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AUTOLOGOUS CONDITIONED SERUM IN ORTHOPEDIC THERAPY: A SYSTEMATIC REVIEW OF CLINICAL AND EXPERIMENTAL DATA IN HUMAN AND EQUINE MUSCULOSKELETAL LESIONS

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Trabalho de Conclusão apresentado ao Programa de Residência em Área Profissional de Saúde em Medicina Veterinária da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo.

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Autologous conditioned serum in orthopedic therapy: a systematic review of clinical and experimental data in human and equine musculoskeletal lesions

Summary

Musculoskeletal lesions are among the major causes of daily functional and athletic disabilities with high rates of occurrence and relapse. Injectable hemoderivatives, such as autologous conditioned serum (ACS), have become usual treatment options for these conditions despite conflicting results presented in the scientific literature regarding their efficacy and mechanisms of action.

The objective of this review is to compile and present information retrieved from studies that evaluated the effectiveness of ACS in the healing of tendon, ligament and articular lesions in men and horses, and to compare results from experimental and observational studies in both species. This review aimed to provide critical information and assist clinicians in the choice of appropriate therapeutic blood-based interventions for the treatment of orthopedic lesions.

A systematic review of articles using Medline, PubMed, Embase, Bireme and Google scholar databases was conducted, searching for observational studies (case control and cohort) and RCTs on the use of ACS in human orthopedic lesions. In equine medicine, all studies found on the subject were included independently of their design, given their scarcity. Experimental laboratorial studies were selected if controlled and when providing useful information on the therapeutic role of ACS in tendon, ligament or joint injuries. A total of 1218 results were found; 250 articles were fully accessed and 22 studies met the inclusion criteria. Predefined data fields were employed in order to present features of selected ACS' articles in tables, presented as experimental or observational. Tables included study design, population, lesion, ACS characterization (acquisition, composition and administration protocol), outcome measures and results, and were finalized with comments and a brief evaluation of the ACS's performance.

Experimental studies, particularly conducted *in vitro*, have struggled to demonstrate consistent positive properties and effects without success, but most clinical trials indicate a beneficial response associated with ACS administration. That was true for both species, despite the small numbers, disparities and flaws in study design. Observational studies, however, failed to find beneficial long-term effects regarding this therapy.

The use of ACS in the treatment of musculoskeletal lesions, although safe, promising and appealing, still cannot be recommended without due caution in face of the observed

inconsistencies. Overcoming these incongruences will demand efforts to construct well-designed studies and, most importantly, to regard ACS as an autologous compound that encompass the diverse composition and wide range of therapeutic potential that characterizes their originating raw material: blood.

1 Introduction

Musculoskeletal lesions are commonly associated with high physical demands. Injuries in joints, tendons or ligaments greatly impact on common daily activities and athletic performance of human and equine subjects. Such tissues have demonstrated an intrinsic poor healing potential with inadequate reorganization and usual relapse of the original lesions. The current scarcity of effective proven therapies for the treatment of such lesions poses a challenge for clinicians and raise the focus for research in this field (Ziltener et al., 2012; Dehghani & Rodeo 2019)

The use of blood-products as therapy for musculoskeletal lesions has emerged as a type of regenerative medicine that aims to control the degenerative disease process and restore the structural and functional capacity of such tissues (Dehghani & Rodeo 2019). These therapies intend to supply the demand for cost-effective, efficient and safe forms of treatment and although not well established yet, have been explored by clinicians, regardless of conflicting data on their therapeutic potential.

The employment of autologous blood preparations as therapy relies in the fundamentals of exploring the beneficial mechanisms of body's natural response to tissue damage. Autologous conditioned serum is a cell-free product harvested after the exposition of blood to activating surfaces which prompt the production of several anti-inflammatory cytokines and growth factors by leukocytes (Fjordbakk et al., 2015; Strümper 2017; Geburek et al., 2015). Reported as the major anti-inflammatory cytokine in the ACS, the interleukin-1 receptor antagonist (IL-1Ra) is a natural inhibitor of the interleukin-1, which has been implicated as the key pro-inflammatory mediator of some pathologic conditions (e.g. osteoarthritis) (Evans et al., 2016). Further anti-inflammatory cytokines, such as IL-10 and IL-4 and growth factors as insulin-like growth factor 1 (IGF-1), transforming growth factor β (TGF-β) and others are also found in high concentrations in ACS (Genç et al., 2018; Evans et al. 2016). In addition, antioxidant properties regarding this therapy were described by Brossi et al. (2012) where a decrease in the production of free radicals was demonstrated in *in vitro* synovial fluid cells treated with IRAP.

Another attractive feature of ACS treatment is its reported safety. Weinberger (2008) reported absence of side effects related to ACS joint injections, after multiple treatments of 262 horses. In addition, complications involving ACS therapy in horses has been retrospectively studied by Warner and Lischer (2017), where intra-articular ACS injections were carried out in 1445 joints of 387 horses. The study concluded that the intra-articular ACS treatment was classified as low risky with no serious or life-threatening conditions being observed. In human medicine, Damjanov et al (2018) reported no adverse effects after 4 weekly supraspinatus paratenon injections in 16 patients. Tassara et al (2018) made similar remarks after treatment of 28 cases where 4 weekly treatments of ACS were administered into hip and knee joints of osteoarthritic patients.

Although beneficial effects have been associated with this treatment in a considerable amount of studies, results are not unanimous and occasionally, conflicting.

A systematic review of experimental and observational studies performed in human and equine medicine was conducted to assess the ACS' effectiveness in the treatment of tendon, ligament and joint lesions. Furthermore, evidence was critically evaluated in order to assist clinicians in their choice of treatment for orthopedic disorders.

2 Materials and Methods

This systematic review was conducted according to the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) statement published by the CONSORT group (Moher et al, 2009). A broad literature search was conducted for all relevant articles in English, French, German, Spanish and Portuguese up to December 2018, addressing the therapeutic use of ACS in orthopedic lesions in humans and equine. The database of Embase, Bireme, Medline, PubMed, and Google Scholar were consulted, searching the terms "autologous conditioned serum", "ACS", "tendon", "joint", "articular", "ligament", "musculoskeletal injuries", "human" and "equine". The reference lists of the selected articles were also examined for identification of further studies. Articles where ACS was used in conjunction with other blood-derivatives, or as anti-inflammatory therapy targeting tissues other than the ones described above or in other medical fields (e.g. reproduction) were excluded. Studies testing products with similar function or composition of ACS were not included if not derived from blood.

Experimental studies *in vivo* and *in vitro* investigating the ACS' effects and mechanisms of action in the target tissues were selected if controlled. Clinical trials performed in human species which reported the use of ACS in joints, tendons or ligaments were included if double-blinded and randomized (RCTs). Cohort studies (prospective or retrospective) and case series with a control group and describing ACS therapy in these musculoskeletal tissues were also admitted for this species (Figure 1). Abstracts presented at conferences or oral communications and other study designs not described above were not included for human species.

Due to the paucity of equine clinical research currently available in this area, studies describing the effectiveness of the ACS application in tendons, ligaments or articular injuries were included regardless of their design or evidence level.

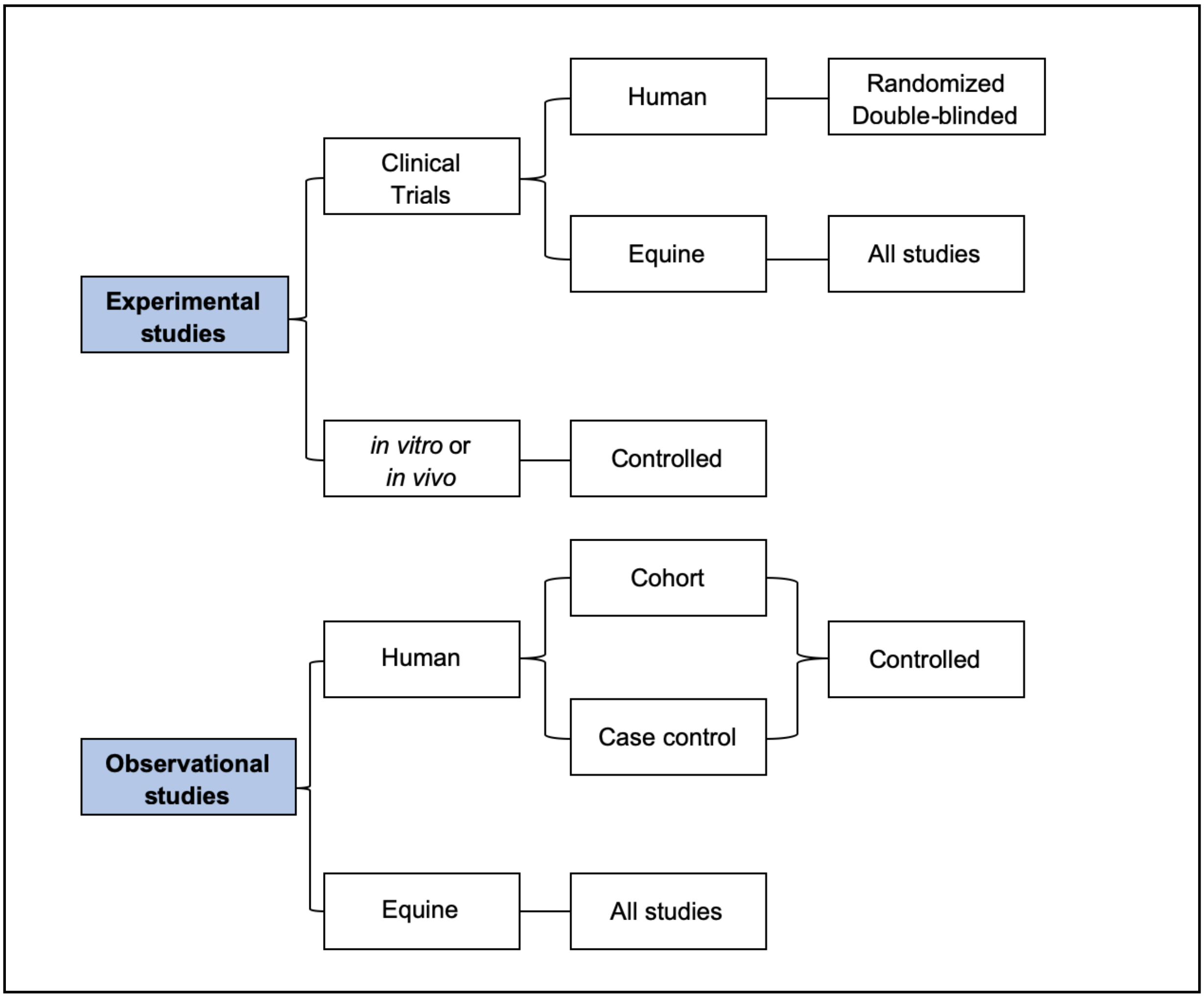

Figure 1. Inclusion criteria according to study design.

Primarily, all titles generated by the literature search, up to December 2018, were analyzed for selection of the articles for full-text review. Studies that did not fit in the inclusion criteria were excluded and after eliminating duplicates, relevant data was extracted from elected articles and organized in tables in order to facilitate identification of the study design, population included, follow-up time, control, outcome measures, the evaluated lesion, hemoderivative characteristics, the intervention applied, the blinding and the results. The results were further identified as positive (+), partially positive (±) or negative/impartial (-) regarding the ACS effects.

In addition, potential weak points and bias of the selected studies were presented in the "observations" column of the table. Lack of randomization and control or true control (placebo) group; poor characterization of the hemoderivative and incomplete information about its processing method; insufficient blinding; small enrolled sample (described by the author based on power analysis of the selected population); adoption of a short follow-up period; inadequacy of, or limited outcome measures were classified as weak points.

Two main tables were constructed in order to accommodate experimental and observational studies (Appendix 1 and 2).

3. Results

The researched databases generated a total of 1218 results of which 371 were from PubMed, 292 from Medline, 273 from Google Scholar, 171 from Embase and 111 from Bireme. Nine hundred seventy-three results were excluded after screening of titles and abstracts. Full access of 245 articles and further 5 studies retrieved from references, led to a total of 22 selected pieces (Figure 2). The exclusion of 228 studies were based on removal of duplicates and articles that did not meet the inclusion criteria. From the 22 selected articles on ACS, 20 were experimental and 2 were observational studies. Two experimental studies encompassed *in vivo* and *in vitro* ramifications, yielding a total of 23 experiments.

3.1 Experimental studies

Twenty experimental studies were selected and further classified as clinical trials, controlled *in vivo* or controlled *in vitro*.

3.1.1 Clinical trials

Among the clinical trials, five studies were performed in human species and four enrolled equine subjects. Selected human clinical trials were all randomized and double-blinded (RCTs) and investigated the effectiveness of ACS in the treatment of osteoarthritis (two studies) (Yang et al., 2008; Baltzer et al., 2009), for postoperative treatment of anterior cruciate ligament reconstruction (two studies) (Darabos et al., 2009; Darabos et al., 2011) and for supraspinatus tendinopathy (one study) (Damjanov et al., 2018). Equine clinical trials comprised four studies, being one randomized controlled clinical trial evaluating ACS as a treatment option for SDFT forelimb tendinopathies (Geburek et al., 2015) and three non-randomized clinical trials investigating naturally occurring osteoarthritis (Weinberger, 2008; Chiaradia et al., 2012; Schneider & Veith, 2013)

In general, positive results were reported in all equine studies and in four out of five human RCTs (88,8% in total). One partially positive result originated from a human clinical RCT where osteoarthritis affected human subjects were evaluated after a series of ACS injections. Despite improvement in specific scores for evaluation of human knee osteoarthritis, significant improvement in other parameters was not verified, and the primary objective of this study, established by the authors, was not met (Yang et al., 2008).

Three equine clinical trials had a weaker evidence level as were not randomized (Weinberg et al, 2008; Chiaradia et al., 2012 and Schneider & Veith, 2013) and although 30% of all experimental studies were classified as RCTs, study design was not always ideal. It was possible to observe that in one equine and two human RCT small samples were enrolled (Geburek et al., 2015; Darabos et al., 2009; Darabos et al., 2011) and in four studies outcome measures relied on one parameter only (questionnaires in three studies and IL-1β levels in another study) (Yang et al., 2008; Baltzer et al., 2009; Damjanov et al., 2018; Darabos et al. 2009). Additionally, a short follow up period was adopted in one study (Darabos et al., 2009).

In eight out of nine clinical trials, ACS was prepared with the Orthokine® method and differences within it were observed on the protocols regarding incubation period and centrifugation. In the majority of the studies a 24-hours incubation period was employed but a shorter incubation time was also encountered (6 to 9 hours). Centrifugation was described as relative centrifugal force in two studies (2100x g; 3000 x g) that employed different incubation periods (Chiaradia et al., 2012; Damjanov et al., 2018). The rotations per minute (rpm) adopted was reported in one clinical trial (4000 rpm) although no information about the radius of the rotor was provided (Geburek et al., 2015). The remaining studies described preparation according to manufacturer's instructions.

A different method of cytokine production induction (Goldic®) based on blood exposure to gold particles for conditioning serum was employed by Schneider & Veith (2013), prepared according to the product's manufacturer guidelines. This study was performed with equine subjects and evaluated the Goldic® ACS as a treatment for several different disorders

(OA, tendon and ligament injuries and sesamoiditis). Although positive results were found, this was a non-randomized, uncontrolled and unblinded study in which a small sample (n = 36) was enrolled and the outcome measures were limited and subjective (lameness grading and swelling/effusion evaluation).

In another equine clinical trial, 262 subjects were enrolled, but a lack of randomization, blinding and a control group was observed. Additionally, in this study, there was marked sample heterogeneity (different osteoarthritis-affected joints, at different stages of the disease, not age or activity matched patients) and improvement in these patients was judged only based in lameness evaluation (Weinberger, 2008). Another equine clinical trial performed by Chiaradia et al. (2012) was, in fact, part of a study where the main objective was to perform proteomic analysis of equine synovial fluid of osteoarthritis (OA) and osteochondritis dissecans (OCD) affected patients. Ten OA patients were treated with ACS and were evaluated regarding synovial fluid proteomic analysis and athletic performance.

Regarding outcome measures, clinical evaluation, questionnaires and patient-attributed scores for pain and function were the predominant tools utilized to verify ACS efficiency. More objective means of evaluation (such as imaging, immunohistochemical and histologic tests) were rarely employed. As scant examples of objective outcome measures adopted, in one study CT scans were used to evaluate bone tunnel widening, in two studies, Il-1β concentrations were measured and in one proteomic analysis of the synovial fluid was performed.

Further hemoderivative characterization regarding its anti-inflammatory concentrations in the applied ACS was not performed in clinical trials.

3.1.2 Controlled *in vivo* and *in vitro*

Eleven experimental controlled studies were selected according to stablished criteria and they contained fourteen experiments. Among these fourteen experiments, three (21,5%) yielded positive results and were conducted *in vivo*. Negative results were observed in four experiments (28,5%), two *in vivo* and two *in vitro.* Seven experiments (50%) yielded partially positive results in which four were conducted *in vivo* and three *in vitro*.

From the five experiments conducted *in vitro* none demonstrated positive results associated with ACS intervention; two had negative results and three had partially positive outcomes. From the nine *in vivo* experiments, 33,3% yielded positive results, 22,2% resulted in negative outcomes and 44,5% demonstrated partially beneficial effects of ACS administration.

Ten trials were on articular tissues: one yielded positive results (Lasarzik et al.,2018), five yielded partially positive results (Frisbie et al., 2005; Frisbie et al., 2007; Rutgers et al., 2010; Carlson et al., 2013; Moreira et al., 2015) and four yielded negative results (Rutgers et al., 2010 *in vivo* and one *in vitro* part; Tatarniuk, 2015; Garbin, 2017). Tendons were employed in three experiments, two with positive outcomes (Heinsterbach et al., 2012; Genç et al., 2018) and one other with a partially positive outcome (Majeski et al., 2009). One ramification *in vitro* part of a study performed by Tatarniuk (2015) compared the prepared ACS to incubated unconditioned serum and an unincubated serum.

In the experimental studies, the Orthokine® and Arthrex® commercial kits, and further two different serum conditioning methods were employed as acquisition of ACS. Therefore, heterogeneous means of obtaining ACS throughout different studies were used, hampering comparison of the results. Fifty-seven per cent of the controlled experimental studies employed the Orthokine® system, with incubation periods that varied from six to 24 hours. This variation also occurs between the manufacturer's guidelines depending on the chosen product (e.g. Orthokine®vet IRAP10, Orthokine®vet, EOT®II syringes). There were two positive (Heisterbach et al., 2012; Genç et al., 2018), four partially positive (Frisbie et al., 2005; Frisbie et al., 2007; Majewski et al., 2009; Rutgers et al., 2010) and two negative results (Rutgers et al., 2010 *in vivo* and one *in vitro* part) among experimental studies employing the Orthokine® preparation method.

The Arthrex® system (IRAP II™) was employed in four studies in which one yielded positive result (Lasarzik et al., 2018), two were partially positive (Carlson et al., 2013; Tatarniuk, 2015 *in vitro*) and the last one generated a negative result (Tatarniuk, 2015 *in vivo*).

Moreira et al. (2015) observed a partially positive result where a different method of processing the blood was employed. In such study, blood was collected in a sodium heparin containing tube followed by 24-hours incubation period and two centrifugation steps in order to remove all cellular debris. Further filtration of the product was performed with a 0.22μm milipore filter.

Garbin (2017) employed borosilicate glass beads processed in their laboratory at The Colorado State University for the ACS preparation and compared different presentations of the product (frozen; freeze-dried and filtered freeze-dried) and negative results with these treatments were observed when tested *in vitro*.

Experiments were performed with equine chondrocytes (1) (Carlson et al., 2013), rat Achilles tendons (3) (Majewski et al., 2009; Heisterbach et al., 2012; Genç et al., 2018), synovial fluid of osteoarthritic patients (4) (Rutgers et al., 2010; Tatarniuk, 2015; Moreira et al., 2015; Lasarzik et al., 2018), human osteoarthritic cartilage explants (2) (Rutgers et al., 2010), equine osteoarthritic cartilage and synovium explants (1) (Garbin, 2017) and equine induced-carpal osteoarthritis (2) (Frisbie et al., 2005; Frisbie et al., 2007) and equine serum (1) (Tatarniuk, 2015). Cell cultures (4 trials) resulted in partially positive outcomes in two trials and negative results in other two studies.

Furthermore, it was observed that identification of IL-1Ra increases in synovial fluid of ACS- treated subjects was not straightforward and that in cartilage explant cultures, ACS did not consistently benefit cell metabolism. In *in vivo* experiments, ACS intervention did not positively affect cartilage morphology and metabolism. The two trials that evaluated mechanical properties of treated tissues (a rat Achilles tendon rupture model) showed a partially positive influence of ACS treatment.

3.2 Observational studies

A total of two observational studies were selected according to inclusion criteria. They were both cohort studies and evaluated the clinical effects of ACS treatment in two years and ten years, respectively (Warner & Lischer., 2016; Zarringam et al., 2018).

One retrospective cohort study enrolled 26 equine subjects and took owners' reports of improvement and return to previous athletic performance into consideration. Negative outcomes were observed in this study which found that in 54% of horses a long-term effect (two years) was not observed. Population did not include a control group.

A prospective study performed by Zarringam et al. (2018) collected information through interviews or electronic health records about surgical interventions taken by patients within ten years after participating in a RCT (Yang et al., 2008). Similar percentages of both ACS and placebo treated groups (40,3% and 46,3%) that had undergone surgical intervention within the proposed period were observed. This study concluded that ACS treatment had failed in preventing or delaying surgical treatment for knee osteoarthritis albeit possible reduction of intake of pain-relief medications or improvement in quality of life due to the ACS treatment were not taken into account.

Several human observational studies were not included in this review due to the lack of a group of patients non-exposed to treatment (Garcia-Escudero & Trillos, 2015; Tassara et al., 2018; Stümper, 2017).

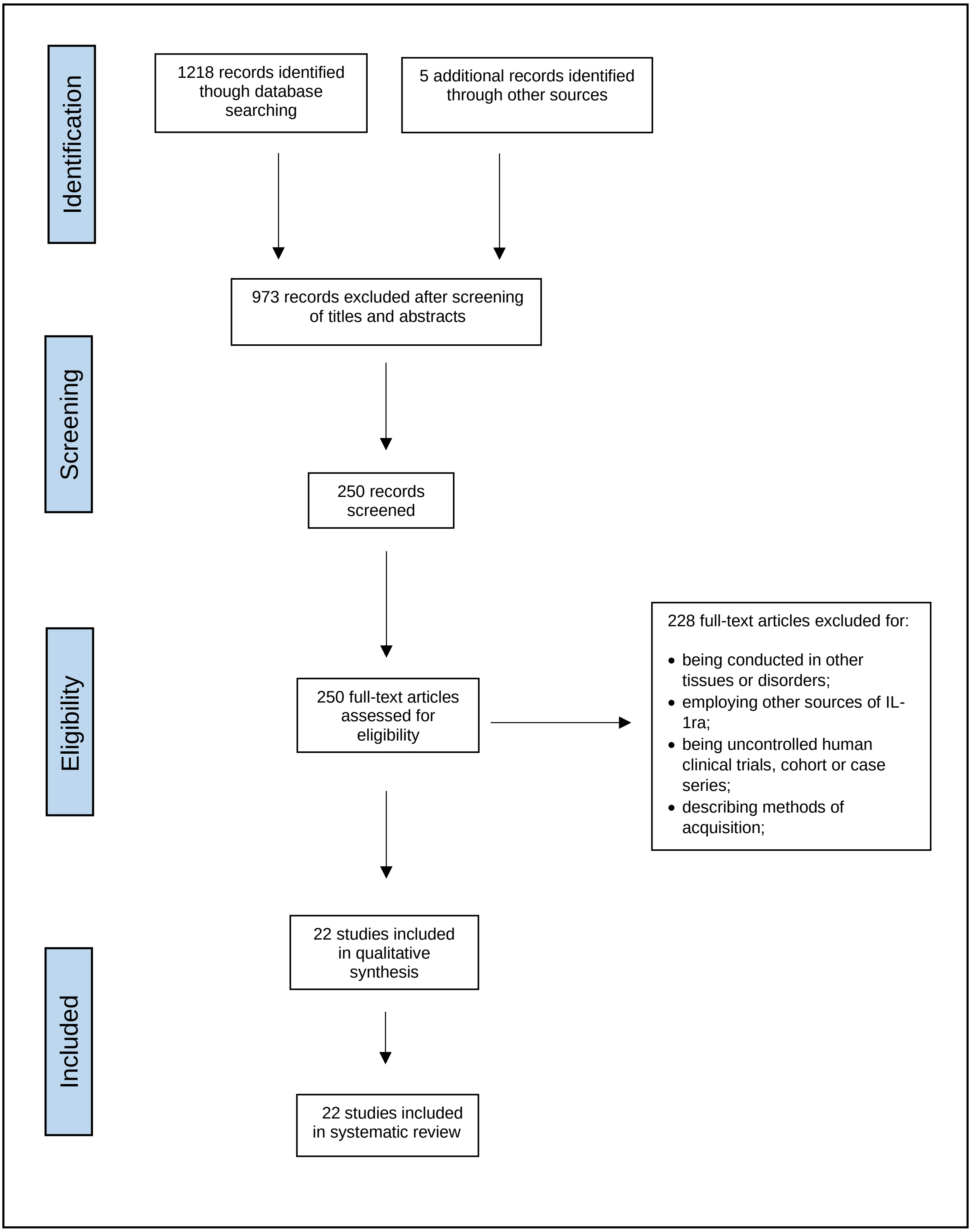

Figure 2. Flow diagram for identification of published studies. Prisma 2009.

4 Discussion

Regenerative therapies and the use of blood-products have become popular treatment options for musculoskeletal disorders in human and equine species. Despite the lack of solid scientific fundamentals, ACS has earned its place within this range of options for tissue healing in orthopedic lesions (Chevalier, 2010; Fox and Stephens, 2010; Malemud, 2010; Burnouf et al, 2013; Gross and Hoffmann, 2013; Wehling et al., 2017; Bogers, 2018; Ortved, 2018).

Different study designs evaluating ACS' effects in the treatment of tendon, ligament and articular injuries were included in this review. Regarding the heterogenicity of study samples, this strategy was implemented due to scarce published data regarding this subject and aimed to provide a more comprehensive amount of information in this article. As human studies were more abundant, it was possible to demand a more rigorous inclusion criteria. Equine studies, on the other hand, were included regardless of their design because a great disparity and scarcity in the number of available articles in this species was found. Furthermore, the paucity of studies included in this review poses a question on whether the observed results are likely to be reproduced in further investigations and on the reliability of ACS's efficacy, advising for care in the interpretation of the results.

Experimental controlled *in vitro* or *in vivo* and clinical trials were divergent, although in a different manner. While experimental results were not able to consistently demonstrate beneficial properties of this blood-derived intervention, outcomes from clinical trials were mostly positive. Furthermore, *in vivo* experimental studies yielded better results than *in vitro* experiments, suggesting that maybe studies have failed in reproducing all the mechanisms involved the studied lesions and that perhaps, ACS has been acting through different pathways from the ones investigated in the particular experiments.

Observational studies selected in this review were very limited. Only two studies were included and aimed to investigate the long-term effects of ACS therapy. Although different species were enrolled and distinct follow up times were adopted in these studies, both reached the same conclusion, where no long-term positive effects after ACS therapy could be observed. Nevertheless, caution should be taken when interpreting these results as in observational studies the risk of bias is higher when compared to RCTs or controlled experimental studies.

Regarding acquisition methods of ACS, the Orthokine® system was adopted in 68% of experimental and observational studies (15/22), however, comparison of results from the selected articles was hampered due to adoption of other different acquisition systems. Other two commercial and two laboratory methods for ACS preparation were described. The Arthrex® system was employed in 18,5% (4/22) of all studies and the Goldic® method was employed in one equine study. Further two methods were described by other two experimental studies.

In general, Orthokine®, Arthrex® and the acquisition method described by Garbin (2017) were similar, and together reached 45% positive, 35% partially positive and 30% negative results in all studies. In the Goldic® kit, golden particles are employed as the activating surface for anti-inflammatory cytokines and growth factors production, differing highly from the usual borosilicate treated glass beads. Only one study used this method for ACS acquisition and positive outcomes were observed albeit a poor study design was implemented. The last distinct process for ACS preparation was performed by Moreira et al. (2015) and was previously described above. Although defined by the author as autologous processed plasma (APP), the technique includes two centrifugations and removal of cell debris with a further filtration step resulting in a cell-free cytokine-enriched product. Partially positive results were obtained by the author in this study which compared two treatment protocols (short and long-term protocols). A mild and transient inflammation was observed after the short-term protocol which comprises on a single APP injection, whereas better outcomes were observed in the long-term protocol when compared with saline injections.

Primarily, the preconized incubation period of blood to effectively produce maximal amounts of the IL-1ra in the ACS was 24-hours (Meijer et al., 2003). However, incubation periods ranging from 6 to 9 hours are also recommended by the manufacturer depending on the chosen product (EOT®II-syringe and Orthokine®vet IRAP10) (Arbel, 2017). Incubation time was proven to be one of the key factors in the induction of IL-1ra synthesis, and the increase in IL-1ra concentration occurred in a time-dependent manner, even in the absence of the glass beads (Hraha et al, 2011). Not only IL-1ra concentration increases, but also an increase in the synthesis of the anti-inflammatory cytokine interleukin-10 was observed after the 24-hour incubation period (Hraha et al, 2011).

Although following the manufacturer's instructions would be ideal, the incubation period adopted by Tatarniuk (2015) differed from it. The Arthrex® VetSystems recommends a 24-hour period of incubation for their product IRAP II® but the author described a 18-20 hours of incubation in the ACS processing. Hypothetically, this could justify the negative results found in the *in vivo* part of the experiment. The remaining authors described similar incubation periods recommended by the adopted products.

The majority of lesions treated in human RCTs referred to osteoarthritis, and anterior cruciate ligament injuries, and at times, in human clinical trials, these could be considered as a sole entity, under the assignment of knee osteoarthritis. They are both intra-articular knee disorders, adversely affected by IL-1 (Höher et al, 1998; Irie et al, 2003) and frequently occur concomitantly. Nevertheless, consistently reported beneficial results after administration of ACS warrant further investigation, given the scant number of clinical trials and inconsistent and less encouraging experimental results.

The ACS was also explored as a treatment option for supraspinatus tendinopathy in one human RCT (Damjanov et al., 2018) which demonstrated through subjective patient reported outcomes (PROs), that better results were achieved with this hemoderivative when compared to betamethasone injections.

The adoption of scarce and subjective outcome measures (relying in patients' perception of improvement; lameness subjective evaluation) was, in fact, a feature of the majority of clinical studies. This could have favored the finding of positive outcomes, even in the absence of a true positive effect. Objective evaluation tools adopted in ACS' clinical trials were the proteomic analysis of synovial fluid proteins before and after treatment with ACS (Chiaradia et al., 2012), the measurement of IL-1β concentrations in synovial fluid of treated patients and controls (Darabos et al., 2009; Darabos et al., 2011), CT scans for measurement of width of tibial bone tunnel after ACL reconstruction (Darabos et al., 2011) and ultrasonographic, histological and immunohistochemical evaluation of SDFT (Geburek et al., 2015). Inclusion of diverse and objective outcome measures are of great importance in future clinical trials aiming to evaluate ACS's efficacy as a multimodal evaluation promotes a more comprehensive understanding of the treatment's effects.

Enrollment of small samples was also frequently encountered in ACS clinical trials, in both species. Uncontrolled (or treatment-controlled) and non-blinded studies were less frequent among human studies. Nevertheless, poor study design was a characteristic of equine clinical trials. Despite the large number of horses enrolled in one trial and the sufficient sample size defined by power analysis in another, both studies lacked control groups, randomization of patients and blinding. Moreover, samples were not homogeneous, and patients were evaluated only with a clinical exam (Weinberger, 2008; Schneider & Veith, 2013). In other study, although a desirable control sample was available, a small number of patients was enrolled, without randomization, and was observed for a short period of time. Besides, outcome assessors were not blinded, and a true placebo control group was absent (Chiarardia, 2012). Finally, one equine study presented a better design, where albeit a small number of equine subjects were enrolled, randomization of treated and control groups was performed. Also, in this RCT, evaluation of the ACS treatment on SFDT lesions, relied not only on clinical evaluation but also on ultrasonographic assessment, histological study and immunohistochemical expression of collagen I and III. In addition, the ultrasound exam evaluator and the histological assessor were blinded to treatment.

In *in vitro* studies, increases in IL-1ra concentrations after intra-articular ACS injection, as well as positive effects on cartilage morphology and metabolism, were not consistently detected. These observations lead us to speculate on the possible explanations for these failures. First, undetermined bioactive components present in the hemoderivative could be responsible for the beneficial effects reported in clinical trials. Secondly, different articular tissues, other than cartilage, could be the primary target for ACS actions. In summary, ACS could be exerting its beneficial effects through undetermined mechanisms of action.

The eventual participation of molecules other than IL-1ra in the observed effects of ACS has been described. Conditioning of blood for ACS acquisition results in synthesis of anti-inflammatory cytokines, such as Il-4 and IL-10 (Meijer et al, 2003; Hraha et al, 2011; Textor 2011). These cytokines can contribute to the positive results observed when these preparations are administered but adopted outcome measures are not specifically selected to detect their presence or effects. Interleukin-10, for example, is considered an antioxidant and a potent anti-inflammatory cytokine (Dokka et al, 2001; Haddad & Fahlman, 2002; Adib-Conquy & Cavaillon, 2009) with several potential therapeutic indications (Opal and DePalo, 2000). The reduction of oxidative burst generated in inflammatory processes could, therefore, contribute to the clinical improvements described in clinical trials. Brossi et al. (2012) investigated the antioxidant effects of blood-products on equine synovial fluid cells *in vitro*. In such study, the authors were able to demonstrate a markedly reduction of free radicals generated by synovial leukocytes, restoring the redox equilibrium of these cells.

Moreira et al. (2015) evaluated PGE_2 concentration, a primarily biomarkers of inflammation, after application of processed plasma in healthy equine joints and demonstrated a reduced concentration of this biomarker compared to saline injected joints. This reduction was not observed, however, when compared to baseline levels and this can be justified by the fact that the baseline values where extracted from healthy – non-osteoarthritic - joints. Further studies evaluating this biomarker in ACS treated osteoarthritic joints should, therefore, be investigated.

Studies have also found that in the processing of ACS, an increase of concentrations of several growth factors such as IGF-1, TGF-β can be observed (Heisterbach et al., 2012).

Increased concentrations of such factors have been associated with improvements in tendon healing (Geburek et al., 2015). Additionally, Wei et al. (2017) have demonstrate that a decreased level of IGF-1 is associated with cartilage catabolism.

Different mechanisms and pathways through which ACS may exert its beneficial actions, aside from its properties to block interleukin-1-mediated responses, have not been explored yet, and this could be a reason for the difficulty in demonstrating its effects. These alternative mechanisms are under investigation, as are appropriate outcome measures to demonstrate their participation – if any - in ACS's efficacy, and it could be provided with a few examples. Proteomic analysis of synovial fluid from osteoarthritic equine joints, revealed a positive effect of ACS administration on protein profile, regarding inflammatory state, coagulation pathways, oxidative stress, and matrix damage (Chiaradia et al, 2012). It has been demonstrated that ACS, as well as a similarly processed blood-derived product, exert strong inhibiting effects on the oxidative burst of stimulated synovial fluid cells. Addition of ACS, as well as of processed plasma, to chemically and biologically stimulated equine synovial fluid cells prevented the generation of oxygen reactive species (Brossi et al, 2012). The deleterious effects of reactive oxygen species on cartilage homeostasis and joint inflammation have already been demonstrated (Henrotin et al, 2003).

Regarding articular components, other than cartilage, being the target of ACS' actions, Frisbie et al (2007) demonstrated significant reduced synovial membrane hyperplasia after intra-articular administration on ACS in an *in vivo* experiment. The synovial membrane has been recognized as a potent source on inflammatory mediators and proteolytic enzymes that fuel the cycle of deleterious intra-articular events leading to cartilage damage (Sutton et al, 2009). Maybe a longer-term evaluation would be necessary to demonstrate beneficial effects on cartilage structure, given the slow metabolic rate of this tissue.

There is a tendency to disregard ACS as blood sub-product and in this fact may reside the reason for the difficulty to demonstrate and appreciate their effects. The processing of blood may result in several different preparations, which should not be defined by a singular component or effect. At least, a combination of inflammatory, anti-inflammatory and anabolic cytokines, not to mention other molecules present in plasma, is to be expected, together with possible additive or suppressive effects resulting from their interaction. Beyond, the multitude of factors present in a blood-derived product may be more effective than a high concentration of a single protein (Woodell-May et al, 2011). In other cases, hormones present in donor's plasma may affect musculoskeletal metabolism or haemoderivative's efficacy in itself, in an unknown manner. Non-identified plasma proteins and other bioactive factors likely contribute to the biologic healing process (Boswell et al, 2012) and regarding ACS as a source of the interleukin-1 receptor antagonist protein offers an over-simplistic approach for hemoderivatives in orthopedic therapeutics. We are surely administering much more than interleukin -1 receptor antagonist protein when ACS are preconized, and the role of other undetermined factors within their composition has to be taken into consideration to correctly and fully appreciate their effects and limitations.

The use of blood derivates for tissue healing is an in important concept in the development of personalized medicine, where patient´s own biological materials are used for therapeutic purposes. Although understanding the full range of biological functions from plasma components is a complex task, there have been several advances in this field, fueled by proteomic analysis (Anitua & Orive, 2012). The most intriguing question in plasma proteomics today is how to make use of the therapeutic potential of these million plasma proteins (Lathrop et al, 2003). Human plasma is one of the richest tissues in body and potentially contains representatives of every protein produced in an individual. Despite decades of study, less than 1% of the estimated 1000000 proteins and antibodies in plasma have been characterized and only a few are currently used clinically (Hammond & Lathrop, 2005).

5 Conclusion

This systematic review found positive results expressed in 47,8% of all experimental study designs. Among them, RCTs showed the highest positive results (88,8%), followed by experimental controlled *in vivo* studies (33,3%). *In vitro* experimental only presented partially positive (60%) and negative (40%) outcomes.

Well-designed observational studies, on the other hand, were scarce and concordant regarding long-term effects of this hemoderivative treatment, pointing to negative results.

The scarcity of well-constructed RCTs regarding the effectiveness of ACS therapy prevented the production of a homogeneous systematic review. Different study designs were included and therefore, the level of evidence of the included studies were diverse. This should highlight the need for well-designed RCTs, which would prevent a high risk of bias in its construction and avoid the association of favorable results to a low evidence level.

6 References

Adib-Conquy, M., & Cavaillon, J. M. (2009). Compensatory anti-inflammatory response syndrome. *Thrombosis and haemostasis*, *101*(01), 36-47.

Anitua, E., & Orive, G. (2012). Endogenous regenerative technology using plasma-and platelet-derived growth factors. *Journal of Controlled Release*, *3*(157), 317-320.

Arbel R. (2017) Orthokine. In: Gobbi A., Espregueira-Mendes J., Lane J., Karahan M. (eds) Bio-orthopaedics. Springer, Berlin, Heidelberg

Baltzer, A. W. A., Moser, C., Jansen, S. A., & Krauspe, R. (2009). Autologous conditioned serum (Orthokine) is an effective treatment for knee osteoarthritis. *Osteoarthritis and Cartilage*, *17*(2), 152-160.

Bogers, S. H. (2018). Cell-based therapies for joint disease in veterinary medicine: what we have learned and what we need to know. *Frontiers in veterinary science*, *5*, 70.

Boswell, S. G., Cole, B. J., Sundman, E. A., Karas, V., & Fortier, L. A. (2012). Platelet-rich plasma: a milieu of bioactive factors. *Arthroscopy: The journal of arthroscopic & related surgery*, *28*(3), 429-439.

Brossi, P. M., Baccarin, R. Y., & Massoco, C. O. (2012). Do blood components affect the production of reactive oxygen species (ROS) by equine synovial cells in vitro?. *Pesquisa Veterinária Brasileira*, *32*(12), 1355-1360.

Burnouf, T., Goubran, H. A., Chen, T. M., Ou, K. L., El-Ekiaby, M., & Radosevic, M. (2013). Blood-derived biomaterials and platelet growth factors in regenerative medicine. *Blood reviews*, *27*(2), 77-89.

Carlson, E. R., Stewart, A. A., Carlson, K. L., Durgam, S. S., & Pondenis, H. C. (2013). Effects of serum and autologous conditioned serum on equine articular chondrocytes treated with interleukin-1 β. *American journal of veterinary research*, *74*(5), 700-705.

Chevalier, X. (2010). Intraarticular treatments for osteoarthritis: new perspectives. *Current drug targets*, *11*(5), 546-560.

Chiaradia, E., Pepe, M., Tartaglia, M., Scoppetta, F., D'Ambrosio, C., Renzone, G., ... & Beccati, F. (2012). Gambling on putative biomarkers of osteoarthritis and osteochondrosis by equine synovial fluid proteomics. *Journal of proteomics*, *75*(14), 4478-4493.

Damjanov, N., Barac, B., Colic, J., Stevanovic, V., Zekovic, A., & Tulic, G. (2018). The efficacy and safety of autologous conditioned serum (ACS) injections compared with betamethasone and placebo injections in the treatment of chronic shoulder joint pain due to supraspinatus tendinopathy: a prospective, randomized, double-blind, controlled study. *Medical ultrasonography*, *20*(3), 335-341.

Darabos, N., Haspl, M., Moser, C., Darabos, A., Bartolek, D., & Groenemeyer, D. (2011). Intraarticular application of autologous conditioned serum (ACS) reduces bone tunnel widening after ACL reconstructive surgery in a randomized controlled trial. *Knee Surgery, Sports Traumatology, Arthroscopy*, *19*(1), 36-46.

Darabos, N., Hundric-Haspl, Z., Haspl, M., Markotic, A., Darabos, A., & Moser, C. (2009). Correlation between synovial fluid and serum IL-1β levels after ACL surgery–preliminary report. *International orthopaedics*, *33*(2), 413-418.

Dehghani, B., & Rodeo, S. (2019). Cell Therapy—a Basic Science Primer for the Sports Medicine Clinician. *Current reviews in musculoskeletal medicine*, 1-10.

Dokka, S., Shi, X., Leonard, S., Wang, L., Castranova, V., & Rojanasakul, Y. (2001). Interleukin-10-mediated inhibition of free radical generation in macrophages. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, *280*(6), L1196-L1202.

Evans, C. H., Chevalier, X., & Wehling, P. (2016). Autologous conditioned serum. *Physical Medicine and Rehabilitation Clinics*, *27*(4), 893-908.

Fjordbakk, C. T., Johansen, G. M., Løvås, A. C., Oppegård, K. L., & Storset, A. K. (2015). Surgical stress influences cytokine content in autologous conditioned serum. *Equine veterinary journal*, *47*(2), 212-217.

Fox, B. A., & Stephens, M. M. (2010). Treatment of knee osteoarthritis with Orthokine®-derived autologous conditioned serum. *Expert review of clinical immunology*, *6*(3), 335-345.

Frisbie DD, Kawcak CE, McIlwraith CW. (2005) Evaluation of autologous conditioned serum using an experimental model of equine osteoarthritis. AAEP Proceedings.

Frisbie, D. D., Kawcak, C. E., Werpy, N. M., Park, R. D., & McIlwraith, C. W. (2007). Clinical, biochemical, and histologic effects of intra-articular administration of autologous conditioned serum in horses with experimentally induced osteoarthritis. *American journal of veterinary research*, *68*(3), 290-296.

Garbin, L. C. (2017) *Evaluation of allogeneic freeze-dried platelet lysate and conditioned serum in joint tissues under inflammatory condition in vitro* (Doctoral dissertation, Colorado State University. Libraries).

García-Escudero, J. B., & Trillos, P. M. H. (2015). Treatment of osteoarthritis of the knee with a combination of autologous conditioned serum and physiotherapy: a two-year observational study. *PloS one*, *10*(12), e0145551.

Geburek, F., Lietzau, M., Beineke, A., Rohn, K., & Stadler, P. M. (2015). Effect of a single injection of autologous conditioned serum (ACS) on tendon healing in equine naturally occurring tendinopathies. *Stem cell research & therapy*, *6*(1), 126.

Genç, E., Beytemur, O., Yuksel, S., Eren, Y., Çağlar, A., Küçükyıldırım, B. O., & Güleç, M. A. (2018). Investigation of the biomechanical and histopathological effects of autologous conditioned serum on healing of Achilles tendon. *Acta orthopaedica et traumatologica turcica*, *52*(3), 226-231.

Gross, G., & Hoffmann, A. (2013). Therapeutic strategies for tendon healing based on novel biomaterials, factors and cells. *Pathobiology*, *80*(4), 203-210.

Haddad, J. J., & Fahlman, C. S. (2002). Redox-and oxidant-mediated regulation of interleukin-10: an anti-inflammatory, antioxidant cytokine?. *Biochemical and biophysical research communications*, *297*(2), 163-176.

Hammond, D., & Lathrop, J. (2005). FIoNA: The Discovery of Trace Therapeutic Proteins from Complex Biological Mixtures. *Innov Pharm Technol*, *17*, 38-41.

Heisterbach, P. E., Todorov, A., Flückiger, R., Evans, C. H., & Majewski, M. (2012). Effect of BMP-12, TGF-β1 and autologous conditioned serum on growth factor expression in Achilles tendon healing. *Knee Surgery, Sports Traumatology, Arthroscopy*, *20*(10), 1907-1914.

Henrotin, Y. E., Bruckner, P., & Pujol, J. P. (2003). The role of reactive oxygen species in homeostasis and degradation of cartilage. *Osteoarthritis and cartilage*, *11*(10), 747-755.

Höher, J., Möller, H. D., & Fu, F. H. (1998). Bone tunnel enlargement after anterior cruciate ligament reconstruction: fact or fiction?. *Knee Surgery, Sports Traumatology, Arthroscopy*, *6*(4), 231-240.

Hraha, T. H., Doremus, K. M., McIlwraith, C. W., & Frisbie, D. D. (2011). Autologous conditioned serum: the comparative cytokine profiles of two commercial methods (IRAP and IRAP II) using equine blood. *Equine veterinary journal*, *43*(5), 516-521.

Irie, K., Uchiyama, E., & Iwaso, H. (2003). Intraarticular inflammatory cytokines in acute anterior cruciate ligament injured knee. *The Knee*, *10*(1), 93-96.

Lasarzik, J., Bondzio, A., Rettig, M., Estrada, R., Klaus, C., Ehrle, A., ... & Lischer, C. J. (2018). Evaluation of two protocols using autologous conditioned serum for intra-articular therapy of equine osteoarthritis—a pilot study monitoring cytokines and cartilage-specific biomarkers. *Journal of equine veterinary science*, *60*, 35-42.

Lathrop, J. T., Anderson, N. L., Anderson, N. G., & Hammond, D. J. (2003). Therapeutic potential of the plasma proteome. *Curr. Opin. Mol. Ther*, *5*(3), 250-257.

Majewski, M., Ochsner, P. E., Liu, F., Flückiger, R., & Evans, C. H. (2009). Accelerated healing of the rat Achilles tendon in response to autologous conditioned serum. *The American journal of sports medicine*, *37*(11), 2117-2125.

Malemud, C. J. (2010). Anticytokine therapy for osteoarthritis. *Drugs & aging*, *27*(2), 95-115.

Meijer, H., Reinecke, J., Becker, C., Tholen, G., & Wehling, P. (2003). The production of anti-inflammatory cytokines in whole blood by physico-chemical induction. *Inflammation Research*, *52*(10), 404-407.

Moher D., Liberati A., Tetzlaff J. (2009) Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *BMJ* 339: b2535.

Moreira, J. J., Moraes, A. P. L., Brossi, P. M., Machado, T. S., Michelacci, Y. M., Massoco, C. O., & Baccarin, R. Y. (2015). Autologous processed plasma: cytokine profile and effects upon injection into healthy equine joints. *Journal of veterinary science*, *16*(1), 47-55.

Opal, S. M. (2000). DePalo VA. *Anti-inflammatory cytokines. Chest*, *117*, 1162-1172.

Ortved, K. F. (2018). Regenerative Medicine and Rehabilitation for Tendinous and Ligamentous Injuries in Sport Horses. *Veterinary Clinics: Equine Practice*, *34*(2), 359-373.

Rutgers, M., Saris, D. B., Dhert, W. J., & Creemers, L. B. (2010). Cytokine profile of autologous conditioned serum for treatment of osteoarthritis, in vitro effects on cartilage metabolism and intra-articular levels after injection. *Arthritis research & therapy*, *12*(3), R114.

Schneider, U., & Veith, G. (2013). First Results on the Outcome of Gold-induced, Autologous-conditioned Serum (GOLDIC) in the Treatment of Different Lameness-associated Equine Diseases. *J Cell Sci Ther*, *5*(151), 2.

Strümper, R. (2017). Intra-Articular Injections of Autologous Conditioned Serum to Treat Pain from Meniscal Lesions. *Sports medicine international open*, *1*(06), E200-E205.

Sutton, S., Clutterbuck, A., Harris, P., Gent, T., Freeman, S., Foster, N., ... & Mobasheri, A. (2009). The contribution of the synovium, synovial derived inflammatory cytokines and neuropeptides to the pathogenesis of osteoarthritis. *The veterinary journal*, *179*(1), 10-24.

Tassara, M., De Ponti, A., Barzizza, L., Zambelli, M., Parisi, C., Milani, R., & Santoleri, L. (2018). Autologous conditioned serum (ACS) for intra-articular treatment in Osteoarthritis: Retrospective report of 28 cases. *Transfusion and Apheresis Science*, *57*(4), 573-577.

Tatarniuk, D. (2015). Autologous conditioned serum and subsequent synovial fluid concentrations of cytokines, matrix metalloproteinases, and tissue inhibitors of metalloproteinases after injection into equine osteoarthritic distal interphalangeal joints. tendinopathies. *Stem Cell Res Therapy* 6(1):1

Textor, J. A., Norris, J. W., & Tablin, F. (2011). Effects of preparation method, shear force, and exposure to collagen on release of growth factors from equine platelet-rich plasma. *American journal of veterinary research*, *72*(2), 271-278.

Warner K., Lischer C. J. (2017) Komplikationen nach intraartikulärer Anwendung von ACS (IRAP®) beim Pferd – Retrospektive Studie. *Pferdeheilkunde* 33, 356-362;

Warner K., Schulze T., Lischer C. J. (2016) Behandlung von Osteoarthritis mit ACS (IRAP®) bei 26 Pferden – Retrospektive Studie. *Pferdeheilkunde* 32, 341-248

Wehling, P., Evans, C., Wehling, J., & Maixner, W. (2017). Effectiveness of intra-articular therapies in osteoarthritis: a literature review. *Therapeutic advances in musculoskeletal disease*, *9*(8), 183-196.

Wehling, P., Moser, C., Frisbie, D., McIlwraith, C. W., Kawcak, C. E., Krauspe, R., & Reinecke, J. A. (2007). Autologous conditioned serum in the treatment of orthopedic diseases. *BioDrugs*, *21*(5), 323-332.

Wei, F. Y., Lee, J. K., Wei, L., Qu, F., & Zhang, J. Z. (2017). Correlation of insulin-like growth factor 1 and osteoarthritic cartilage degradation: a spontaneous osteoarthritis in guinea-pig. *European review for medical and pharmacological sciences*, *21*(20), 4493.

Weinberger T. (2008) Klinische Erfahrumgen mit der anwendung von ACS/Orthokin/IRAP beim pferd. *Pferde Spiegel* 2008;

Woodell-May, J., Matuska, A., Oyster, M., Welch, Z., O'Shaughnessey, K., & Hoeppner, J. (2011). Autologous protein solution inhibits MMP-13 production by IL-1β and TNFα-stimulated human articular chondrocytes. *Journal of Orthopaedic Research*, *29*(9), 1320-1326.

Yang, K. A., Raijmakers, N. J. H., Van Arkel, E. R. A., Caron, J. J., Rijk, P. C., Willems, W. J., ... & Saris, D. B. F. (2008). Autologous interleukin-1 receptor antagonist improves function and symptoms in osteoarthritis when compared to placebo in a prospective randomized controlled trial. *Osteoarthritis and cartilage*, *16*(4), 498-505.

Zarringam, D., Bekkers, J. E., & Saris, D. B. (2018). Long-term effect of injection treatment for osteoarthritis in the knee by orthokin autologous conditioned serum. *Cartilage*, *9*(2), 140-145.

Ziltener JL, Allet L, Sclison P, et al. (2012) How effective are injections of platelet-rich plasma (PRP) for the treatment of sports injuries: a critical review of the literature. *J Sport Medic Doping Studie*; 1-6.

Appendix 1. Experimental Studies included in this systematic review – Summarized data 1/6

Author/ year	Study Design	Population	Follow up	Control	Outcome Measures	Lesion	Hemoderivative Acquisition/ Analysis	Intervention	Blinding Patients (P)/ Outcome Accessors (OA)	Results	Observations	
FRISBIE et al, 2005	CONTROLLED *in vivo*	Horses; n=16	70 days	Saline inj. (6ml) in sham operated joints and OA joints on days 14, 21, 28 and 35; n = 8	Histologic examination (HE, SOFG), GAG, $^{35}SO_4$ incorporation; Gross evaluation post-mortem	Experimentally induced OA in middle carpal joint	Orthokine – 24 hours incubation	6 ml ACS inj. in OA joints n= 8 PBS inj.in sham operated joints n=8 on days 14, 21, 28, 35 + exercise from day 15 on	OA	No adverse effects; Signif. ↓ of intimal hyperplasia in synovial membrane in ACS group and improvement in lameness. No signif. improvement in articular cartilage morphology, GAG content and $^{35}SO_4$ incorporation detected in ACS treated joints. [IL-1Ra] in ACS not detected	Funded in part by the Arthrex® Bioservices; Poor ACS characterization	±
FRISBIE et al, 2007	CONTROLLED *in vivo*	Horses; n=16	70 days	Saline inj. (6ml) in sham operated joints and OA joints on days 14, 21, 28 and 35; n = 8	Clinical and radiologic evaluation; Synovial fluid analysis (TP, cytology, total WBC, aspect, mucin clot, GAG, PGE2, IL-1Ra and macroscopic evaluation)	Experimentally induced OA in middle carpal joint	Orthokine - 24 hours incubation + centrifugation	6 ml ACS inj. in OA joints n= 8 PBS inj.in sham operated joints n=8 on days 14, 21, 28, 35 + exercise from day 15 on	OA	Late signif. improvement in lameness (day 70) in ACS group. ACS treated OA joints had ↓ synovial membrane hyperplasia. IL-1Ra mean levels from both joints of ACS treated horses were signif. ↑ only at days 35 and 70. IL-1 RA identified only with mouse antibody [IL-1Ra] ↑ in ACS compared to levels in unprocessed blood.	Poor ACS characterization	±
YANG et al, 2008	RCT	Human patients; n=153	12 months	Saline inj. (2 ml) on days 0, 3, 7, 10, 14, 21 days; n= 73	30% superiority on WOMAC index at 3, 6, 9, 12 months, VAS, KOOS, KSCRS	KOA; I-III KL index	Orthokine - 24 hours incubation	ACS inj. (2 ml) on days 0, 3, 7, 10, 14, 21 days; n= 80	OA/P	Primary objective not met. Significant improvement in KOOS in ACS group. Consistently higher, but not signif., improvement in other parameters.	Outcome measures limited; poor ACS characterization	±
WEINBERGER, 2008	non-Randomized Clincal trial	Horses; n=262	12 weeks	---	Lameness evaluation at 6 and 12 weeks	OA, different joints affected	Orthokine - 24 hours incubation + centrifugation at 3700 rpm for 10 min	2 -3 inj. of ACS; 8 - 12 days apart	---	At 6 weeks, 199 horses were lame free and 22 showed improvement. After 12 weeks, 178 horses were sound and in normal training.	Poor study design. Outcome measures limited and subjective. Short follow-up. Uncontrolled, unblinded.	+
MAJEWSKI et al, 2009	CONTROLLED *in vivo*	Sprague-Dawley Rats; n=80	8 weeks	No treatment; n= 40	Immunohistochemistry, biomechanical and histologic evaluation, fluorometric assay (Lysil oxidase activity);	Transected and surgically repaired Achilles tendons	Orthokine - 9 hours incubation + centrifugation/ TGF-β1, PDGF-BB, VEGF	ACS inj. 24, 48 and 72 h postop.; n= 40	OA	Tendons exposed to ACS had greater expression of COL1AI gene, had greater type 1 collagen content, were thicker and regained stiffness and histologic maturity earlier. Maximum load to failure was not significantly affected by ACS.	---	±

Appendix 1. Experimental Studies included in this systematic review – Summarized data 2/6

Author/ year	Study Design	Population	Follow up	Control	Outcome Measures	Lesion	Hemoderivative Acquisition/ Analysis	Intervention	Blinding Patients (P)/ Outcome Accessors (OA)	Results	Observations	
BALTZER et al, 2009[205]	RCT	Human patients; n=376	2 years	Saline 1 inj. weekly for 3 weeks; n= 107	WOMAC, VAS, Short-Form 8, GPA; baseline and weeks 7, 13 and 26 and at 2 years	KOA/ KL II-III	Orthokine (according to instructions)	A) ACS, 2 inj., weekly for 3 weeks; n=134 B) HA, 1 inj., weekly for 3 weeks; n= 135	OA/P	Effects of ACS were signif. superior to those of HA and saline for all outcome measures. HA and saline had similar effects.	Only clinical evaluation. Discrepancy between number of inj. of ACS, Saline and HA.	+
DARABOS et al, 2009[206]	RCT	Human patients; n=20	10 days	Physiological solution inj. (2ml) on day of surgery and postop. days 1, 6 and 10; n= 10	IL-1β in synovial fluid and serum on day of surgery and postop. days 1, 6 and 10	ACL reconstruction	Orthokine (according to instructions)	ACS (2ml) on day of surgery and postop. days 1, 6 and 10; n= 10	OA/P	ACS group had a steady ↓ in IL-1β levels over time. At 10 days values were lower than those reported for normal joints and signif. lower than in control group.	Small n. Only one outcome measure. Short follow up.	+
RUTGERS et al, 2010[12]	CONTROLLED *in vivo*	Human KOA patients; n=22	21 days	SF analysis before treatment	Synovial fluid cytokine profile (Multiplex ELISA)	KOA	Orthokine – 6 hours incubation; Autologous blood ↑IL-1Ra; IL-10, TGF-β1; IL-6; IL-1β; OSM; TNF-α and ↓ OPG	Total of 6 ACS inj. (2ml) on days 0, 3, 7, 10, 14, 21	---	No signif difference in cytokine profile of synovial fluid before and after ACS treatment was noted.	Small n; clinical outcomes not evaluated; short follow-up.	-
RUTGERS et al, 2010[12]	CONTROLLED *in vitro*	Human KOA cartilage explants; n=48	16 days	Cartilage explants cultured in non-conditioned serum; n= 24	PG metabolism	KOA/ KL III-IV	Orthokine – 6 hours incubation + centrifugation at 1000x g for 10 min; heterologous blood	Explants cultured with ACS; n=24	---	Treatments did not affect PG metabolism of cartilage explants. I-L10, IL-6, IL-1 and TGF-β1 ↑ signif. after conditioning.	Heterologous blood employed; control treatment may not be inert	±
RUTGERS et al, 2010[12]	CONTROLLED *in vitro*	Human KOA cartilage explants; n=24	16 days	Cartilage explants cultured with 25% control serum; n= 8	$^{35}SO_4$ incorporation at days 4, 8 and 12; release of newly synthesized PGs at days 4, 8, 12 and 16; DNA and GAG content at day 16.	KOA/ KL III-IV	Orthokine – 6 hours incubation + centrifugation at 1000x g for 10 min; heterologous blood	A) Explants cultured with Etanercept; n= 8; B) Explants cultured with ACS; n= 8	---	No difference in PG release, PG content or ^{35}S incorporation between different cultures of cartilage explants. Etanercept did not affect outcome measures.	Heterologous blood employed; control treatment may not be inert	-

Appendix 1. Experimental Studies included in this systematic review – Summarized data 3/6

Author/ year	Study Design	Population	Follow up	Control	Outcome Measures	Lesion	Hemoderivative Acquisition/ Analysis	Intervention	Blinding Patients (P)/ Outcome Accessors (OA)	Results	Observations	
DARABOS et al, 2011[204]	RTC	Human patients; n=62	12 months	Saline (2ml) at day of surgery, and postop. days 1, 6 and 10; n= 31	Width of tibial bone tunnel through CT at 1 day, 6 and 12 months postop.; WOMAC, IKDC 2000 at 6 and 12 months; IL-1β concentration in synovial fluid at days 1, 6 and 10 postop.	Bone tunnel widening after ACL reconstruction	Orthokine prepared according to references	ACS (2ml) on day of surgery and postop. days 1, 6 and 10; n= 31	OA/P	Bone tunnel enlargement was signif.↓ in ACS group compared to control. Clinical outcomes were signif. better in patients treated with ACS and [IL1-β] signif. lower in ACS group at day 10.	Different surgical procedures performed on patients	+
CHIARADIA et al., 2012	non-Randomized Clinical Trial	Horses; n=10	40 days	SF proteomic analysis before treatment	SF proteomic analysis after treatment; Return to previous athletic level	OA of MCPJ and MTFJ	Orthokine - 24 hours incubation + centrifugation at 2100 x g for 10 min	Four ACS inj. (3 - 5ml) 7-10 days apart	---	↓ acute phase proteins (C4A, α2MG and CP) after treatment; 7/10 horses returned to their previous levels of athletic activities	Small n.; limited outcome measures. Short follow-up; Non-randomized; different joints assigned	+
HEISTERBACH et al, 2012[209]	CONTROLLED *in vivo*	Sprague-Dawley Rats; n=60	8 weeks	Natural expression of GFs; n= 15	Immunohistochemistry	Transected and surgically repaired Achilles tendons	Orthokine - 9 hours incubation + centrifugation	A) BMP-12; n=15 B) TGF-β1; n=15 by gene transfer C) ACS; n=15; 1 inj. daily for 3 days	OA	ACS treatment increased bFGF and BMP-12 signif. after 8 weeks. Expression of bFGF, BMP-12, TGF-β1 were signif. greater in ACS at all time points. ACS had the greatest effect on GF expression	---	+
CARLSON et al, 2013[208]	CONTROLLED *in vitro*	Equine chondrocyte pellets; n= 115	---	Chondrocytes treated with AES 10%	GAG synthesis, COL II and ciclooxigenase-2 mRNA expression and MMP-3 activity	---	AES = equine blood in borosilicate tubes with clot activator, centrifuged ACS= IRAP II®, processed ac. To manufacturer	A) 10% AES+ rhIl-1β B) 20% AES+ rhIl-1β C) 10% ACS+ rhIl-1β D) 20% ACS+ rhIl-1β	---	No difference in mRNA expression, GAG release, GAG and DNA content or MMP-3 activity between ACS or AES+rhIl-1β Medium from ACS+rhIl-1β had ↑ [IL-1ra] compared to AES+ rhIl-1β 20% ACS+ rhIl-1β resulted in ↑ [IGF-1] than 10% AES+ rhIl-1β	---	±
SCHNEIDER & VEITH, 2013	non-Randomized Clinical trial	Horses; n=36 (37 cases)	24 weeks	---	Lameness evaluation (AAEP Grading system); swelling/effusion before treatment, at weeks 1, 2, 3, 12 and 24.	19 horses OA/OCD; 16 horses tendon/ligam.t injuries; 1 horse sesamoiditis	Goldic® produced according to manufacturer's guidelines	4 applications of GOLDIC ACS; First ACS injection one day after incubation period. Further three injections - dates of application not specified.	---	Significant ↓ of lameness and effusion/swelling within 2 weeks of treatment. Horses free of symptoms up to 3-6 months after treatment. No major side effects observed within 24 weeks.	Non-randomized; uncontrolled and unblinded. Interval of inj. not specified; multiple lesions included; limited and subjective outcome measures.	+

Appendix 1. Experimental Studies included in this systematic review – Summarized data 4/6

Author/ year	Study Design	Population	Follow up	Control	Outcome Measures	Lesion	Hemoderivative Acquisition/ Analysis	Intervention	Blinding Patients (P)/ Outcome Accessors (OA)	Results	Observations	
TATARNIUK, 2015	CONTROLLED *in vivo*	Horses; n= 11	21 days	SF analysis before treatment; Lameness score before treatment.	SF analysis and soundness on D0, D7, D14 and D21	Naturally occurring unilateral forelimb DIP joint OA	IRAP-II incubated for 18-20 hours + centrifugated at 1,800xg for 10 min; ACS ↑ [ ] IL-1Ra, MMP-1: TIMPs ratio and MMP-9: TIMP (-1, -2 and -4) compared to unincubated serum. IL-4, IL-6 and IL-8 ↓ compared to unconditioned serum.	3 weekly IA injection of ACS	OA	No changes of SF [ ] IL-1Ra; not observed improvement in lameness scores.	Lack of placebo intervention; doubtful inclusion criteria, short follow up; incubation period different from manufacture's recommendations	-
TATARNIUK, 2015	CONTROLLED *in vitro*	Conditioned and uncond. serum from 11 horses	----	Unincubated and unconditioned serum	Cytokine profile IL-1Ra, IL-1β, TNF-α; IL-4, IL-6, IL-8, IL-10; MMP (-1, 13, -9, -13) and TIMPs	---	IRAP-II incubated for 18-20 hours + centrifugated at 1,800xg for 10 min	Incubation for 18-20 hours and freeze-thawing	---	Conditioning has no effect on IL-1Ra [ ]; incubation effectively ↑ [ ] IL-1Ra; Freeze-tawing ↓ aprox. 10% IL-1Ra [ ] compared to fresh ACS. ACS ↑MMP-1: TIMPs ratio and MMP-9: TIMP (-1, -2 and -4) . Incubated unconditioned serum ↓ [ ] of IL-6.	incubation period different from manufacture's recommendations	±
GEBUREK et al., 2015	RCT	Horses; n=17	190 days	Intralesional single injection 1-3ml Saline (n=2) or untreated (n=5)	Clinical and B-MODE US examination (D0 D11 D22 D36 D50 D78 D106 D134 D162 and D190); Histologic and Immunohistochemical expression of collagen I and III (D0, D36 and D190)	Acute naturally occurring tendinopathy of forelimb SDFT	IRAP®-10 (Orthogen) (6-9 hours of incubation) + centrifugated at 4,000 rpm for 10 min	Intralesional single injection 1-3 ml ACS; n= 10	US assessor (treatment) Histologic assessor (treatment and horse)	**Lameness**: all horses sound by D36 - ACS group showed a faster improvement (D11) ; **Signs of inflammation:** Swelling ↓ by D50-D78 and remained (ACS); Sensitivity and surface temperature: ↓ both groups by D22. **B-MODE US:** mean %T-lesion ↓ and echogenicity ↑ in ACS group. **Histology:** more spindle shaped tenocytes and more uniform cell density on D36 and improvement of fibers organizations compared to baseline on ACS group. **Immunohistochemistry**: ↑ collagen-I btw D36 - D190 in ACS group - not changed in Control group	Small n; Control group not homogeneous (untreated x saline injections); clinical evaluation not specified as blinded	+

Appendix 1. Experimental Studies included in this systematic review – Summarized data 5/6

Author/ year	Study Design	Population	Follow up	Control	Outcome Measures	Lesion	Hemoderivative Acquisition/ Analysis	Intervention	Blinding Patients (P)/ Outcome Accessors (OA)	Results	Observations	
MOREIRA et al., 2015	CONTROLLED *in vivo*	Horses n=10 Healthy MCPJ n=20	1) 168h 2) 28 days	1) Single 4ml injection of Saline (contralateral joint) n=10 2) Three weekly applications of 4ml Saline (contralateral joint)	1) SF [ ] WBC; TP; IL-1β; IL-1Ra; PGE_2; TNF-α and GAG prior to first injection and at 3, 6, 24, 48 and 168 hours; 2) SF [ ] WBC; TP; IL-1β; IL-1Ra; PGE_2; TNF-α and GAG on D0, D7, D14, D21 and D28.	---	Horses' blood + sodium heparin, inc. 24 hours + centrif. at 300x g for 10 min at 24°C + transferred to another tube and centrifugated at 900x g for 10 min at 24°C and filtered with 0. 22μm Millipore filter	1) Single 4ml Injection of APP into one randomly assigned MCPJ; n=10 2) Weekly IA injections of APP in randomly assigned MCPJ for 3 consecutive weeks; n=6	---	1) APP ↑ counts of WBC at 3, 6, 24 and 48h compared to saline; ↑ [ ] of PGE_2 and TP at 3h and 6h; ↑ [ ] CS and IL-1Ra at 24h - IL-1Ra start increasing at 3h; mild transient inflammatory response; Saline ↑ [ ] of WBC and CS at 24h and 48h; ↑ [ ] IL-1Ra at 24h and ↑ [ ] of PGE_2 at 6h; 2) APP showed no relevant changes in measured factors compared to baseline values; but Saline ↑ [ ] of WBC at 14, 21 and 28 days and↑ [ ] of PGE_2 at 28days compared to APP	Small n	±
GARBIN, 2017	CONTROLLED *in vitro*	Horses; n=16 Articular cartilage n= 8 Synovium n=8	10 days	Cartilage and synovium explants cultured with ITS or FBS exposed to IL-1β	Total GAG and $^{35}SO_4$-labeled GAG in cartilage and media; Gene expression on cartilage (ADAMTS-5, ADAMTS-4, MM-1, COX-2 and IL-1β) and on synovium (COX-2 and IL-1β)	Stimulation of explants with IL-1β	**1) Frozen ACS**: blood in conical tubes containing coated beads, inc. for 18-24h + centrif. at 4,000 rpm for 10 min + stored at -80°C; **2) Freeze-dried**: frozen ACS lyophilized for 18h and stored at -80°C; **3) Filtered freeze-dried**: filtration of serum with a 45μm filter before lyophilization and stored at -80°C; **4) Allogenic CS:** Aliquots from 4 different horses pooled together. **Platelet-lysate not of interest**	IL-1β + CS treatment* on D0 and D4; *Frozen, freeze-dried, filtered freeze-dried or allogenic CS	---	Total GAG and $^{35}SO_4$-labeled GAG in cartilage and media + CS* not statistically different from controls; Gene expression: CS induced upregulation of MM-1 in cartilage and IL-1β in synovium; pro-inflammatory gene expression differed btw tissues but not btw different treatments.	Possible sponsorship industry bias, Small n	-

Appendix 1. Experimental Studies included in this systematic review – Summarized data 6/6

Author/ year	Study Design	Population	Follow up	Control	Outcome Measures	Lesion	Hemoderivative Acquisition/ Analysis	Intervention	Blinding Patients (P)/ Outcome Accessors (OA)	Results	Observations	
GENÇ et al., 2018	CONTROLLED *in vivo*	Sprague-Dawley rats; n=40 Obs: Blood-donor rats for ACS n=5	30 days	Placebo saline injection on the repair zone n= 20	**Histopathological:** Bonar and Movin scales. **Immunohistochemical:** staining of collagen-III. **Biomechanical test:** tensile testing.	Surgically transected and sutured rats' Achilles tendons	Heterologous blood. Orthokine - incubation time not elucidated + centrif. at 3,500 rpm for 10 min	Three injections of Saline or ACS into the repair zone after surgery - dates of application not specified n= 20	---	ACS group: histopathological results significantly better on D15 and D30; Immunohistochemical density of collagen-III reduced at D30. Biomechanical: maximal load to failure values higher at D15	Short follow-up; Small n; application times not specified	+
LASARZIK et al., 2018	CONTROLLED *in vivo*	Horses; n= 12; randomly allocated in two groups of 6 horses.	42 days	Synovial fluid pre-treatment; (OA biomarker concentrations)	1) SF before each ACS injection, 1 hour after each ACS injection and 42 days after treatment start; 2) SF before each ACS injection, 4 hours after each ACS injection and 42 days after treatment start. Concentrations of IL-1Ra, IL-1β; C12C; CS 846 and CPII	Advanced naturally occurring OA. 2 MCPJ, 2 MTPJ, 2 TCJ and 1 MTFJ included for each group.	ABPS Arthrex Vet Systems - incubation for 24h	Application of ACS 14 days after arthroscopy; 1) 3 applications at weekly intervals; 2) 3 applications at 2-day intervals. 2ml for MCFJ/MTFJ and 6ml for TCJ or MFTJ	---	[ ] of IL-1Ra ↑ 1 and 4h after ACS but ↓ back to baseline levels within 48h - half-life of ACS btw 4 and 48h. [ ] of IL-1Ra, IL-1β, C12C, CS 846 and CP II ↓ significantly 42days after 2-day protocol - parameters closer to normal joints.	Small n; non uniform pop.; groups compared at different moments (1h and 4h after ACS application; high number of horses with previous OCD	+
DAMJANOV et al., 2018	RCT	Humans; n = 32	24 weeks	Betamethasone 3 weekly applications followed by one placebo injection on the fourth week	VAS and CSS on weeks 0, 4 and 24; Safety profile of treatment	Supraspinatus tendinopathy	EOT II incubated for 7 hours + centrifugated at 3,000xg for 10 min	1) Four ACS weekly applications at entheses and paratenon of supraspinatus;	OA/P	Improvement of shoulder pain after ACS treatment on 4 and 24 weeks compared to glucocorticoid group; CSS improved greatly after 24 weeks; No major side effects of ACS treatment	Small n; control not placebo; outcome measures limited	+

ACS= Autologous conditioned serum; CS= Conditioned serum; APP= Autologous processed plasma; AES= Autologous equine serum; RCT= randomized clinical trial; OA= Osteoarthritis; KOA= Knee osteoarthritis; ACL = Anterior cruciate ligament; OCD= Osteochondritis dissecans; SDFT= Superficial digital flexor tendon; MCPJ= Metacarpophalangeal joint; MTPJ= Metatarsophalangeal joint; TCJ= Tarsocrural joint; MFTJ= Medial femorotibial joint; DIP= Distal interphalangeal; SF= Synovial fluid; HA= Hyaluronic acid; HE= Hematoxylin and eosin staining; SOFG= Safranin-O fast green; PBS= Phosphate buffered saline; FBS= Fetal bovine serum; ITS= Insulin transferrin-selenium; TP= Total protein; WBC= White blood cells; WOMAC= Western Ontario and McMaster Universities Osteoarthritis Index; VAS= Visual analogue scale; KOOS= Knee and Osteoarthritis Outcome Score; KSCRS= Knee Society Clinical Rating System; GPA= Grade Point Average; IKDC 2000= International Knee Documentation Committee; CSS= Constant Shoulder Score; KL= Kellgren- Lawrence classification; GAG= Glycosaminoglycan; PG= Proteoglycan; PGE_2= Prostaglandin E2; IL-1Ra= interleukin 1 receptor antagonist; TGF-β1= Transforming growth factor β1; PDGF= Platelet-derived growth factor; VEGF= Vascular endothelial growth factor; bFGF= Basic fibroblastic growth factor; GFs= growth factors; BMP= Bone morphogenetic proteins; OPG= Osteoprotegerin; OSM= Oncostatin M; TNF-α= Tumor necrosis factor alpha; C4A= Complement component C4; α2MG= Alpha2 macroglobulin; CP= Ceruloplasmin; MMP= Matrix Metalloproteinases; TIMP= Tissue inhibitors matrix metalloproteinases; C12C= Catabolic collagenase-cleaved type II collagen epitope; CS 846= Aggrecan chondroitin sulfate 846 epitope; CPII= anabolic procollagen II C-propeptide; CT= Computerized tomography; US= Ultrasonography; %T-lesions= Percent total lesion.

Appendix 2. Observational Studies included in this systematic review – Summarized data

Author/ year	Study Design	Population	Follow up	Control	Outcome Measures	Lesion	Hemoderivative Acquisition/ Analysis	Intervention	Blinding Patients (P)/ Outcome Accessors (OA)	Results	Observations	
WARNER et al., 2016	Retrospective Cohort	Horses; n=26	2 years	---	Owners reports of improvement, horses' return to performance level.	DIP joint OA	IRAP®	2 - 4 inj. of ACS, within 7-21 days	---	31% of horses returned to work at a better or equal performance level; 15% of horses returned to work at lower level; 54% of horses did not present long-term improvement.	Small n; possible recall bias.	**–**
ZARRINGAM et al., 2018	Prospective Cohort	Humans; n=126	10 years	Placebo treatment (saline injection)	Surgical intervention taken in the previously studied knee in 10 years - after ACS or Placebo treatment	KOA	Orthokine (24 hours incubation)	Interview with patients treated in Yang et al. (2008) ACS and placebo groups; or information retrieved from electronic health records.	---	46,3% of the placebo and 40,3% of ACS group had been treated surgically within follow-up period. ACS does not seem to prevent or delay surgical intervention at 10 years after treatment for end-stage knee osteoarthritis.	non-surgical treatments were not considered;	**–**

DIP = Distal interphalangeal; OA= Osteoarthritis; KOA= Knee Osteoarthritis.