to the anti-inflammatory cytokines and/or growth factors investigated in the current study, these results should raise some concern when using ACS in clinical practice as to date there is no method of identifying animals with poor *de novo* synthesis of these proteins in ACS and which subsequently will respond poorly to treatment. Determination of cytokine content in ACS by ELISA-analyses prior to treatment could provide this information; however, such an arrangement would be impractical and cost prohibitive in most instances.

Serum from blood incubated in plastic vacutainer tubes containing Z Serum Clot Activator[a] contained similar levels of the cytokines and growth factors investigated as serum incubated in 2 specialised ACS containers. These results corroborate those of a previous study [6] and indicate that cytokine production may be a consequence of whole blood incubation rather than an effect of specialised ACS containers. One of the ACS containers used in the current study is marketed for human use whereas the other ACS system is a prototype container not yet commercially available. Both containers are, however, designed using the same principles as ACS systems marketed for use in equine practice. Significant differences in cytokine production between commercially available ACS systems have previously been demonstrated [6]. There were only minor differences between ACS I and ACS II in the current study and the clinical relevance of these may be questioned. However, the purpose of our study was not to compare the efficacy of these products and we do not recommend using products not marketed for veterinary use in clinical practice.

In conclusion, blood collection for ACS preparation during an acute-phase response may influence the resultant ACS quality and should be done with caution. Specialised ACS containers may not be necessary for preparation of a cytokine-rich serum.

Authors' declaration of interests

No competing interests have been declared.

Ethical animal research

Owners gave informed consent for their horses inclusion in the study. All procedures were approved by the Norwegian School of Veterinary Science and in accordance with national legislation concerning ethical animal research (FOR-1996-01-15–23).

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Authorship

C.T. Fjordbakk contributed to study design, study execution, data interpretation and preparation of the manuscript. A.K. Storset contributed to study design, data interpretation, and preparation of the manuscript. A.C. Løvås contributed to study execution, data analysis, data interpretation and preparation of the manuscript. K.L. Oppegård contributed to study execution, data analysis and data interpretation and preparation of the manuscript. G.M. Johansen contributed to data analysis and data interpretation. All authors have given final approval of the manuscript.

Manufacturers' addresses

[a]Greiner Bio-One Ltd, Stonehouse, UK.
[b]Actavis; Oslo, Norway.
[c]MSD Animal Health; Bergen, Norway.
[d]CNC Medical Devices, Mumbai, India.
[e]Onoccomed; Plasmaconcept AG, Köln, Germany.
[f]Em-Cyte Corporation, Fort Myers, Florida, USA.
[g]Braun, Meslungen, Germany.
[h]R&D Systems Europe Ltd; Abingdon, Oxfordshire, UK.
[i]Kingfisher Biotech; St. Paul, Minnesota, USA.
[j]Stata Corp, College Station, Texas, USA.

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