Surgical stress influences cytokine content in Autologous Conditioned Serum

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Keywords: horse; regenerative medicine; autologous conditioned serum

Summary

Reasons for performing the 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) to identify haematological markers for prediction of cytokine production in ACS; 3) to 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 pre-operatively and 22 - 24 h post-operatively and incubated in ACS containers and plastic vacutainer tubes.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/evj.12277
containing Z Serum Clot Activator. Concentrations of IL-1Ra, IL-10, IL-1β, TNF-α, IGF-1 and 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 was compared between these groups by ANOVA.

**Results:** Delta IGF-1 was significantly lower in post-operative samples compared to pre-operative. 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 hematology 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.

**Introduction**

In recent years, ‘biologic 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 biologic therapy for joint disease in horses [1].
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 biologic treatment of joint disease. Autologous conditioned serum refers to the cytokine-rich serum harvested after exposing circulating leukocytes to activating surfaces, thereby stimulating de novo synthesis of several anti-inflammatory cytokines and growth factors [4,5]. Compared to un-manipulated horse blood, ACS contains increased levels of anti-inflammatory cytokines such as IL-1Ra and IL-10, as well as the growth factors TGF-β and 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 Activator³ 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 OA 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 versus unconditioned serum on chondrocyte metabolism [8]. Despite obvious paucities in clinical evidence, ACS is popular amongst 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 to ACS prepared from blood drawn pre-operatively. 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) to identify potential hematological or inflammatory markers for prediction of cytokine production in ACS; and 3) to 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 to ACS prepared pre-operatively; 2) cytokine levels in ACS are associated with leucocyte levels; and 3) blood incubation in plastic vacutainer tubes containing Z Serum Clot Activator results in comparable cytokine and growth factor levels as incubation in specialised ACS systems containing medical-grade glass beads.

Materials and methods

Horses

15 stallions (age 1 - 8 years, mean 3.1 years) admitted to the hospital for elective castration or cryptorchidectomy were included in the study; there were 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 on physical...
examination and pre-operative 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 i.v.) and flunixin meglumine (Finadyne\(c\), 1.1 mg/kg i.v.) were administered peri-operatively.

Sample collection and preparation

Blood was collected aseptically pre-operatively and 22 – 24 h post-operatively from an indwelling jugular vein catheter (BD Secalon 14G catheter)\(d\) placed using standard hospital routines. Pre-operatively, one plastic EDTA vacutainer tube\(a\) was submitted for routine hematology analysis within 2 h of sample collection. At both sampling times, the remainder of the blood was distributed into different containers as follows: One plastic vacutainer tube containing Z Serum Clot Activator\(a\) was allowed to coagulate at room temperature for one hour 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)\(f\) both containing medical-grade spherical 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 (24h 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); 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 photometric immunoassay. Commercially available ELISA kits were used to determine serum concentration of cytokines and growth factors in baseline serum, 24h serum, ACS I and ACS II. Equine-specific kits for IL-1Ra$^h$, IL-10$^h$, IL-1β$^h$, and 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 was calculated for all variables. Due to positive skewness, variables were transformed using either log transformation (pre-operative SAA) or square root transformation (post-operative SAA, cytokines and growth factors) until normal distribution was confirmed visually and by the Shapiro-Wilkes 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, 24h 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 pre-operative samples were determined by Pearson
correlation coefficients with 95% confidence intervals and by linear regression modelling.
Variables associated with each of the cytokines/growth factors in a univariate regression
analysis with $P \leq 0.20$ 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.

Results

Routine castration was performed in 11 stallions. Unilateral inguinal cryptorchidectomy was
performed in 2 stallions, and unilateral abdominal cryptorchidectomy was 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 (pre-operative 0.31
mg/L, 95% CI 0.2 – 0.4 mg/L; versus 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 – 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).

- Pre-operative serum analyses

Due to kit availability, ACS I was used in 8 horses only. Levels of cytokines and growth factors determined in pre-operative serum samples are displayed in Fig 1. Compared to 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). 24h serum contained significantly more IGF-1 than baseline serum (P = 0.03). There were no significant differences between the preparation methods (24h 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 Fig 2. Baseline cytokine levels were comparable to pre-operative levels except for IGF-1 which was significantly lower (26.5 pg/ml, 95% CI -1.4 – 54.5 pg/ml post operatively versus 241.2 pg/ml, 95% CI 65.4 – 404.2 pg/ml pre-operatively, P = 0.007). Compared to 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). ACS 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 (24h serum, ACS I and ACS II) in cytokine or growth factor content or in IL-1Ra:IL-1β ratio.

- **Effect of surgical stress**

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

Considerable inter-individual differences in cytokine levels were found in all serum preparations. No association between cytokine levels and the haematology variables or post-operative SAA were identified. A strong positive correlation was found between thrombocyte count and the level of TGF-β in all pre-operative 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 x 10⁹/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 humans [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 versus horses with lower post operative SAA. A proposed mechanism of SAA is alterations in leukocyte recruitment and function [11] that might explain these findings. However, the exact physiologic effects of SAA are poorly understood as contradicting effects regarding leukocyte 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 to the castration method performed; not surprisingly, cryptorchidectomy elicited higher SAA levels than regular castrations. However, high SAA levels were seen in 2 stallions that were 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 inter-individual 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 there to date 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 after 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).

Sources of funding
The Swedish/Norwegian Foundation for Equine Research, the Norwegian School of Veterinary Science, and VetLine Bio AB.

Authorship
C.T. Fjordbakk contributed to study design, study execution, data interpretation, and preparation of the manuscript. A. 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.

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References


**Figure legends**

**Fig 1:** Levels of cytokines and growth factors (mean and 95% confidence interval) determined by commercially available ELISA tests in pre-operative serum samples of 15 healthy stallions admitted for elective castration/cryptorchidectomy. Different letters indicate significant differences (P<0.05) between the preparation methods.
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.

Fig 3: Change in cytokine- and growth factor content induced by incubation (delta cytokine = incubated level – baseline level) in pre-operative and post-operative serum samples (mean and 95% confidence interval). Different letters indicate significant difference (P<0.05) between the time points.

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 SAA levels; mild surgical stress = post-op SAA <21 mg/L; moderate surgical stress = post-op SAA 21 – 200 mg/L; marked surgical stress = post-op SAA > 200 mg/L. Different letters indicate significant differences (P<0.05) between groups.