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Final report

Autologous biological treatments – content and effect on joint inflammation

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Part 1: Detailed summary

Hälta, till följd av osteoartrit (OA), är en av de vanligaste orsakerna till veterinärbesök hos våra sporthästar. Vid OA uppträder alltid en inflammation i någon fas av processen samt en successiv nedbrytning av ledbrosket. Autologt konditionerat serum (ACS) har blivit en mycket vanlig intra-artikulär behandling vid ledinflammation hos häst och människa trots att det inte är känt exakt vad i detta serum som skulle ge en positiv effekt. ACS framställs från patientens eget blod med hjälp av kommersiella produkter (IRAP, ABPS). Konditioneringen antas öka innehållet av anti-inflammatoriska cytokiner såsom Interleukin-1 receptor antagonist (IL-1Ra) samt tillväxtfaktorer. Platelet-rich plasma (PRP) är en annan produkt framställd från patientens blod som används för intra-artikulär behandling. I denna produkt ansamlas olika tillväxtfaktorer men även anti-inflammatoriska substanser.

Syftet med projektet var att studera innehållet i behandlingar framställda från hästens eget blod samt att studera dess effekter i väl etablerade *in vitro* modeller.

Blod samlades in från halta hästar och behandlades enligt följande:

- ACS: Insamling av blod i kommersiella ABPS rör innehållande ”borosilicate” glaskulor (Arthrex Vet Systems) enligt tillverkarens instruktioner inklusive inkubering 24 h i 37ºC följt av centrifugering.
- Autologt ekvint serum inkuberat 24 h (AES 24h): Blod insamlat i serumrör utan tillsats av ”borosilicate” glaskulor.

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- Autologt ekvint serum (AES): Blod insamlat i serumrör och centrifugerat utan inkubering, utan direkt alikvoterat.
- PRP: Insamling av blod i kommersiella ACP rör (Arthrex Vet Systems) enligt tillverkarens instruktion.

Innehållet i produkterna analyserades och en ökning av IL-1Ra kunde ses i både ACS och AES 24h jämfört med AES. En potentiell anti-inflammatorisk effekt studerades *in vitro* i ett pelletsystem där en inflammation inducerades i kondrocyter. Behandlingarnas (AES, AES 24h och ACS) förmåga att stoppa inflammationen studerades med hjälp av microarray och PCR av genuttryck samt innehåll av det nedbrytande enzymet MMP-13 i det omgivande mediet.

Analyserna visade inte någon förmåga av behandlingarna att stoppa inflammationen då ett minskat uttryck av matrix molekyler samt en ökning av nedbrytande enzymer kunde ses oavsett behandling. Behandlingarnas förmåga att regenerera brosk studerades i en *in vitro* modell där explant från brosk med mild ytlig OA användes. Brosket utvärderades med histologi och visade inte någon förmåga av behandlingarna AES, ACS eller PRP att regenerera brosket. Fortsatta analyser med hjälp av immunohistokemi skulle kunna identifiera andra effekter på brosket.

Sammanfattningsvis innehåller inkuberat serum ökade nivåer av IL-1Ra oavsett tillsats av glaskulor (ACS) eller inte (AES 24h) jämfört med serum som inte inkuberats, det var även stora individuella skillnader. Någon anti-inflammatorisk effekt på inflammerade kondrocyter eller någon regenerativ effekt på explant från ledbrosk kunde inte identifieras i *in vitro* försöken. Andra effekter än de studerade kan inte uteslutas och fortsatta studier är av stor vikt.

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Part 2: Main report (max. 10 pages)

Introduction

Background

Osteoarthritis (OA), a condition involving inflammation and joint destruction, is a common cause of lameness in horses. There is a need for safe and effective disease-modifying treatments against OA. Autologous biological products such as autologous conditioned serum (ACS) and platelet-rich plasma (PRP) are currently used as intra-articular treatments even though the scientific evidence of positive effects is limited. The products are derived from the patient's own blood and the complete content and mechanisms of action are not fully understood. ACS and PRP both contain growth factors and anti-inflammatory cytokines. ACS is considered the main anti-inflammatory treatment through increased expression of IL-1Receptor antagonist (IL-1Ra), whereas PRP is suggested to be more anabolic.

Objective

The purpose of this project was to define the individual components of the preparations *ex vivo* and characterise their effects on inflamed articular cartilage in well-established *in vitro* systems.

The specific aims were:

Ex vivo: a) To determine the concentration of well-defined cartilage-modifying substances (e.g. growth-stimulating and anti-inflammatory factors) in ACS and PRP preparations from different horses; and b) to define the inter-individual variations in concentrations of these factors.

In vitro: To test the hypothesis that ACS and PRP has a disease-modifying effect on inflamed articular cartilage in well-defined *in vitro* models.

Material and methods

Preparation of samples

Sampling 1

Blood from five lame horses was collected and prepared as follows:

- Autologous conditioned serum (ACS): Blood collected in commercial ABPS tubes containing borosilicate glass beads (Arthrex Vet Systems) according to the manufacturer's instruction including incubation for 24 h in 37ºC prior to centrifugation and collection of serum.
- Autologous equine serum with 24 h incubation (AES 24h): Blood collected in serum tubes without glass beads and prepared as ACS.
- Autologous equine serum (AES): Blood collected in serum tubes which was allowed to clot in room temperature for 1h prior to centrifugation and collection of serum.

Sampling 2

Blood from twenty lame horses was collected and prepared as follows:

- ACS: see sampling 1
- AES 24h: see sampling 1
- AES: see sampling 1
- PRP: Blood collected in commercial ACP tubes (Arthrex Vet Systems) according to the manufacturer's instruction.

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Ex vivo methods

Sampling 1

The concentration of IL-1Ra in the samples from sampling 1 was measured using Equine IL-Ra DuoSet sandwich ELISA (RnD Systems) and the concentration of IL-1β was measured using Equine IL-1β VetSet™ sandwich ELISA (Kingfisher Biotech Inc.).

Proteins in the samples were measured with Proximity Extension Assay (PEA) technology using Olink® Inflammation panel v. 3004 and Olink® Oncology II panel v. 7002 (Olink Proteomics AB) according to the manufacturer's instructions. PEA technology enables 92 analytes to be analyzed simultaneously, using 1 µL of each sample. Data are quality controlled and normalized using an internal extension control and an inter-plate control, to adjust for intra- and inter-run variation. The final assay read-out is presented in Normalized Protein Expression (NPX) values, which is an arbitrary unit on a log2-scale; a high value corresponds to greater protein expression. All assay validation data are available on the manufacturer's website (www.olink.com). The assay is directed against human proteins and values below limit of detection can therefore indicate either low levels or an inability to detect the equine protein.

Sampling 2

The samples from sampling 2 were analysed using equine specific commercially available solid phase sandwich ELISA kits (RnD Systems) for determining concentration of IL-1Ra (DY2466), IL-1β (DY3340), IL-10 (DY1605) and TNFα (DY1814), whereas human-specific kits were used for TGF-β (DY240) and IGF-1 (DY291), the latter two having confirmed equine cross-reactivity.

In vitro methods

The effects of the preparations were investigated in two *in vitro* models, chondrocyte pellet cultures and cartilage explants.

Pellet culture

Chondrocytes were isolated from macroscopically normal equine cartilage from five euthanized, non-lame Icelandic horses (30 months old) as part of another study (Ley et al. 2013). The chondrocytes were expanded and cultured into 3D pellets and thereafter stimulated with recombinant equine IL-1β (RnD Systems) to mimic the inflammation in OA. The pellets were treated in the following treatment groups using samples from sampling 1 above:

- Unstimulated control
- IL-1β (5 ng/ml)
- IL-1β (5 ng/ml) + AES (40% V/V)
- IL-1β (5 ng/ml) + AES24h (40% V/V)
- IL-1β (5 ng/ml) + ACS (40% V/V)

The pellets and media were collected after 2 and 48h. RNA was isolated from the pellets and gene expression of aggrecan and collagen type II alpha I was analysed with qRT-PCR using TaqMan® Gene Expression Assays (Applied Biosystems). Global gene expression was analysed with microarray using GeneChip Equine Gene 1.0 ST Arrays (Affymetrix Inc.). The content of the matrix degrading enzyme MMP-13 was measured in pellet medium using Fluorokine® E Human Active MMP-13 Fluorescent Assay (RnD Systems).

Cartilage explants

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Cartilage tissue for explant culture was collected at a local abattoir from 7 horses (7-16 years old) with mild structural OA in the fetlock joint. Macroscopic staging was done according to McIlwraith *et al.* 2010.
The explants were treated in the following treatment groups using samples from sampling 2 above:

- Unstimulated control
- AES (40% V/V)
- ACS (40% V/V)
- PRP (40% V/V)

The treatments were added for 72 h at day 0, 6 and 12 and explants were harvested at day 24. The explants were embedded in paraffin and the sections were stained with hematoxylin & eosin (H&E), 0.1% aqueous safranin-O counterstained with 0.1% aqueous fast green (SOFG) and graded microscopically according to McIlwraith *et al.* 2010.

Statistical methods

Statistical analyses of PEA technology
A paired Wilcoxon test was used for testing sinificance between groups. The *p*-values were corrected for multiple hypothesis testing using the Benjamini and Hochberg method (Benjamini & Hochberg 1993). Differences were considered significant when $p < 0.05$.

Statistical methods for analysing IL-1Ra in serum preparations
The data were analysed as a one-factor randomized block experiment (Olsson 2011) with "horse" as block and using the Mixed procedure in the SAS package according to the SAS/Stat User's Guide Version 9.4. (SAS Institute Inc. Cary, NC, USA). Assumptions underlying the analysis were checked using diagnostic plots. No apparent deviations from normality or homoscedasticity were detected. Multiple comparisons were adjusted for multiplicity using Tukey's method. Differences were considered significant when $p < 0.05$.

Statistical comparisons in sampling 2
The content of IL-1Ra, IGF-1 and TGF-β in AES, AES 24h, ACS and PRP was compared using a 95% confidence interval (CI).

Statistical methods for MMP-13 in culture media and qRT-PCR of gene expression in pellets
Experimental data were analyzed using a repeated-measures mixed model (Littell *et al.* 2006) and the Mixed procedure in the SAS package according to the SAS/Stat User's Guide Version 9.4. (SAS Institute Inc.). The fixed part of the model included treatment, time, and the interaction between these. Relations between observations within unit were modeled using a compound symmetric covariance structure. Assumptions underlying the analysis were checked using diagnostic plots. No apparent deviations from normality or homoscedasticity were detected. Differences were considered significant when $p < 0.05$.

Microarray data analysis
All raw data from the microarray analyses were normalized in an Expression Console (Affymetrix Inc.) using the robust multi-array average method suggested by Li and Wong (Li & Wong 2001, Irizarry *et al.* 2003) and subsequent analyses of gene expression data were performed using the statistical computing language R (www.r-project.org) and packages available from the Bioconductor project (www.bioconductor.org). In order to search for the differentially expressed genes between unstimulated, IL-1β stimulated pellets and the other groups (unstimulated, AES, AES24h and ACS), a paired empirical Bayes moderated t-test was applied using the 'limma' package (Smyth 2004 and 2005). To address the problem with

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multiple testing, the *p*-values were adjusted using the method of Benjamini and Hochberg (Benjamini & Hochberg 1993). Differences between IL-1β stimulated pellets and the other groups (unstimulated, treated with AES, AES 24h or ACS) were considered significant if the logarithmic fold change (log2 FC) was ≥ 1 or ≤ -1 with adjusted $p < 0.05$. The microarray data have been submitted to the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/), accession number GSE152253.

Explant evaluation
The histological staging for control, ACS and AES at day 24 were analysed with GraphPad Prism 7.04 with One-way ANOVA (non-parametric) med Greenhouse-geisser correction. Data were presented as mean (±SD) and significance was set at $p < 0.05$.

Ethical permission
The study was approved by the Ethical Committee on Animal Experiments, Uppsala, Sweden (No.C62/13 and 5.8.18-02896/2018). Collection of cartilage for pellet culture was done as part of another study (Ley et al. 2013) with ethical approval from the Iceland National Animal Research Committee 2007.

Results and discussion

Ex vivo
Sampling 1
IL-1Ra was detected in all samples and the mean concentrations and results of statistical comparisons are shown in table 1.

Table 1. IL-1Ra concentration in AES, AES 24h and ACS and statistical comparisons between samples. Values are presented as mean ± standard deviation (SD) and $p<0.05$ is considered significantly different. n=5.

	Concentration (ng/ml ± SD)	**Significantly different than:**	
AES	0.412 ± 0.493	AES24h	p=0.0002
AES 24h	31.1 ± 11.2	ACS	p=0.0162
ACS	15.8 ± 9.94	AES	p=0.0153

Increased levels of IL-1Ra after incubation of serum with or without glass beads have previously been detected in equine (Hraha *et al.* 2011, Fjordbakk *et al.* 2014, Lasarzik de Ascurra *et al.* 2019, Linardi *et al.* 2019), canine (Sawyere *et al.* 2016), and human (Meijer *et al.* 2003, Rutgers *et al.* 2010) samples. The significantly higher concentration in AES 24h (incubated without commercial system) is interesting and questions the need for commercial products to obtain high levels of IL-1Ra.

IL-1β was not detected in any AES or ACS sample, and was only detected in the AES 24h sample from one horse.

The PEA technology allowed analysis of 184 protein assays. The following table depicts the detectability range of the 184 assays (Table 2). Detectability 1 means that the assay is detected in all samples and detectability 0 means the assay is not detected in any sample.

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Table 2. Detectability distribution of assays

Detectability range	Number of assays
0 - 0.25	78
0.25 – 0.5	23
0.5 – 0.75	8
0.75 - 1	74

The statistical test found no assays that significantly (adjusted *p*-value < 0.05) separated the groups AES, AES 24h and ACS from each other. It is not possible to know if this is due to the small sample size or if there really are none to be found.

Sampling 2

The content of IL-1Ra, IGF-1 and TGF-β in samples from sampling 2 is presented in table 3. Comparisons of the 95% CI showed that the concentration of IL-1Ra was higher in AES 24h than in the other groups and that the content in ACS was higher than in AES and PRP. The content of TGF-β was higher in PRP than in the other goups. The majority of IL-10, IL-1β and TNF-α measurements fell below or near the lower limit of detection even at the lowest possible dilution.

Table 3. Concentration of IL-1Ra, IGF-1 and TGF-β in AES, AES24h, ACSand PRP. Values are presented as mean (95% Confidence interval (CI)), n=20.

	Concentration IL-1Ra (ng/ml) Mean (95% CI)	**Concentration IGF-1 (ng/ml) Mean (95% CI)**	**Concentration TGF- β (ng/ml) Mean (95% CI)**
AES	2.38 (1.24 – 3.52)	25.92 (21.69 – 30.13)	1.85 (1.76 – 1.95)
AES 24h	148.26 (102.44 – 194.09)	27.11 (23.78 – 30.44)	1.74 (1.71 – 1.77)
ACS	17.58 (5.78 – 29.38)	25.32 (22.33 – 28.30)	1.31 (1.13 – 1.49)
PRP	5.14 (-0.60 – 10.89)	21.36 (19.33 – 23.39)	11.65 (9.57 – 13.73)

In vitro

Pellet culture

The concentration of matrix degrading enzyme MMP-13 in cell culture media was not significantly different in the treatment groups after 2 h. The concentration was significantly higher in the cell medium from IL-1β stimulated pellets compared to the unstimulated pellets after 48h but there were no significant differences between IL-1β stimulated pellets and pellets treated with AES, AES 24h or ACS.

Gene expression of the matrix molecule aggrecan was significantly lower in all IL-1β treated pellets after 2 h and 48 h, with and without treatments with AES, AES 24h and ACS. A significant reduction in gene expression was also seen for collagen type II alpha I after 48h in IL-1β treated pellets with and without treatments.

Microarray analysis of the pellets revealed differences in gene expression of 672 genes between IL-1β stimulated pellets and unstimulated pellets at 2 h and of 279 genes at 48 h. The number of significantly different genes between IL-1β and the treatment groups AES, AES 24h and ACS were 11, 13 and 12 respectively at 2 h and 82, 80 and 75 respectively at 48 h.

An overall catabolic state of the pellets were identified as increased concentration of MMP-13 and decreased gene expression of matrix components such as aggrecan and collagens. Gene

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expression of the degrading enzymes *ADAMTS1* and *ADAMTS5* was increased in treated pellets. Genes involved in inflammation such as *IL-6* and *IL-11* were identified among the significantly different genes. These genes has a regulative function and can be either beneficial or detrimental. Increased expression of several growth factors such as *FGF7, PDGFD, BMP2, GDF10, INHBA* and *INHBE* was identified which may be favourable for regenerating cartilage.

Cartilage explants
The cartilage explants were histologically graded after 24 days of culture. There were no significant differences between the groups unstimulated, AES and ACS which were graded as 8 (±2.4), 8.7 (±1.8) and 8.5 (±2.2) respectively. A regenerative effect on cartilage of the treatment can therefore not be supported in this study. The explants treated with PRP has been graded but not published and will be further analysed. All explants will be studied further with immunohistochemistry including markers for pain such as NGF (nerve growth factor), and their receptors (TrkA, p75).

Conclusions

In conclusion, this study is the first to display the global gene expression in inflamed chondrocytes and histological staging of OA cartilage to study the treatment effects of autologous serum. There are large inter-individual differences in the content but an increase in IL-1Ra was observed in incubated samples, regardless of borosilicate glass beads were present (ACS) or not (AES 24h). Additionally, AES 24h contained significantly higher concentration of IL-1Ra than ACS, which questions the use of commercial systems to achieve high levels of IL-1Ra. However, neither, AES 24h nor ACS did show any evidence of anti-inflammatory effects on the inflamed chondrocyte pellets or any evidence of regeneration in the OA cartilage explants. Hence the presented results do not support disease modifying properties of ACS.

Relevance for the practical horse sector incl. recommendations

The project has increased the knowledge about autologous biological treatments but cannot support disease modifying properties of the treatments. ACS and AES 24h contain higher levels of IL-1Ra than unincubated serum but an anti-inflammatory effect cannot be confirmed in the *in vitro* systems. Large inter-individual differences in the content was observed and the need for commercial systems can be questioned due to the higher concentration of IL-1Ra in AES 24h, not treated with borosilicate glass beads, compared to ACS. Further studies of the content has to be performed in order to exclude increased content of pro-inflammatory substances.

The lack of evidence for anti-inflammatory effects in the pellet system and lack of regeneration in the OA cartilage explants do not support disease-modifying properties but further studies are needed to potentially find other positive effects. In order to study potential pain relieving effects we would like to continue the analysis of the *in vitro* material using immunohistochemistry for pain mediators such as nerve growth factor (NGF) and its receptors TrkA and p75.

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Part 3: Result dissemination

Scientific publications, *published*	**Marques-Smith P, Kallerud AS, Johansen GM, Boysen P, Jacobsen AM, Reitan KM, Henriksen MM, Löfgren M & Fjordbakk CT. (2020) Is clinical effect of autologous conditioned serum in spontaneously occurring equine articular lameness related to ACS cytokine profile?. BMC Veterinary Research. 16(1):1-9. https://doi.org/10.1186/s12917-020-02391-7**
	Löfgren M, Ekman S, Ekholm J, Engström M, Fjordbakk CT, Svala E, Holm Forsström K, Lindahl A, Skiöldebrand E. (2022) Conditioned serum in vitro treatment of chondrocyte pellets and osteoarthritic explants. Equine Veterinary Journal. https://doi.org/10.1111/evj.13852
Scientific publications, *submitted*	
Scientific publications, *manuscript*	*Author(s), title*
Conference publications/ presentations	*Author(s), year, title, conference name, location and date, (link if applicable)*
Other publications, *media etc.*	*Title, year/date, place of publication (link if applicable)*
	Ledbehandling med autologt konditionerat serum, 2020-11-20, Newsletter: Forskningsnytt Djurhälsa och djurvälfärd, SLU, https://www.slu.se/acshast
Oral communication, *to horse sector, students etc.*	*Title, year/date, group presented to (link if applicable)*
	Introduktion Hippologprogrammet, 2019-09-06, hippologstudenter åk 1, Flyinge
	Ledforskning, 2019-09-18, Beridarprogrammet åk 1-2, Flyinge
	Vetenskap, forskning, källor, 2020-02-29, Öppet hus hippologprogrammet Flyinge
	Ledforskning, 2020-09-11, Hippologigymnasiet åk 1-3, Flyinge
Student theses	*Author/Student, co-authors/supervisors, year, title, type of thesis (doi/link if applicable)*
Other	

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