The effect of exercise on cytokine concentration in equine autologous conditioned serum

Hale, Josephine N; Hughes, Kristopher J; Hall, Sarah; Labens, Raphael

Published in:
Equine Veterinary Journal

DOI:
10.1111/evj.13586

First published: 15/05/2022

Document Version
Peer reviewed version

Hale Josephine N. (Orcid ID: 0000-0002-4080-5319)
Hughes Kristopher (Orcid ID: 0000-0002-8405-6268)

The effect of exercise on cytokine concentration in equine autologous conditioned serum

Josephine N. Hale*[1], Kristopher J. Hughes[1], Sarah Hall[2] and Raphael Labens[1]

[1]School of Agricultural, Environmental and Veterinary Sciences, Charles Sturt University, Wagga Wagga, New South Wales, Australia and [2]Animal and Veterinary Sciences Department, Scotland's Rural College, Edinburgh, Scotland, UK.

***Corresponding author email:** Josephine Hale johale@csu.edu.au

Keywords: horse, osteoarthritis, IL-1Ra

Running head: Exercise effect on cytokine concentration in equine autologous conditioned serum

1. Abstract

Background: Autologous conditioned serum (ACS) is a commonly administered intra-articular treatment for the management of osteoarthritis in athletic horses.

Objectives: To investigate the influence of exercise on the concentration of cytokines in a non-commercial method of ACS production.

Study design: Non-randomised cross over design.

Methods: Whole blood was obtained from 8 healthy Standardbred horses immediately prior to, 1 hour and 24 hours following a single bout of exhaustive exercise. Blood was processed using a non-commercial method of ACS production. Fluorescent microsphere immunoassay (FMIA) analysis was performed to quantify Interleukin 1 receptor antagonist (IL-1Ra), Interleukin-10 (IL-10), Interleukin 1β (IL-1β) and tumour necrosis factor α (TNF-α) concentrations at each time point. Mixed effect repeated

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.13586

measures analysis of variance (ANOVA) was used to compare the pre-exercise and post-exercise cytokine concentrations. Significance was set at $P < 0.05$.

Results: A reduced concentration of IL-1Ra (median 584.4, IQR 81.9 – 5098 pg/mL, P = 0.004) and an increased concentration of TNF-α (11.92, 9.28 – 39.75 pg/mL, P = 0.05) at 1 hour post-exercise was observed when compared to baseline values (IL-Ra 7349, 1272 – 10760 pg/mL; TNF TNF-α 11.16, 8.36 – 32.74 pg/mL). No difference in cytokine concentrations of IL-10 or IL-1β were found between any of the time points.

Main limitations: The large biological variability and small sample size represents limitations of this study.

Conclusions: These results suggest that a single bout of intense exercise can reduce the concentration of the anti-inflammatory cytokine IL-1Ra and increase the concentration of the pro-inflammatory cytokine TNF-α, reducing the 'anti-inflammatory' cytokine composition of ACS. Our findings suggest that collection of blood for ACS production should be performed no sooner than 24 hours following a single episode of intense exercise.

2. Introduction

Osteoarthritis is one of the most common causes of lameness in the equine athlete [1]. Endogenous pro-inflammatory cytokines play an important role in the onset and progression of osteoarthritis [2]. Expression of tumour necrosis factor α (TNF-α) and Interleukin 1 β (IL-1β) is increased in synovial tissue and cartilage of equine osteoarthritic joints [2]. In the normal joint, these pro-inflammatory or catabolic cytokines are balanced by the presence of inhibitory or anti-inflammatory cytokines [3]. Interleukin 1 receptor antagonist (IL-1Ra) is an anti-inflammatory cytokine that has been shown to competitively inhibit IL-1β [4,5]. Autologous conditioned serum (ACS), produced through the aseptic collection and incubation of whole blood for 24 hours, followed by centrifugation and extraction of the serum fraction [6] contains an increased concentration of the anti-inflammatory cytokines IL-1Ra, interleukin 10 (IL-10) and growth factors including, transforming growth factor β (TGF-β), insulin-like growth

factor 1 (IGF-1) and platelet derived growth factor [7-9]. In vivo administration of IL-1Ra has been shown to result in concentration-dependent anti-inflammatory and chondroprotective effects [6,10,11]. In horses, ACS is commonly used as an intra-articular treatment for osteoarthritis [12]. Despite the widespread use of ACS, there are little available scientific data for evidence-based optimisation of the production and use of this biological product.

Surgical stress has been shown to decrease the concentration of IGF-1, TGF-β and IL-1Ra in ACS [13]. In humans, exercise is known to cause an acute inflammatory response including an increase in concentrations of the pro-inflammatory cytokines IL-6, TNF-α and IL-1β [14]. In horses, exercise has been shown to increase systemic mRNA expression of IL-1β and TNF-α [15]. This exercise induced inflammatory response is thought to be caused by overtraining which is associated with muscle, skeletal and joint trauma [16].

Intra-articular administration of ACS is most often used in athletic horses. However, the timing of collection of blood for ACS in association with training has not been previously investigated. The aim of this study was to investigate the influence of exercise on concentrations of cytokines in a previously described non-commercial method of ACS production [17]. We hypothesised that exercise prior to the collection of blood for ACS production would decrease the concentrations of IL-1Ra and IL-10 and increase the concentrations of TNF-α and IL-1β in ACS products.

3. Materials and Methods

3.1 Animals

Eight healthy Standardbred horses (4 females and 4 geldings), with a mean (± SD) age of 3.5 ± 2.1 years (range 2-8), trained for harness racing were included in this study. Horses were sourced from a single standardbred pacing training facility, local to the university teaching hospital.

3.2 Exercise protocols

Horses were determined to be free of lameness by examination at the trot prior to commencement of exercise by a single observer (JH). No horse had a history of recent poor performance. The horses were worked on a 1000 m sand track, by one of two experienced pacing drivers and exercised to maximum threshold depending on the fitness level of each horse. Four horses (2 mares and 2 geldings) were considered fit to race and four horses (2 fillies and 2 geldings) were in full time work but not considered fit to race. All horses worked at peak speeds between 34.3 km/h and 44.6 km/h for 1.6 km following a warm-up period (~13 km/h for 5 minutes) which was measured utilising a stopwatch. Heart rate was measured via cardiac auscultation prior to the commencement of exercise and immediately following exercise.

3.3 Sample collection

Blood sample collection was performed under aseptic conditions. Venipuncture sites were clipped, after which a 5-minute scrub was performed using sterile swabs in 0.05% chlorhexidine solution before methanol-soaked sterile swabs were used to finalise preparation of the site. Using sterile gloves and a 21-gauge butterfly catheter, blood was collected via a single venipuncture of the left jugular vein. Blood was collected over 1–2 minutes, with minimal traction applied to the syringe plunger. A total of 54 mL of venous blood was collected for each horse prior to exercise and 1 hour post-exercise. Additionally, a further 50 mL of venous blood was collected at 24 hours post-exercise. At each time point, 50 mL of blood was transferred into a sterile 50 mL polypropylene conical falcon tube (Screw cap tube 50mL 114 x 28mm, Sarstedt AG & Co. KG) for ACS preparation. At the pre-exercise and 1 hour post-exercise sampling points, the remaining 4 mL of blood was distributed evenly between an EDTA (plastic whole blood tube, BD Vacutainer, BD) and a plain tube (plastic whole blood tube, BD Vacutainer, BD) and used for haematological examination, measurement of serum concentrations of serum amyloid A, total protein, albumin, globulin and cortisol and determination of the A:G ratios. Measurements of concentrations of lactate (Accutrend® Plus system, Roche) were performed on whole non-clotted blood immediately following collection.

3.4 Sample preparation

The blood samples collected into Falcon tubes were incubated (Melag 80, Melag) for 24 hours at 37°C followed by centrifugation (Hettich Zentrifugen Rotofix 32 A, Hettich Franke GmbH & Co) at 3500 rpm for 10 min. An 8 ml serum sample was then aspirated from each Falcon tube using a sterile 100 mm 18-gauge needle and sterile 30 mL polypropylene syringe. Samples were subsequently sterilised by passage through a single use filter (Millex-GP Syringe Filter Unit, Merck Millipore Ltd) with 0.22 µm pore size and aliquoted into four, sterile 2 mL Eppendorf tubes (SafeSeal micro tubes, Sarstedt), and snap frozen at -80°C until analysis. Total storage time for frozen samples did not exceed 6 months and samples remained frozen during transportation to the analysing laboratory.

3.5 Serum analysis

A fluorescent microsphere immunoassay (FMIA) was performed on all ACS samples. Cytokine measurements were optimised as a fourplex for IL-1Ra, IL-1β, IL-10 and TNF-α. The optimisation of the multiplex plates was performed using the validation method described elsewhere [17,18], using polyclonal capture and detection antibodies (R&D Systems) and magnetic carboxylated microspheres (BioRad). Information regarding the capture and detection antibodies used is outlined in Table 1. A pooled serum sample was run undiluted and at dilutions of 1:2 and 1:4 for added control of linearity and recovery. The results for IL-1Ra at a dilution of 1:2 was greater than the detection range; however, all dilutions for IL-1β, IL-10, TNF-α were within the standard curve range. To allow all cytokines to be measured in undiluted samples, the range of standards was set at 41.0-10,000 pg/mL for IL-1β, IL-10, TNF-α and 81.9-20,000 pg/mL for IL-1Ra. Repeat FMIA analysis was performed on samples with results less than the detectable range (<OOR) and samples with a coefficient of variation (CV) of > 9.5%. Repeated analyses were performed following one freeze/thaw cycle. The samples with the lowest CV% of both results were included in the statistical analysis. Minimum range standards for the repeat samples were set at 226 pg/mL for IL-1Ra, 82 pg/mL for IL-1β, 40 pg/mL for IL-10 and 32 pg/mL for TNF-α.

3.6 Data analysis

To compare the cytokine concentrations between time points, mixed effect repeated measures analyses of variance (ANOVA) were performed with separate models fitted to each of the dependent variables (IL-1Ra, IL-10, IL-1β and TNF-α). Time was declared the repeated variable and "Horse" was set as a random effect. Prior to tests being performed, normality of raw data and residuals was assessed according to Shapiro-Wilk. Residuals were plotted against predicted values to evaluate model fit and heteroscedasticity. If the data were nonparametric, log transformations were performed to meet test assumptions. Non-transformable data were analysed using Friedman and Wilcoxon signed rank tests. Post-hoc, multiple comparisons were adjusted using the Bonferroni method ($P < 0.02$). Tests were performed (On Demand for Academics, SAS) with significance set at $P \leq 0.05$.

4 Results

4.1 Haematology and Blood Biochemistry

Haematological and blood biochemical analyses determined that a systemic inflammatory response was not present in any of the horses. In all horses, concentrations of serum amyloid A (pre-exercise and post-exercise 0 ± 0 mg/L, normal range <7), total protein (pre-exercise 62.6 ± 4.3 g/L, post-exercise 63.5 ± 6.0, normal range 53-75), albumin (pre-exercise 34.3 ± 2.0 g/L, post exercise 34.8 ± 2.2, normal range 27-39) and globulin (pre-exercise 28.4 ± 4.5 g/L, post-exercise 28.8 ± 5.3, normal range 20-40) levels were within normal reference ranges. One horse had mild leukopenia pre-exercise ($4.9x10^9$/L; pre-exercise 7.9 ± 1.5 x10; post-exercise 8.4 ± 1.8, normal range 5.5-12.5). However, there was no additional evidence of a systemic inflammatory response, and the data from this horse were retained in the analyses. All 8 horses had resting or pre-exercise lactate concentrations within the normal reference range (1.2 ± 0.8 mmol/L, normal range < 2). All horses had increased concentrations of lactate in blood following exercise (4.9 ± 2.4), representing a 3.5 ± 1.3-fold increase in lactate concentration from pre-exercise concentrations. All 8 horses had both pre-exercise and post-exercise cortisol concentrations within the normal reference range (pre-exercise 118.9 ± 37 nmol/L, post-exercise 135 ± 31.9, normal range 71-240). Seven horses had a post-exercise cortisol concentration

greater than pre-exercise, resulting in a 1.2 ± 0.4-fold increase in cortisol levels following exercise. One horse had a 2.2-fold decrease in post-cortisol concentration compared to pre-exercise.

4.2 Heart Rate

All horses had an increase in heart rate when comparing resting values (pre-exercise 36 ± 4.8 bpm) to values immediately following exercise (post-exercise 103.5 ± 11.4 bpm). This resulted in a mean 2.9 ± 0.6-fold increase in heart rate.

4.3 Pro and anti-inflammatory cytokines

The data for IL-1Ra, IL-1β and IL-10 were non-normally distributed and required log transformation to achieve normal distribution prior to analysis by ANOVA. Time following exercise was associated with a difference in IL-1Ra concentration ($P = 0.004$). Adjusted post hoc comparisons revealed a difference between pre-exercise and 1-hour samples ($P = 0.03$). No difference was found in IL-1Ra concentration between pre-exercise and 24 hours post-exercise samples ($P = 0.1$) or 1 hour post-exercise and 24 hours post-exercise samples ($P = 1.0$). No effect of time was observed on IL-1β ($P = 0.1$) or IL-10 concentrations ($P = 0.5$). Analysis of non-parametric and non-transformable TNF-α data revealed no effect of time ($P = 0.2$); however, when pre-exercise and 1 hour post-exercise data were compared, an increase in TNF-α concentration was found ($P = 0.05$). No difference was found in TNF-α concentration between 1 hour and 24 hours post-exercise samples ($P = 0.8$) or pre-exercise and 24 hours post-exercise samples ($P = 0.6$). No difference in concentration of all cytokines was found between horses that were considered fit to race and those not considered fit to race. Descriptive statistics for each cytokine and time point are provided in Table 2.

5 Discussion

5.1 Anti-inflammatory Cytokines

Our results support the hypothesis that the concentration of the anti-inflammatory cytokine IL-1Ra is reduced by strenuous exercise; however, our assumption that a concurrent decrease in IL-10 would

also be observed did not hold true. Autologous conditioned serum results in increased concentrations of IL-1Ra and IL-10 [7] compared to normal serum, which when administered as an intra-articular treatment results in both anti-inflammatory and chondroprotective effects [6]. Increased concentrations of IL-1Ra within an ACS product have been associated with an improved clinical response when used to treat naturally occurring osteoarthritis [11]. External factors that influence the concentrations of these cytokines in ACS, are of particular interest, as manipulation of the timing of blood collection for ACS preparation may improve clinical efficacy and optimisation of production practices. The negative influence of surgical 'stress' on concentrations of IL-1Ra in an ACS product has been documented [13]; however, the effect of exercise 'stress' on cytokine concentrations within an ACS preparation has not been assessed. The cytokine response to systemic inflammation is well documented: TNF-α is released after an inflammatory stimulus, followed closely by an increase of IL-1β and IL-6 [19,20]. These cytokines perpetuate the inflammatory response through leucocyte recruitment via stimulation of release of granulocyte colony stimulating factor and chemotactic cytokines [19,20]. A rebound anti-inflammatory response, characterised by the release of IL-10, IL-4 and IL-1Ra, is subsequently induced to re-establish basal conditions [21-23]. Prolonged strenuous exercise, such as endurance racing in human athletes, has been shown to upregulate systemic inflammatory responses [23,24], resulting in peaks in the concentrations of TNF-α, IL-1β and IL-6 at the cessation of exercise and marked increase in IL-1Ra concentration 1-2 hours later [23-26]. This inflammatory sequence has also been reported in horses undergoing various intense exercise regimens [27]. However, short duration anaerobic exercise has not been associated with the same magnitude of cytokine upregulation, suggesting that cytokine responses are influenced by exercise intensity and duration [19,20,28]. The transient reduction in concentration of IL-1Ra at 1 hour which returned to normal by 24 hours following a single episode of intense exercise in our study suggests that this initial reduction in systematic circulating anti-inflammatory cytokines can influence concentrations within an ACS product. However, sampling at a greater number of time points post-exercise would have allowed improved characterisation of this rebound anti-inflammatory effect, and better guided ACS collection

times to maximise any increase in IL-1Ra concentration compared to basal values. These findings have important implications for the timing of collection of ACS in relation to exercise regimens, as ACS collection that occurs close to the cessation of intense exercise will result in an ACS preparation with a reduced IL-1Ra concentration possibly reducing the therapeutic efficacy of the product.

5.2 Pro-inflammatory Cytokines

Our hypothesis that the pro-inflammatory cytokine TNF-α would be increased by strenuous exercise was supported by the data. However, our hypothesis that a simultaneous increase in IL-1β would also be observed was not supported by the data. The cytokines TNF-α and IL-1β play an important role in the pathophysiology of degenerative joint disease through the upregulation of inflammation and cartilage degradation, respectively [29-31]; and in the cytokine stress response [19,20]. Interleukin 1-β exerts a potent negative effect at low concentrations, resulting in a higher concentration of IL-1Ra required to counter the action of this cytokine [32]. Therefore, factors that influence the concentration of TNF-α and IL-1β within an ACS product are important and may influence refinement of collection recommendations for ACS preparation. The negative systemic effect of exercise stress on inflammatory cytokine concentrations is well documented; however, effects of exercise on an ACS preparation are unknown. Low intensity exercise in untrained people has been shown to cause an upregulated systemic inflammatory cytokine response [33], which is thought to be increased with prolonged exercise such as marathon running [24,34]. The combined effects of fatigue and increased metabolic demand further activate circulating monocytes to produce and release proinflammatory cytokines [35]. In addition, cytokines produced in peripheral tissues can exacerbate localised inflammatory pathways in muscles and joints resulting in an amplified systemic response [19]. Thus, the magnitudes of systemic increases of TNF-α and IL-1β are associated with the intensity, type, and duration of exercise [24,25,36]. Intensity-dependent increases in mRNA expression of IL-1β and TNF-α have been documented in horses 2 hours following exercise [15], whilst gene expression for TNF-α has also been shown to increase for up to 24 hours following various exercise schedules (graded, interval and repeat sprint exercise tests) in standardbred mares [27]. Similarly, in our study, TNF-α concentration

increased 1 hour after a single bout of intense exercise in both trained and untrained horses, followed by a return to basal values by 24 hours after exercise. Thus, it can be concluded that gene expression of TNF-α likely results in an increase in systemic TNF-α concentration which is then observed in an ACS product. Gene expression, however, is an indirect method of cytokine quantification and a corresponding increase in serum concentrations of cytokines is not always detected. Gene expression of IL-1β has previously been reported to increase for up to 2 hours following exercise in horses prior to a return to baseline concentrations within 24 hours [27]. The lack of change in IL-1β concentration in our population of horses when compared to other studies [27] which have used gene expression of IL-1β to quantify cytokine concentration may be explained by the early sampling times (1 hour post-exercise) in this study. An increased number of sampling intervals in the current study would have provided a more comprehensive characterisation of the response of both TNF-α and IL-1β to exercise stress. Varying levels of exercise intensity may also account for the differing results between studies. The up regulation of the pro-inflammatory cascade following exercise in our study suggests careful consideration should be given to the timing of blood collection for ACS preparation, ACS collection close to the end of an episode of intense exercise will lead to a product containing increased concentrations of TNF-α possibly reducing its therapeutic efficacy.

In the current study, horses exercised at aerobic threshold, characterised by post-exercise mean blood lactate concentrations of > 4 mmol/L [37], had increased cortisol concentrations when compared to baseline values [38], and increased heart rates immediately following exercise when compared to baseline values. Marked surgical stress, characterised in horses by a serum amyloid A (SAA) concentrations of > 200mg/mL has been shown to decrease IL-1Ra concentration in ACS [13]. In this study no increase in SAA concentration was present when comparing pre-exercise to post-exercise samples, suggesting that despite the negative influence of exercise stress on cytokine concentrations, no effect on this acute phase protein occurred. These findings indicate that the exercise protocol used was sufficient to cause a 'stress' related exercise event.

5.3 Limitations

Biological variability in a small number of horses used, represents a limitation of this study, preventing broad generalisations of the results. The limited sample size and sampling frequency was determined by available funding. While individual differences in physiologic responses were addressed by the mixed modelling approach, a larger sample size would be required to minimise the potential for type 2 errors. Further limitations are reflected by our sampling schedule and the limited capacity to extrapolate our findings to horses in other disciplines as the effect of submaximal exercise was not considered. To reduce the effect of breed on the results, only standardbred horses were included in this study, however generalisation of these results to different horse breeds undergoing maximal exercise is not possible.

6 Conclusion

Our findings suggest that collection of blood for ACS production should be performed no sooner than 24 hours following a single episode of intense exercise. Further investigations of the influence of blood sampling time and exercise type on cytokine concentrations in ACS are warranted.

Authors' declarations of interest

No conflicts of interest have been declared.

Ethical animal research

This study was approved by the Charles Sturt University Animal Care and Ethics Committee.

Approval number A19023.

Informed consent

Informed client consent was obtained prior to the commencement of this study

Data availability statement

The data that support the findings of this study are openly available in Dryad at

https://doi.org/10.5061/dryad.7sqv9s4tj

Sources of funding

Faculty of Science and Health, Charles Sturt University

Acknowledgements

Veterinary Diagnostic Laboratory, Charles Sturt University

Authorship

J. Hale is the first author and was responsible for primary research, including sample collection and manuscript preparation. R. Labens is the senior author and primary doctorate supervisor and assisted with study design and manuscript preparation. K. Hughes is the secondary doctorate supervisor and assisted with study design and manuscript preparation. S. Hall performed cytokine analysis on serum samples.

Table 1: Information on capture and detection antibodies used in this study including source and concentration.

Cytokine	**Bead Region (BioRad catalogue number)**	**Capture Antibody (Concentration µg/ml)**	**Detection Antibody (Concentration µg/ml)**	**Source catalogue number**
IL-1ra	65 (MCA-0065-01)	Goat anti-equine 20	Biotinylated goat anti- equine 0.5	R&D Systems DY2466
IL-1β	77 (MCA-0077-01)	Goat anti-equine 20	Biotinylated goat anti- equine 0.5	R&D Systems DY3340
IL-10	35 (MC1-0035-01)	Goat anti-equine 20	Biotinylated goat anti- equine 0.5	R&D Systems DY1605
TNF-α	55 (MC1-0055-01)	Goat anti-equine 20	Biotinylated goat anti- equine 0.5	R&D Systems DY1814

Table 2: Median (interquartile) concentrations for cytokines measured for each time point.

Cytokine	Pre-exercise	1 hour Post-exercise	24 hours Post-exercise
IL-1Ra (pg/mL)	7349 (1272 – 10760)	584.4 (81.9 – 5098)	533.9 (271.7 – 7946)
IL-1β (pg/mL)	460.6 (195.3 – 885.6)	358 (121.4 – 2338)	268.2 (42.33 – 360.8)
IL-10 (pg/mL)	154.5 (99.71 – 488.6)	104.5 (61.86 – 488.6)	170.3 (71.87 – 649.5)
TNF-α (pg/mL)	11.16 (8.36 – 32.74)	11.92 (9.28 – 39.75)	17.99 (6.98 – 41)

References

1. Rossdale PD, Hopes R, Digby NJ, Offord K. Epidemiological study of wastage among racehorses 1982 and 1983. Vet Rec. 1985;116(3):66-9.
2. Kamm JL., Nixon AJ, Witte TH. Cytokine and catabolic enzyme expression in synovium, synovial fluid and articular cartilage of naturally osteoarthritic equine carpi. Equine Veterinary Journal. 2010; 42(8):693-699.
3. Goldring M. Osteoarthritis and cartilage: The role of cytokines. Current Rheumatology Reports. 2000; 2(6):459-465.
4. Granowitz EV, Clark BD, Mancilla J, Dinarello, CA. Interleukin-1 receptor antagonist competitively inhibits the binding of interleukin-1 to the type II interleukin-1 receptor. The Journal of Biological Chemistry. 1991;266(22):14147-14150.
5. Arend WP, Malyak M, Guthridge CJ, Gabay C. INTERLEUKIN-1 RECEPTOR ANTAGONIST: Role in Biology. Annual Review of Immunology. 1998;16(1):27-55.
6. Frisbie DD, Kawcak CE, Werpy NM, Park RP, McIlwraith CW. Clinical, biochemical, and histologic effects of intra-articular administration of autologous conditioned serum in horses with experimentally induced osteoarthritis. American Journal of Veterinary Research. 2007;68(3):290-296.
7. Meijer H, Reinecke J, Becker C, Tholen G, Wehling P. The production of anti-inflammatory cytokines in whole blood by physico-chemical induction. Inflamm Res. 2003;52(10):404-7.
8. Hraha TH, Doremus KM, McIlwraith CW, Frisbie DD. Autologous conditioned serum: The comparative cytokine profiles of two commercial methods (IRAP and IRAP II) using equine blood. Equine Veterinary Journal. 2011;43(5):516-521.
9. Wehling P, Moser C, Frisbie D, McIlwraith CW, Kawcak C, Krauspe R, Reinecke J Autologous Conditioned Serum in the Treatment of Orthopedic Diseases. BioDrugs. 2007;21(5):323-332.
10. Frisbie D, Ghivizzani S, Robbins P, Evans C, McIlwraith C Treatment of experimental equine osteoarthritis by in vivo delivery of the equine interleukin-1 receptor antagonist gene. Gene Therapy. 2002;9(1):12.
11. Marques-Smith P, Kallerud AS, Johansen GM, Boysen P, Jacobsen AM, Reitan KM, Henriksen MM, Löfgren M, Fjordbakk CT. Is clinical effect of autologous conditioned serum in spontaneously occurring equine articular lameness related to ACS cytokine profile? BMC Veterinary Research. 2020;16(1):1-9.
12. Ferris DJ, Frisbie DD, McIlwraith CW, Kawcak CE. Current joint therapy usage in equine practice: A survey of veterinarians 2009. Equine Veterinary Journal. 2011;43(5):530-535.
13. Fjordbakk CT, Johansen GM, Lovas AC, Oppegard KL, Storset AK. Surgical stress influences cytokine content in autologous conditioned serum. Equine Vet J. 2015;47(2):212-7.
14. Huldani, Pattelongi I, Massi MN, Idris I, Bukahari A, Widodo ADW, Achmad H. Research Reviews on Effect of Exercise on DAMP's, HMGB1, Proinflammatory Cytokines and Leukocytes. Systematic Reviews in Pharmacy. 2020;11(4).
15. Horohov DW, Sinatra ST, Chopra RK, Jankowitz S, Betancourt A, Bloomer RJ. The Effect of Exercise and Nutritional Supplementation on Proinflammatory Cytokine Expression in Young Racehorses During Training. Journal of Equine Veterinary Science. 2012;32(12):805-815.
16. Smith LL. Cytokine hypothesis of overtraining : a physiological adaptation to excessive stress? Medicine and Science in Sports and Exercise. 2000;32(2):317-331.
17. Hale J, Hughes K, Hall S, Labens R. Effects of Production Method and Repeated Freeze Thaw Cycles on Cytokine Concentrations and Microbial Contamination in Equine Autologous Conditioned Serum. Frontiers in Veterinary Science. 2021;8(1426).
18. Hall SA, Stucke D, Morrone B, Lebelt D, Zanella AJ. Simultaneous detection and quantification of six equine cytokines in plasma using a fluorescent microsphere immunoassay (FMIA). MethodsX. 2015;2:241-248.

19. Suzuki K, Nakaji S, Yamada M, Totsuka M, Sato K, Sugawara K. Systemic inflammatory response to exhaustive exercise. Cytokine kinetics. Exercise Immunology Review. 2002;8:6-48.
20. Suzuki K. Characterization of Exercise-Induced Cytokine Release, the Impacts on the Body, the Mechanisms and Modulations. International Journal of Sports and Exercise Medicine. 2019;5.
21. Suzuki K. Chronic Inflammation as an Immunological Abnormality and Effectiveness of Exercise. Biomolecules. 2019;9(6).
22. Brenner IKM, Natale VM, Vasiliou P, Moldoveanu AI, Shek PN, Sheparad RJ. Impact of three different types of exercise on components of the inflammatory response. European Journal of Applied Physiology and Occupational Physiology. 1999;80(5):452-460.
23. Ostrowski K, Rohde T, Asp S, Schjerling P, Pedersen BK. Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans. The Journal of Physiology. 1999;515(1):287-291.
24. Ostrowski K, Rohde T, Zacho M, Asp S, Pedersen BK. Evidence that interleukin-6 is produced in human skeletal muscle during prolonged running. The Journal of Physiology. 1998;508(3):949-953.
25. Ostrowski K, Hermann C, Bangash A, Schjerling P, Nielsen JN, Pedersen BK. A trauma-like elevation of plasma cytokines in humans in response to treadmill running. The Journal of Physiology. 1998;513(3):889-894.
26. Scott JPR, Sale C, Greeves JP, Casey A, Dutton J, Fraser WD. Effect of exercise intensity on the cytokine response to an acute bout of running. Medicine and Science in Sports and Exercise. 2011;43(12):2297-2306.
27. Lamprecht ED, Bagnell CA, Williams CA. Inflammatory responses to three modes of intense exercise in Standardbred mares – a pilot study. Comparative Exercise Physiology. 2008;5(3-4):115-125.
28. Suzuki K. Cytokine Response to Exercise and Its Modulation. Antioxidants. 2018;7(1):17.
29. Bertone AL, Palmer JL, Jones J. Synovial Fluid Cytokines and Eicosanoids as Markers of Joint Disease in Horses. Veterinary Surgery. 2001;30(6):528-538.
30. Frisbie DD, McIlwraith CW, de Grauw JC. Synoival Fluid and Serum Biomarkers; Joint disease in the horse. 2016:179-191
31. Benito MJ, Veale DJ, FitzGerald O, van den Berg WB, Bresnihan B. Synovial tissue inflammation in early and late osteoarthritis. Annals of the Rheumatic Diseases. 2005;64(9):1263-1267.
32. Arend WP, Smith MF Jr, Janson RW, Joslin FG. IL-1 receptor antagonist and IL-1 beta production in human monocytes are regulated differently. J Immunol. 1991;147(5):1530-6.
33. Bruunsgaard H, Galbo H, Halkjaer-Kristensen J, Johansen TL, MacLean DA, Pedersen BK. Exercise-induced increase in serum interleukin-6 in humans is related to muscle damage. The Journal of Physiology. 1997;499(3):833-841.
34. Steensberg A, Keller C, Starkie RL, Osada T, Febbraio MA, Pedersen BK. IL-6 and TNF-α expression in, and release from, contracting human skeletal muscle. American Journal of Physiology - Endocrinology And Metabolism. 2002;283(6):1272-1278.
35. Izquierdo M, Ibanez J, Gorostiaga EM, Calbet JAL, Navarro-Amezqueta I, Gonzalez-Izal M, Idoate F, Häkkinen K, Kraemer WJ, Palacios-Sarrasqueta M, Almar M. Cytokine and hormone responses to resistance training. European Journal of Applied Physiology. 2009;107(4):397-409.
36. Pedersen BK, Hoffman-Goetz L. Exercise and the Immune System: Regulation, Integration, and Adaptation. Physiological Reviews. 2000;80(3):1055-1081.
37. Campbell EH. Lactate-driven equine conditioning programmes. The Veterinary Journal. 2011,190(2):199-207.

38. Nagata S, Takeda F, Kurosawa M, Mima K, Hiraga A, Kai M, Taya K. Plasma adrenocorticotropin, cortisol and catecholamines response to various exercises. Equine Veterinary Journal. 1999;31(30):570-574.