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Use of allogeneic freeze-dried conditioned serum for the prevention of degradation in cartilage exposed to IL-1 β

Livia Camargo Garbin^{1,2}, C. Wayne McIlwraith³ and David D. Frisbie^{3*}

Abstract

Background: Autologous conditioned serum (ACS) has been extensively used in the field of veterinary orthopaedics and sports medicine. Due to the autologous and blood-derived nature of this product, issues such as individual variability, need for storage at low temperatures and non-availability for immediate use are frequently encountered for ACS use in the field. To address those issues, we proposed the evaluation of an off-the-shelf allogeneic freeze-dried version of conditioned serum in an in vitro model of osteoarthritis. In this study, we evaluated if origin (autologous and allogeneic) and preparation (frozen and freeze-dried) of conditioned serum could influence its effect in an in vitro model.

Results: IL-1 β stimulation in cartilage led to a significant increase in media GAG and decreased levels of GAG in cartilage explants at the termination of the experiment. No significant differences were noted in outcomes measured in the cartilage explants with respect to the main effects of treatment (frozen versus freeze-dried serum), autologous versus allogeneic preparations or based on serum concentration.

Conclusions: The study did not observe any substantial differences in the response of cartilage to allogeneic freeze-dried CS when compared to other independent parameters (autologous and frozen preparations). Further investigation using in vivo systems appears warranted.

Background

Inflammation within the joint is a common feature in equine and human osteoarthritis (OA) and it has been associated with its symptoms and as a trigger point for the progressive degeneration of the joint [1]. In traumatically injured joints, acute synovitis and capsulitis may contribute to joint degradation through the release of inflammatory mediators and enzymes, which has been demonstrated firstly in the horse [1]. For this reason, more interest has been given to drugs that could modulate OA development, including the inhibition of target cytokines involved in inflammatory pathways [2]. Although IL-1 is

not the only pro-inflammatory protein enrolled in OA [3] pathogenesis, studies suggest its action is the most promising approach for cytokine blocking [4]. IL-1 receptor antagonist protein (IL-1Ra) is a naturally occurring molecule that competes for occupancy of the IL-1 cell surface receptors, but doesn't initiate the catabolic response typical of IL-1 [5]. Thus IL-1Ra is a natural inhibitor of IL-1 and its efficacy has been shown in vivo, including in an equine model [6]. Autologous conditioned serum (ACS) is produced following culture of whole blood in the presence of medical grade coated glass beads. This process results in serum that is enriched in endogenous IL-1Ra as well as anti-inflammatory cytokines (such as IL-4, IL-10) and growth factors [7].

While significant lameness and histological improvement was observed in experimentally induced osteoarthritic joints treated with ACS commercial kits in horses

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[8] multiple limitations preclude its widespread acceptance and use. One limitation of ACS is the preparation and need for storage at low temperatures (< -20°C), which is inconvenient for most equine practitioners. Additionally, ACS presented variation in both pro- and anti-inflammatory cytokine based on the patient [7]. The current work addresses some of these issues. Specifically, this study evaluated allogeneic freeze-dried conditioned serum (CS), allowing a more homogeneous, stable, quantifiable and practical option for use in the field. Thus, the objective of this research was to determine the influence of origin (autologous and allogeneic) and preparation (freeze-dried and frozen) in the ability of conditioned serum (CS) to palliate degradative changes induced by IL-1β on equine cartilage.

This study is based on three hypotheses tested in a cartilage explant in vitro model. Firstly, that freeze-dried CS would not present significantly different effects compared to frozen preparations. The second is that allogeneic CS would not present significantly different effects compared to autologous preparations and the third, that CS effects would be concentration dependent.

Results

Stimulation of cartilage explants with IL-1β (analysis 1)

The efficiency of using IL-1β as a model to induce a pro-inflammatory environment for cartilage was assessed in this analysis. The presence of IL-1β caused a significant increase in glycosaminoglycan (GAG) released in culture supernatants at Days 2, 4 and 6 (*P* < 0.005) independent of the type of media supplementation used

(Supplementary Table 1). In cartilage explants, the presence of IL-1β (34.32 ± 9.52 μg/μg of DNA) resulted in significant GAG loss at the end of the study compared to control (69.09 ± 9.84 μg/μg of DNA; *P* = 0.0143).

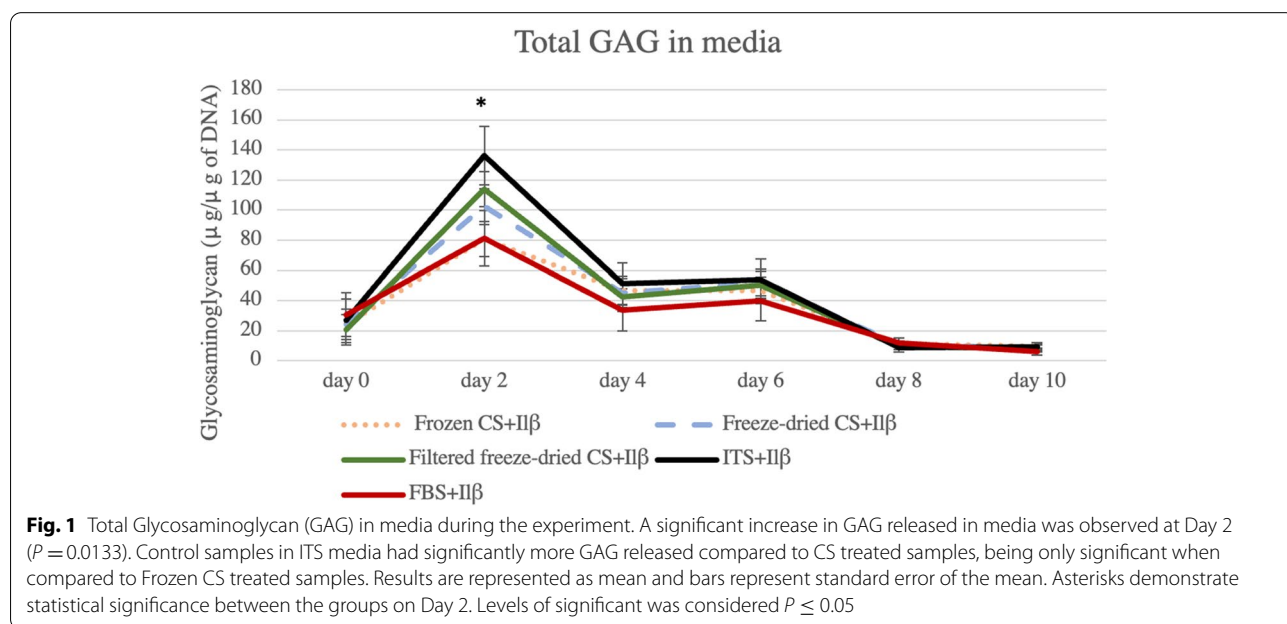
A significant effect of IL-1β was observed for ³⁵SO₄-labeled GAG in culture supernatant at Day 2 and 4 (*P* < 0.0339) of the experiment (Supplementary Table 2). The interaction between type of media used and effect of L-1β was as well significant at Day 2 and 6 (*P* < 0.034).

CS effect on cartilage stimulated with IL-1β (analysis 2)

This analysis was done to assess the effect of CS in cartilage under IL-1β stimulation.

In culture supernatant GAG analysis, a significant effect of Treatment was observed only at Day 2 of the experiment (*P* = 0.0133). Specifically, ITS+IL-1β control presented increase GAG levels in media compared to Frozen CS (Fig. 1). No significant differences between Frozen CS and either Freeze-dried preparation were observed in this comparison. In cartilage explants however, no significant differences were observed in total GAG.

The presence of CS had a significant effect (*P* ≤ 0.0001) on ³⁵SO₄-labeled GAG in media only at Day 2. Specifically, increased levels of ³⁵SO₄-labeled GAG in media were observed in samples treated with Freeze-dried (57.25 ± 4.69 DPM/ug of DNA) and Filtered Freeze-dried CS (61.75 ± 4.75 DPM) in comparison with the ITS+IL-1β (36.48 ± 8.34 DPM/μg of DNA), FBS+ IL-1β (21.63 ± 8.34 DPM/μg of DNA) controls and Frozen CS group (41.37 ± 4.69 DPM/μg of DNA) (Fig. 2). These



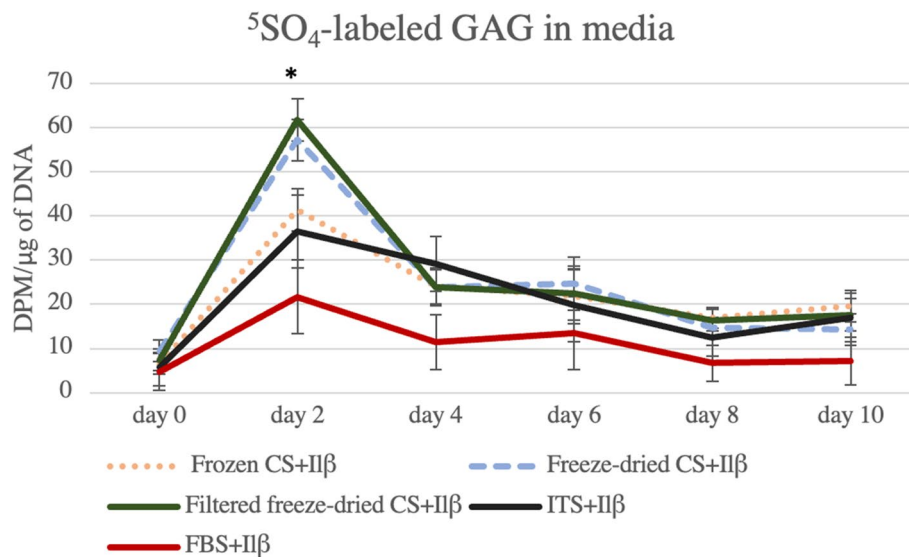


Fig. 2 ³⁵SO₄-labeled GAG released in media during the experiment. ³⁵SO₄-labeled GAG presented significantly higher levels in media only at Day 2 ($P \leq 0.0001$), in samples treated with Freeze-dried and Filtered Freeze-dried CS in comparison with the ITS+IL-1 β and FBS+ IL-1 β controls and Frozen CS groups. Results are represented as mean and bars represent standard error of the mean. Asterisks demonstrate statistical significance between the groups on Day 2. Levels of significant was considered $P \leq 0.05$

changes did not result in any significant differences in the ³⁵SO₄-labeled GAG in cartilage explants at the termination of the study when compared to ITS+IL-1 β control.

Study of the effects of CS different formulations in cartilage explants

Biochemical data analysis (analysis 3)

In this analysis, only CS treated samples were considered to assess the main effects Treatment, Allogenicity and Concentration. Similar to the results observed in Analysis 2 lower media GAG was observed in the media of Frozen CS compared to either Freeze-dried CS treatments; however, in this comparison the P -value was close to significant ($P = 0.0512$) (Fig. 3). On Days 4 and 6 of the experiment, Allogenicity had significant effect ($P < 0.022$), with less GAG being measured in the media of Allogeneic treated samples (Day 4: 36.38 ± 9.43 ; Day 6: 41.98 ± 8.98) when compared to Autologous (Day 4: 53.17 ± 9.39 ; Day 6: 57.38 ± 8.98) on these days (Fig. 4). These changes did not result in any significant differences observed in the total cartilage explants GAG at the end of the experiment.

The amount of ³⁵SO₄-labeled GAG in culture supernatant demonstrated a statistical significant difference on Day 2 based on CS Treatment ($P = 0.0029$) similar to the results presented in Analysis 2. The concentration of ³⁵SO₄-labeled GAG in media on Day 10 demonstrated a significant interaction ($P \leq 0.011$) for individual comparisons between Allogenicity and Treatment effects.

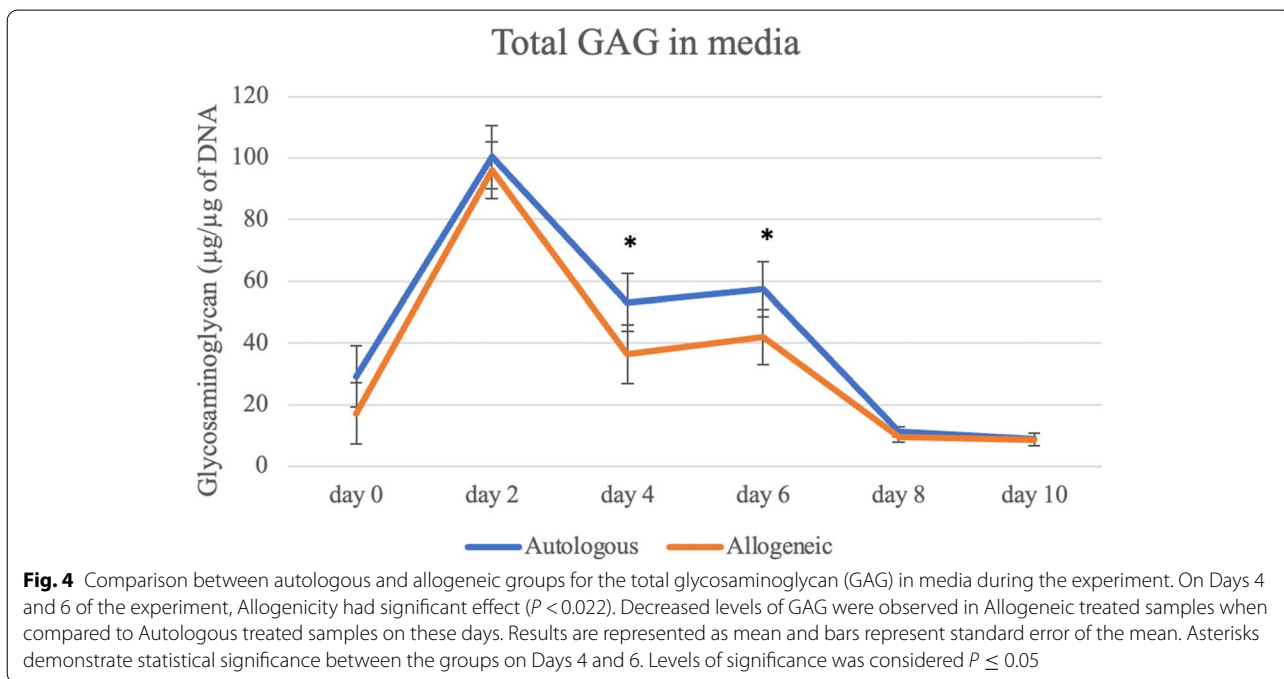
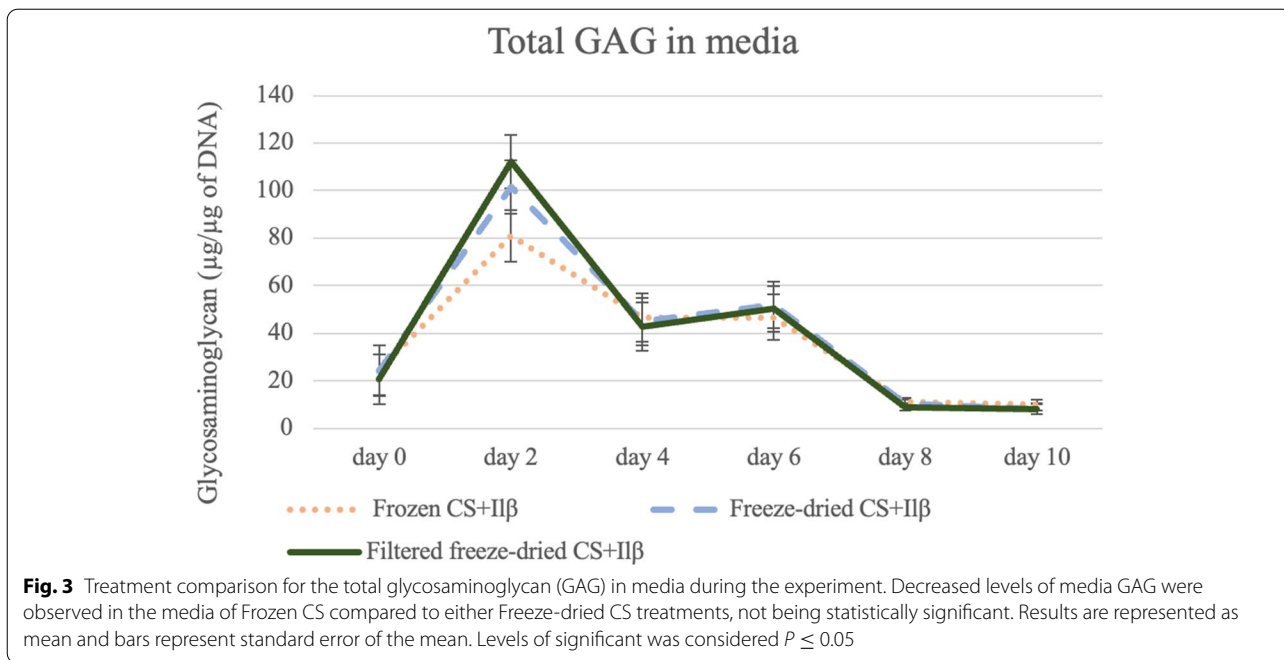
Specifically, it appeared more labelled GAG was released into the media with Allogeneic Frozen CS compared to Autologous Frozen CS as well as Autologous Freeze-dried CS (Fig. 5). However by the termination of the study no significant differences in cartilage ³⁵SO₄-labelled GAG were observed.

Gene expression

Catabolic cytokines and enzymes related to OA were evaluated for gene expression in this study to assess the modulatory effects of CS in cartilage. Gene expression analysis was performed on log transformed values to meet the assumptions of normality. No changes were greater than 2-fold and thus not considered biologically significant for any comparison (Fig. 6).

Discussion

To the authors' knowledge, this was the first published study to compare the effects of an allogeneic freeze-dried version of conditioned serum to autologous frozen conditioned serum, in cartilage under inflammatory conditions. This study utilized IL-1 β to provide an inflammatory environment. IL-1 β resulted in a significant increase in total GAG and ³⁵SO₄-labeled GAG release from cartilage explants, independent of the type of media. When added to media, IL-1 β resulted in less total GAG within the explants compared to non-IL-1 β stimulated samples as expected, although not to the same extent as previously observed when IL-1 β was continually



present in the media [9, 10]. In those studies, cartilage explants were continuously stimulated with IL-1 β as opposed to the acute exposure to the IL-1 β used (Day 0 and 4), which may explain the differences. Although not all outcome parameters showed significant effect of IL-1 β stimulation, the data in this experiment suggests

a significant catabolic effect of this cytokine, supporting the model in this study.

In the second analysis, when comparing the CS treated groups in cartilage to ITS+IL-1 β controls, differences were seen at Day 2 of the study but these changes did not result in differences that affected the

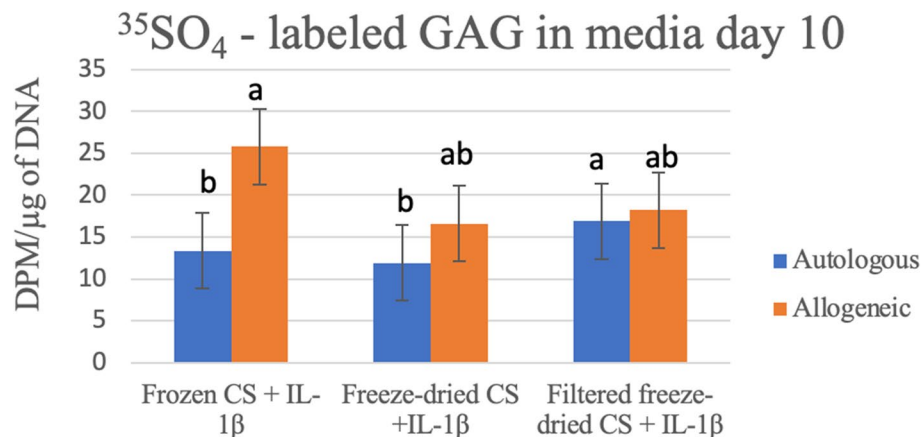


Fig. 5 ³⁵SO₄ - labeled GAG in media day 10 for Treatment*Allogenicity main effect interaction. Allogenic Frozen CS treated samples had significantly more ³⁵SO₄ - labeled GAG in media compared to Autologous Frozen and Freeze-dried CS. Results are represented as mean and bars represent standard error of the mean. Bars with different letters indicate a significant difference between groups. Level of significance used 0.05

cartilage explants at the termination of the study. While little evidence of protective effects of CS in the current in vitro study was observed in cartilage, significant clinical and histological improvement was detected in vivo in joints with OA in previous reports [8, 11]. It has also been reported that ACS combined with physiotherapy demonstrated clinical improvement up to 2 years after treatment in human OA knees [12]. Autologous conditioned serum also improved joint function and reduced shoulder pain more effectively than betamethasone [13]. The model used in the current study lacked the synovium and other in vivo mechanisms that could be key to the ACS effects.

Additionally, it has been reported that IL-1Ra:IL-1 β ratio needs to range between 10 to 100-fold to inhibit the bioactivity of IL-1 β [14]. Based on these numbers IL-1Ra in the current study may have been below this threshold. While the goal of the current study was not to assess raw concentrations of IL-1Ra, it has been measured in previous experiments and found to be 11.122 to 50.11 ng/mL (data not shown). These levels of IL-1Ra would be below what would be expected to block the IL-1 β (10 ng/mL) when one considers the CS was no greater than 30% of the media; thus, providing another possibility for not seeing a robust treatment effect in the current model. It is important to consider that 10 ng/mL of IL-1 β is a supra-physiological concentration beyond of what was found in joints with arthritis (ranging from 25 pg/mL to 175 pg/mL) [15]. Therefore the insufficient protective effects in this in vitro model does not imply in lack of clinical effect in vivo.

For the main effect of Concentration accessed in this experiment, two concentrations of CS were studied (10 and 30%) in media. No significance was found for GAG

or ³⁵SO₄-labeled GAG in cartilage or culture supernatant during the experiment, which suggests that the effects of CS in cartilage were not concentration dependent. These findings are in accordance with previous research in which the total GAG present in chondrocyte pellets stimulated with IL-1 β was not demonstrated to be significant with the use of different CS concentrations [16].

The study did not show any significant differences in the cartilage explant parameters measured at the termination of the study as it related to the effect of Allogenicity, despite some differences at Day 2 and 10. To the authors' knowledge no studies comparing the effects of autologous and allogenic conditioned serum have been published. However, other biological therapies, specifically platelet-rich plasma (PRP), demonstrated similar effects in allogenic compared to autologous versions in in vivo [17] and in vitro [18].

Similarly no significant effects of lyophilization of CS were observed in cartilage at the termination of the study. Although no studies published at this date focused on the effects of lyophilized conditioned serum as potential modulatory treatment, freeze-drying has been performed in other blood derived products [18]. Freeze-drying did not demonstrate to interfere with granulation, proliferation and angiogenic response of platelet-derived products in a wound mouse model [19].

Based on the findings of this experiment, the presentation of the conditioned serum proposed (allogenic freeze-dried) demonstrated to be equivalent to the current version used in the field (autologous frozen). This is the first step for the development of a more practical form of using biologics in the field. An off-the-shelf version of conditioned serum would allow the immediate use of blood derived products, without the need of blood

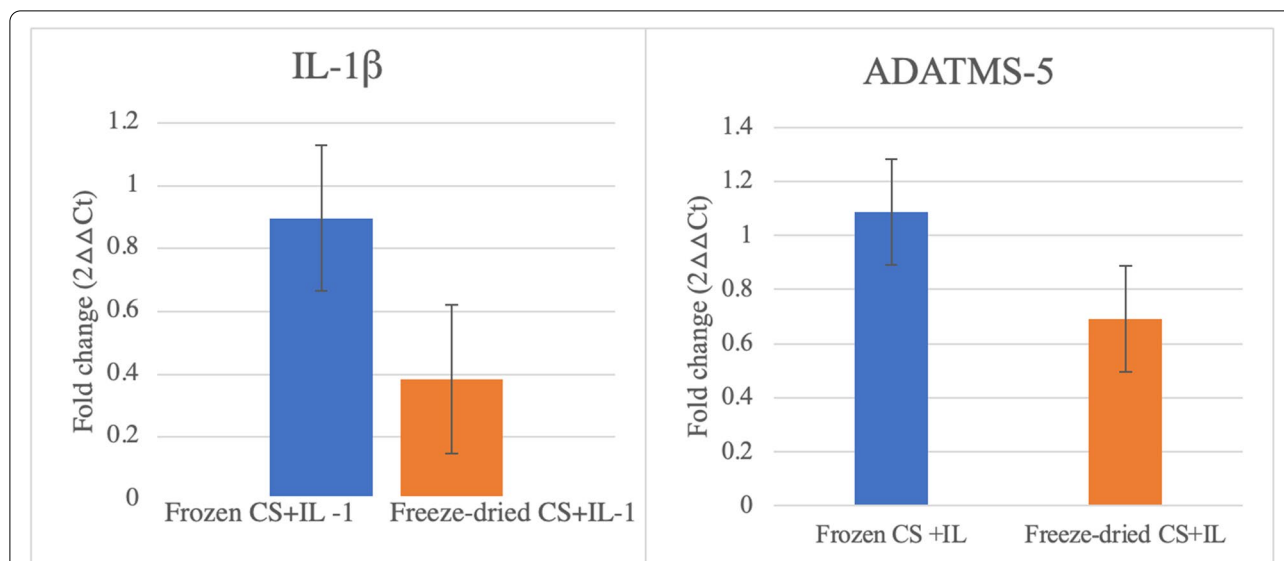


Fig. 6 Relative expression of different genes evaluated in this experiment. ADAMTS-5 and IL-1 β were evaluated in cartilage after the termination of the study. No significant difference was observed between samples treated with difference CS groups, for the main effects in this study including Treatment. The relative expression for the genes studied did not present a normal distribution, and data was log transformed to fit the assumption of normality. The raw fold changes related to ITS+IL-1β controls are presented in the graph for ease of interpretation. Results are demonstrated in means and bars represent standard error of the mean. Level of significance used 0.05

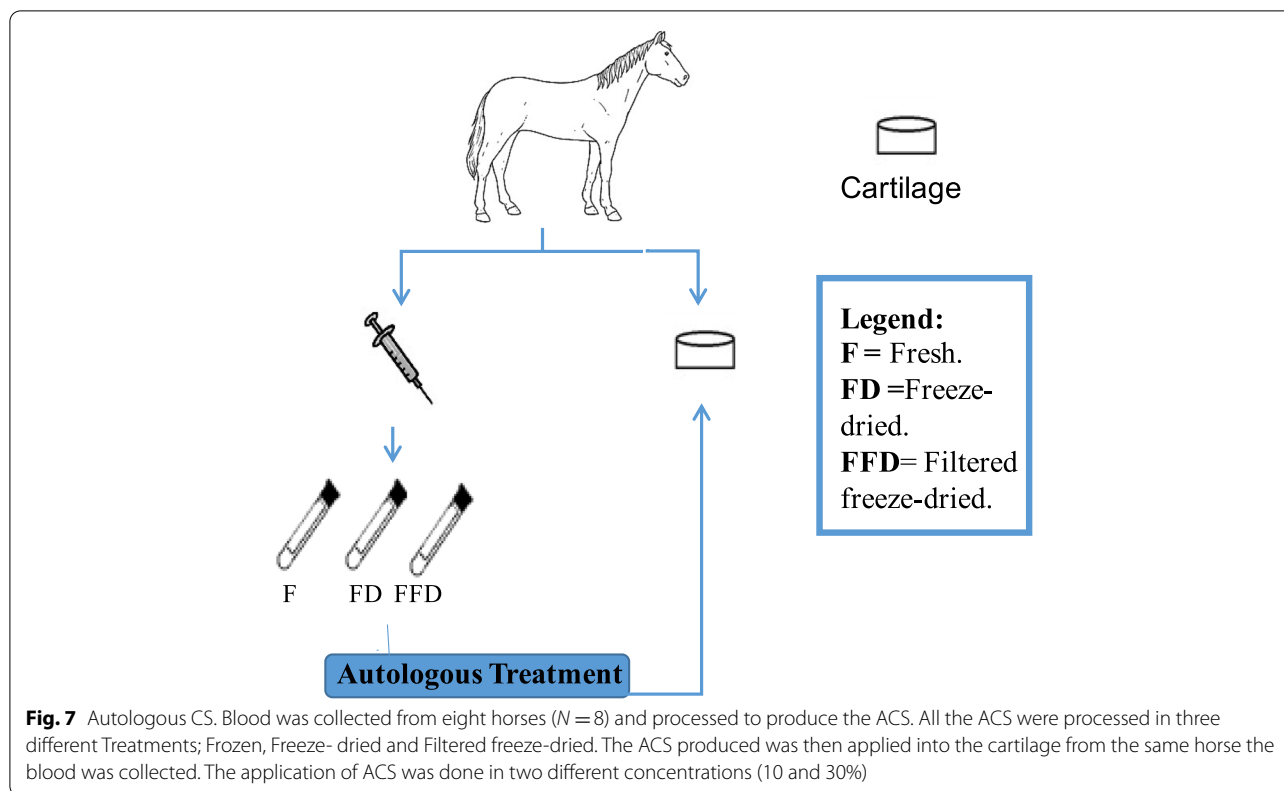
collection and handling, processing, incubation period or storage at low temperatures. This would allow the field clinician, who may not have immediate access to laboratory facilities and equipment to use biologic products in a much more convenient matter.

Some potential pitfalls should be noted in the current study. The complexity and sample numbers of the study design did not allow the collection of all possible outcome parameters. Specifically, histopathologic evaluation was not performed in this study, which could be considered as a potential pitfall. However, GAG analysis in culture supernatant during the experiment and in cartilage allowed the authors to investigate the effects of the proposed formulations of CS in cartilage matrix over time. Another potential pitfall in this study was that growth factors and cytokines present within the different formulations of CS or in the culture supernatant were not measured. It is believed by the authors that each patient’s ACS will be unique and the goal of this study was to compare the freeze-drying technique and overall, allogeneic versus autologous. This lead the investigators to forgo the cytokine composition of the different CS products in the current experiment. We do believe a detailed investigation of the cytokine composition of pooled CS is vital for a better understanding of the product’s effect and potential application in the field, and it is warranted in future research.

Furthermore, in regards to the number of horses used in this experiment for the preparation of CS, we are aware that a greater number of horses could potentially generate a more homogenous product compared to the current study. Previous similar research [20] demonstrated that a minimum of 5 horses would be sufficient to provide statistical significance for this in vitro study. Based on this information and on availability we included 8 horses to be part of this experiment, and divided those in two groups of 4. This was performed to facilitate logistics for blood collection, preparation of CS and cartilage harvest. Although a greater number of horses could be considered ideal, this study provided only an initial step for the investigation of allogeneic freeze-dried version of CS which already demonstrated favourable results. Future studies should include a greater number of horses to provide more robust results and potentially more homogenous allogeneic product.

Conclusion

The effects of an allogeneic freeze-dried version of CS overall did not show significantly different outcomes when compared to autologous frozen CS in an inflammatory environment. Further investigation in in vivo models, as well as the detailed study of the cytokine profile of frozen versus freeze-dried CS are warranted to confirm these results.



Methods

The aim of this study was to evaluate whether the allogeneic freeze-dried version of conditioned serum would present similar effects compared to autologous frozen version in an in vitro model of osteoarthritis. In this model, we induced degradative changes in equine cartilage explants using IL-1 β and evaluated the protective effects of allogeneic freeze-dried conditioned serum compared to autologous version.

Animals

Blood was collected from each of the eight skeletally mature and healthy horses (2–5 years of age) for this in vitro study. The animals used in this study were only used for tissue collection and belonged to a terminal research not related to the present study. Blood was harvest for ACS preparation and cartilage was collected after euthanasia. All the procedures were approved by the Universities’ Institutional Animal Care and Use Committee (ACUC, protocol number: 14-5477A). All research involving the horses were carried out in compliance with the ARRIVE guidelines.

ACS preparation

Blood processing and incubation

The ACS was prepared by combining whole blood with medical grade glass beads. Briefly, blood was collected

from each horse separately and aliquoted into 50ml conical tubes containing coated beads for ACS preparation. Blood was incubated for 24h at 37°C. Subsequently, the tubes were centrifuged for 10 min at 4000 rpm (1716.88 xg) and serum was aliquoted in smaller volumes and stored in a – 80°C freezer until the experiment was initiated.

To create the Frozen, Freeze-dried and Filtered freeze-dried treatments, the ACS aliquots from each horse were divided into three equal parts: one part remained frozen at – 80°C until the experiment was initiated (Frozen treatment), the second portion (Freeze-dried) was lyophilized in specific temperature (< – 45°C) and pressure (< 100 mtorr) for 18 h (Virtis Sentry Lyophilizer) and samples were then returned to – 80°C until the commencement of the experiment, the third portion (Filtered freeze-dried treatment) was processed similarly to the freeze-dried group; however, the serum was filtered with a 45 μ m syringe filter¹ before lyophilizing. This latter group was designed to mimic the procedure done clinically (where ACS is sterile-filtered before application), and to verify if filtrating serum could have an impact in its effect. In this study, the effect of the different ACS formulations will be collectively referred as Treatment.

¹ 33mm syringe low binding filter with PES membrane (45 μ m), Millipore Sigma, Burlington -MA.

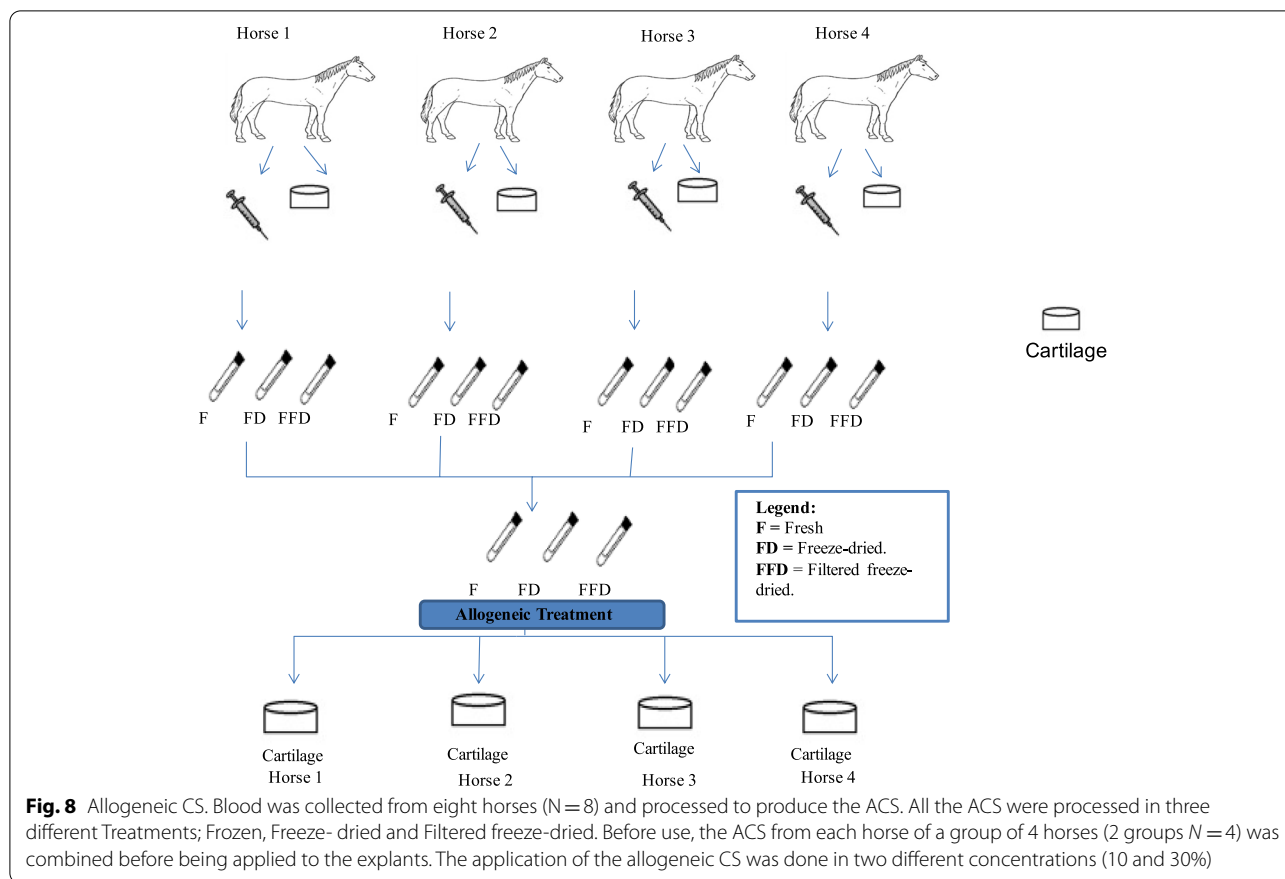


Fig. 8 Allogeneic CS. Blood was collected from eight horses (N=8) and processed to produce the ACS. All the ACS were processed in three different Treatments; Frozen, Freeze- dried and Filtered freeze-dried. Before use, the ACS from each horse of a group of 4 horses (2 groups N = 4) was combined before being applied to the explants. The application of the allogeneic CS was done in two different concentrations (10 and 30%)

For the Autologous CS, the ACS produced were kept separated to be applied into the explants of the same horse from which the blood was collected (Fig. 7), while for the Allogeneic conditioned serum (CS), the aliquots from four horses were pooled together (Fig. 8). The effect of Autologous and Allogeneic CS in this study was referred as Allogenicity. The mentioned preparations of CS utilized in this study were used in two different concentrations in this experiment, 10 and 30% in media (volume /volume). The effect of concentration of CS used in this study was referred as Concentration.

Cartilage explant harvest

Subsequent to euthanasia, cartilage from each horse’s trochlear ridges and condyles of the stifle joint of each horse was harvested using an 8mm punch and weighted between 70 and 100mg (wet weight). Cartilage explants were placed in a 24 well plate containing media [DMEM² supplemented with 1mM nonessential amino acids, 10mM HEPES, 0.4mM proline, 0.11mM ascorbic acid, penicillin (100U/mL), streptomycin (100µg/mL) and 200mML-alanyl-L-glutamine dipeptide in 0.85%

NaCl (GlutaMAXTM)³]. As cytokines and growth factors in routine FBS media could interfere with the effects of the CS used, a 1% volume/volume of insulin transferrin selenium (ITS; ITS Premix 6.25mg of insulin, 6.25mg of transferrin and 6.25µg of selenium acid, 6.26mg of transferrin and 6.25µg of selenium acid)⁴ [21–23] supplement was chosen for this experiment. A FBS media control utilized to allow comparison to other studies. The effects of media supplementation (ITS or FBS) was collectively referred in this study as Media. All explants were allowed to equilibrate in the designated medium for 48h in humidified incubator at 37°C before Treatment application.

Treatment preparation & tissue culture

CS samples representing Frozen, Freeze-dried and Filtered Freeze-dried were thawed and diluted in ITS media based on intended final CS concentration as well as considering autologous or allogeneic conditions when added

² DMEM GIBCO, Thermo Fisher Scientific, Waltham, MA.

³ GlutaMAXTMGibco®.

⁴ ITS Premix 6.25mg of insulin, 6.25mg of transferrin and 6.25µg of selenium acid, 6.26mg of transferrin and 6.25µg of selenium acid – BD Bioscience, San Diego- CA.

to the cartilage explants. All permutations were tested in triplicate within each horse. Control explants were exposed to media supplemented with ITS or FBS. The treatment permutations were applied twice throughout the study on day 0 and day 4. IL-1 β was also added to the media on these days to expose explants to an inflammatory condition (IL-1 β , R&D Systems)⁵, 10 ng/mL [20, 24] diluted in 0.1% bovine serum albumin (BSA)⁶ and PBS]. Media was changed every 48 h and stored at -80°C . On the 10th day of the experiment cartilage explants and the media were collected and frozen at -80°C until analysis.

Biochemical analysis

Cartilage explants were lyophilized and papain digested overnight at 60°C using a crystallized papain suspension [25].

Explant DNA quantification

DNA content in cartilage explants was determined using a fluorescent dye based assay (Hoechst 33258)⁷ [25]. Cartilage samples were run in duplicates and read against a standard curve using calf thymus DNA. The DNA content within the samples was used for normalization of the biochemical data in this study.

Explant and culture supernatant GAG quantification

Cartilage explants and culture supernatant were analysed for total GAG content using a modified method of dimethyl methylene blue assay [26]. In brief, the digested samples were added to a dye/buffer solution and read against a standard curve using chondroitin sulfate C from shark cartilage. The DMMB dye binds to sulphated glycosaminoglycan, forming complexes that result in change of colour within the sample. The colour was measured using spectrophotometry, and samples were read at 530 nm in the plate reader. The change in colour intensity is correlated to the amount of glycosaminoglycan found in the sample. All samples and standards were run in triplicate in this assay. GAG was normalized to DNA content in cartilage and presented as GAG in $\mu\text{g} / \mu\text{g}$ of DNA).

Explant $^{35}\text{SO}_4$ -labeled proteoglycans quantification

Sixteen hours prior to Day 0, $15 \mu\text{Ci}$ of $^{35}\text{SO}_4$ was added to the culture supernatant of all cartilage explants to label the newly synthesized GAG. At the termination of the experiment, $^{35}\text{SO}_4$ within the cartilage was quantified with a modified scintillation count method, using Alcian blue dye as detection system [27, 28]. The samples were run in duplicate and compared to a standard curve

that contained different concentrations of $^{35}\text{SO}_4$ [28]. In brief, samples and standard curve were to each well of a Multiscreen 96 well plate, along with assay buffer and Alcian blue dye. The multiscreen plates were attached to an vacuum manifold which draw the solutions through the screen allowing only the radiolabel bound to GAG and Alcian blue to be catch in the screen. After multiple rinsing steps, the screen of each well of the plate was punched out, and the $^{35}\text{SO}_4$ -labelled GAG retained in the membrane were quantified by liquid scintillation counting [28]. The activity levels of the $^{35}\text{SO}_4$ bound to the GAG molecules in cartilage were measured in disintegrations per minute (DPM), and normalized to DNA content (DPM/ μg of DNA). This was used as a measure of GAG retention and indirect measure of degradation of newly synthesized GAG.

Quantitative polymerase chain reaction (qPCR)

To verify the potential modulatory effect of CS in cartilage under inflammatory conditions, gene expression of catabolic enzymes and cytokines related to OA were assessed.

Cartilage RNA extraction

After experiment termination, cartilage explants were collected, weighed, immediately placed in guanidinium thiocyanate reagent⁸ and stored at -80°C . To perform the RNA extraction, samples were pulverized, homogenized with guanidinium thiocyanate and incubated at room temperature. Nucleic acid was extracted after 20% of chloroform (volume/volume) was added to the samples and followed by centrifugation, according to the manufacturer⁸. Nucleic acids were then precipitated with isopropanol and resuspended in $30 \mu\text{L}$ of nuclease-free water. All centrifugations were performed at 4°C .

Reverse-transcription

After extraction, RNA was treated for genomic DNA contamination and reverse-transcribed simultaneously using a blend of oligodT and random primers according to manufacturer's recommendations⁹ and cDNA was stored at -80°C until use.

qPCR

cDNA solution and Syber[®]Green¹⁰ were mixed with each individual primer for the genes of interest and gene expression levels were determined by quantitative real-time PCR. Conditions were: initial activation step

⁵ IL-1 β , R&D Systems, Minneapolis-MN, USA.

⁶ BSA, Sigma Chemicals, Saint Louis-MO, USA.

⁷ Sigma Chemicals, Saint Louis-MO, U.S.A.

⁸ TRIzol[™] Reagent Invitrogen Life Technologies Corporation, Carlsbad-CA, USA.

⁹ iScript[™] gDNA Clear cDNA synthesis kit, Bio-Rad, Hercules-CA, USA.

¹⁰ iTap[™] Universal Syber[®]Green Supermix, Bio-Rad, Hercules-CA, USA.

3 min at 95 °C, denaturation 10 s at 95 °C, and annealing 30 s at 60 °C and melting temperature 65 °C–95 °C, incrementing 0.5 °C for 0.5 s. Expression levels of IL-1 β , a disintegrin metalloproteinase with thrombospondin motifs ADAMTS-5 and IL-1 β and ubiquitin C (UBC). All primers were designed using PrimerQuest Tool¹¹ and National Center of Biological Information (NCBI)¹² software. Characteristics used for primer designed were; size under 150 nucleotides, primer should span in an exon-exon junction, having CG% approximately in 50%, melting temperature close to 60 °C and the minimum of hairpins or secondary structures. Efficiencies curves were performed using 10-fold dilutions of cDNA from equine chondrocytes exposed to IL-1 β . Primer sequences are shown in Table 1.

Gene expression levels of the target genes were normalized to the ubiquitin C (UBC) as endogenous control (Δ_{CT}). The Δ_{CT} of the target samples were then subtracted from the Δ_{CT} of the calibrator [control samples exposed to IL-1 β (ITS+IL-1 β)] resulting in the $\Delta\Delta_{CT}$. The gene expression was presented in fold ($2^{-\Delta\Delta_{CT}}$) change relative to the calibrator. Samples with a Ct above 38.5 were not considered for analysis.

Data analysis

A mixed-model analysis of variance was performed in this study using SAS 9.3 software¹³ with three different analyses being performed. First, we evaluated main effect of IL-1 β and media supplementation (Media) on explants to test the efficiency of the model. Then, we evaluated the main effect of CS compared to ITS+IL-1 β controls. In this analysis, the effect of CS was averaged over Allogenicity and Concentration. Finally, we compared the effects of Treatment, Allogenicity and Concentration as well as all interaction between these

effects in samples exposed to IL-1 β . This analysis was performed to evaluate the effect of different CS formulations in the model used.

Culture supernatant GAG and explant GAG and ³⁵SO₄-labeled GAG were considered as dependent variables for all three analyses, and horse was considered as random effect. Outliers were identified based in diagnostic residual plots. Values greater than ± 3 standard deviation from the mean were excluded from the data set and statistical analysis was performed again. When sample did not fit assumptions of normality, log transformation was performed and analysis was run again. Restricted Maximum Likelihood was used as estimation technique in this experiment. Individual comparisons supported by the F-test were done using least-squares means procedure. In all statistical comparisons, *P* value ≤ 0.05 was considered significant.

Abbreviations

ADAMTS-5: A disintegrin and metalloproteinase with thrombospondin motifs 5; FBS: Fetal bovine serum; GAG: Glycosaminoglycan; IL-1 β : Interleukin-1 β ; ITS: Insulin transferrin selenium; OA: Osteoarthritis; PBS: Phosphate Buffered Saline; CS: Conditioned serum; MMP-1: Metalloproteinase-1; DMEM: Dulbecco's modified Eagle's medium; μ Ci: Micro Curies.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12917-022-03227-2>.

Additional file 1: Supplementary Table 1. Glycosaminoglycan (GAG) in culture supernatant for control samples. **Supplementary Table 2.** Newly synthesized radiolabelled glycosaminoglycan (GAG) released in culture supernatant for control samples.

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Authors' contributions

DDF was responsible for the study design, the statistical analysis, interpretation of the data and contributed significantly in the edition the manuscript. LCG contributed to the experimental design, and was responsible for optimizing the laboratory assays, performing the sample analysis and interpretation of the data. LCG was also responsible for the preparation of the manuscript. CWM provided critical feedback and contributed with the edition of the manuscript. The author(s) read and approved the final manuscript. The authors read and approved the final manuscript.

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Table 1 Sequences of primers used in the study

Primers used in the study	Primer sequence (5' to 3')
ADAMTS-5	Forward: AAGGTGACTGATGGACCGAATGT Reverse: TTTGAGCCAATGATGCCGTCA
IL-1 β	Forward: CCAGAGGCGGCCGGACATAAC Reverse: GGGAAGGCAGCTGGGCATTGATT
UBC	Forward: GGCTGTTAGCTTTTCAGCTTGTG Reverse: CTAAATTGGGGCTAATGGCTGG

¹¹ PrimerQuest Tool. IDT-Integrated DNA Technologies.

¹² NCBI- National Center of Biotechnology Information.

¹³ SAS/STAT 9.3 user's guide. Carey, NC: SAS Institute Inc.,2011;515p.

Availability of data and materials

The datasets used and/or analysed during current study are available from the corresponding author on reasonable request.

Declarations**Consent or publication**

Not applicable.

Ethics approval and consent to participate

In this experiment, we used cartilage from horses belonging to a terminal study not related to the current study. The Colorado State University Animal Care and Use Committee (IACUC, 14-5477A) approved all procedures, including the blood harvest for ACS preparation. All experiments were performed in accordance with relevant guidelines and regulations of IACUC and were carried out in compliance with the ARRIVE guidelines.

Competing interests

The authors declare that they have no competing interests.

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