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INTRA-ARTICULAR TREATMENT WITH TRIAMCINOLONE ACETONIDE AND PLATELET-RICH PLASMA FOR FETLOCK OSTEOARTHRITIS IN THOROUGHBRED RACEHORSES

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GENERAL INTRODUCTION

Osteoarthritis (OA), also known as osteoarthrosis or degenerative joint disease, of the diarthrodial joint, is the most significant chronic musculoskeletal disorder in horses and a major animal welfare concern [1]. The metacarpophalangeal (MCP) or fetlock joint is the most commonly affected, leading to lameness, lost training days, and the largest economic losses in the equine industry due to osteoarthritic pain [2-7]. OA is a heterogeneous disease with multiple etiologies, clinical phenotypes, and molecular endotypes, which necessitates differential targeting approaches, opening pathways for the development of effective disease-modifying OA drugs (DMOADs) [8].

Currently, the mainstay of intra-articular therapy focuses on alleviating the clinical signs of the disease through temporary reduction of inflammation, often achieved via corticosteroid (CS) injections [2]. While this approach helps manage musculoskeletal pain and lameness, allowing affected horses to continue training and racing, it does not promote regeneration of the affected joint [9-11]. Triamcinolone acetonide (TA) is the most commonly used corticosteroid in high-motion joints [12]. However, concerns remain about the long-term impact of repetitive use of TA and other corticosteroids on cartilage health [12] as either chondroprotective and chondrotoxic effects in vitro have been reported. [13-16]. As a result, alternative treatments are being explored to minimize repetitive corticosteroid use and support the natural healing processes of the tissues [17].

The field of equine regenerative medicine, which includes platelet-rich plasma (PRP), also known as autologous conditioned plasma (ACP), is gaining increasing popularity in the scientific community due to its strategies for the treatment of joint pathologies [9]. Reasons for this increased popularity include the potential to prevent OA progression, reduction in clinical signs and improvement of joint function while reducing the potential of severe adverse event [18]. PRP is an autologous blood product that contains a great number of platelets within a small amount of plasma, but a variable concentration of platelets between individuals due to biological variation [19]. Autologous blood products utilize mechanisms of the natural response to injury, by promoting the production of anti-inflammatory cytokines and release of growth factors [20]. Studies have shown that injecting PRP into equine joints has clinical benefits, including improvement in lameness, synovial effusion, and pain during passive flexion [9,17,21]. Additionally, PRP has been confirmed as a safe option for intra-synovial administration, causing no long-term adverse effects on joint homeostasis, despite a mild early inflammatory response [22,23]. The beneficial effects of PRP are believed to be due to the reduction of inflammatory cytokines and the inhibition of oxidative stress [24,25].

However, despite the prevalence of OA and a phenomenal amount of research is performed each year on OA, yet an exact aetiology has not been elucidated nor an effective treatment discovered [26,27]. Therefore, is necessary to understand the inflammatory cascade behind OA, and to investigate how treatments may affect this cascade. In addition to lameness examination and standard diagnostic procedures, the identification of synovial biomarkers in synovial fluid (SF), to more accurately assess intrasynovial inflammation in cases of joint disease, is an active area of research [28,29]. Biomarkers provide insights into the pathophysiological processes occurring within the joint, offering a non-invasive method to assess disease severity and therapeutic

outcomes [30]. The inclusion of synovial biomarkers in OA studies enhances the ability to evaluate the impact of treatments like intra-articular corticosteroids and biologic products on joint health at a biochemical level, potentially leading to more targeted and effective therapeutic strategies.

The present PhD thesis focused on PRP with the potential of slowing down the disease progression and compared its use to the triamcinolone acetonide since the oldest and most commonly used joint treatments in equine patients. The aim of this study was to investigate the effects of both treatments on chondrocytes, the clinical outcomes in equine patients treated with these products and to investigate their effect on the synovial biomarkers. It was hypothesized that TA and PRP would improve clinical signs of OA and protect the chondrocytes, but PRP would cause stronger anti-inflammatory effect by reducing the concentration of inflammatory cytokines (IL-1β, IL-6), acute phase proteins (APPs), and upregulating the hypoxic condition by activation of hypoxia-inducible factor 1 alpha (HIF1-α) more efficiently than TA treatment.

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THE COMBINED USE OF TRIAMCINOLONE AND PLATELET-RICH PLASMA IN EQUINE METACARPOPHALANGEAL JOINT OSTEOARTHRITIS TREATMENTS: AN IN VIVO AND IN VITRO STUDY.

Abstract

Intra-articular corticosteroids, such as triamcinolone acetonide (TA) help reduce pain related to osteoarthritis (OA), but they may impair cartilage metabolism. In contrast, platelet-rich plasma (PRP) therapy, a regenerative therapy, has shown potential to promote healing and regeneration of articular cartilage. This study investigates the effects of combining PRP with TA to treat osteoarthritis in racehorses. The study proposes that PRP

injection following TA treatment could reduce side effects and improve treatment outcomes. Firstly, in vitro study, chondrocytes were exposed to different TA concentrations, with or without PRP. TA dramatically reduced chondrocyte viability, however, this was prevented by the addition of PRP, which also increased cell proliferation. In the in vivo study, 32 racehorses with metacarpophalangeal (MCP) joint OA were separated into two groups: one received only TA, while the other received TA followed by PRP. After one week since the last treatment, both groups demonstrated improved flexion assessments, but by the second week, the TA+PRP group had reduced lameness and flexion scores, showing a longer-term effect. In conclusion, combining PRP with TA could enhance chondrocyte viability and provide a better long-term therapeutic option for treating OA in racehorses. Further trials are required to thoroughly assess this technique's safety and efficacy.

1. Introduction

Osteoarthritis (OA) in horses is a chronic and degenerative condition with clinical manifestations such as synovitis, varying degrees of lameness, and a progressive loss of joint function. Among equine athletes, the metacarpophalangeal (MCP) joint emerges as a commonly affected joint and can develop both traumatic and degenerative lesions [1]. The impact of OA on the MCP joint holds significant implications for lameness, in particular, resulting in substantial losses in training days and economic burden within the Thoroughbred racehorse industry [2].

In equine orthopaedics, the routine intra-articular (IA) administration of corticosteroid as an initial treatment for osteoarthritis is based on the ability of corticosteroids to provide short-term symptomatic relief, provide potent anti-inflammatory, and improve joint mobility, reduce lameness and joint effusion in horses with synovitis and osteoarthritis [3,4]. Among various corticosteroids, triamcinolone acetonide (TA) is the most widely used due to its medium duration of action which has been associated with beneficial effects on articular cartilage [3,5–8]. However, several studies have also identified potential detrimental effects of corticosteroids on articular cartilage composition and morphology [7,9,10]. While these negative effects are now known to be related to the type and dose of corticosteroid used, the frequency of repeated administration and joint loading after injection, it does imply IA corticosteroids should be used judiciously [3,8]. Results of in vitro and in vivo research indicate the use of betamethasone did not show any detrimental effects on articular cartilage, while the methylprednisolone acetonide had deleterious effects. The impact of the TA is still debated as it seems to elicit positive effects in terms of equine cartilage metabolism [11]. Whereas in vitro studies observed that the treatment with TA in cartilage explants was detrimental to cartilage metabolism [6,12]. However, as with all corticosteroids, the potential for unintentional alteration of cartilage metabolism is also present with TA [3].

Developing techniques for intra-articular therapies in equine athletes that increase tissue regeneration is critical, with PRP emerging as a potentially regenerative treatment [13]. PRP therapy is becoming more widely

accepted [14–16]. PRP contains growth factors capable of stimulating tissue regeneration; these factors promote the proliferation and differentiation of chondrocytes and, possess anti-inflammatory properties [17]. Additionally, PRP has been shown to protect chondrocytes from damage caused by various stressors and drugs [18–20]. Moreover, there are growing evidences to support its potential analgesic and anti-inflammatory properties, notably in the treatment of OA. PRP injections have been found to reduce pro-inflammatory cytokines and increase anti-inflammatory substances in the joint environment; in addition, this action helps to reduce the overall inflammatory response, which contributes to clinical signs relief in conditions of OA [14,15]. PRP is seen as a cost-effective and low-risk therapeutic option that uses the recipient own biological material to reduce the chance of adverse effects [16]. Several in vitro studies on chondrocytes and tenocytes have shown that corticosteroids have negative effects, while the addition of PRP to these medications significantly reduces cytotoxicity by modulating apoptosis and promoting cell proliferation [19-21]. The combination of TA and growth hormones also showed promise in improving anabolic metabolism in the articular cartilage [22]. Human clinical research examined in the literature reveals that PRP can fill cartilage defects, promote cartilage repair, alleviate OA symptoms, and improve joint function, all while maintaining an acceptable safety profile [23]. To the authors' knowledge, very limited information exists regarding the impact of TA and PRP in equine orthopedics. With the extensive use of corticosteroid injections in equine practice, it is critical to fully understand their effects on the targeted tissues. While these injections have well-documented advantages, their related side effects highlight the need for new approaches to long-term joint treatment that try to mitigate potential negative consequences [24].

The aim of this study was to determine how the combination of TA and PRP might improve the clinical signs of MCP joint OA in racehorses. Authors hypothesized that the use of PRP after a single dose of TA may potentially improves the clinical signs of MCP joint OA longer than injection of TA alone. Synovial biomarkers were not investigated in the present study.

2. Materials and Methods

2.1. In vitro study

2.1.1. Primary cultures of equine chondrocytes

The in vitro study was performed using chondrocytes isolated from metacarpo/metatarsophalangeal joints of 6 Thoroughbred horses 4.5 ± 1.3 years old submitted to euthanasia for reasons unrelated to this study and the quality of the biological material was not compromised by their clinical condition. The articular cartilage was harvested post-mortem from the weight-bearing surfaces of the metacarpo/metatarsophalangeal joints, according to Mancini et al. (2017) [25]. Equine tissues were used in accordance with the guidelines of the Animal Care and Use Committee of Perugia University. All the articular surfaces were exposed by making a careful incision around the metacarpo/metatarsophalangeal joint with a sterile scalpel and freeing it from the surrounding tissues. After, macroscopic examination, the cartilage tissues showing structural integrity, consisting in no visible damage, such as tears, cracks, or breaks, and no signs of degenerative changes were collected using scalpel, washed three times in Dulbecco’s phosphate-buffered saline (PBS) without Ca2+ and

Mg2+, containing penicillin (100 U/mL), streptomycin (100 mg/mL), and amphotericin B (250 µg/mL) (EuroClone, Milan, Italy) and then minced. The minced cartilage was digested with 2.5% of trypsin (Sigma Aldrich, Milan, Italy) at 37°C for 10 min and then with 2mg/ml of collagenase (Sigma Aldrich, Milan, Italy) for 16 hours at 37°C. Cells were then collected by using cell strainer 70 □m (EuroClone, Milan, Italy), washed and placed in the culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin in a humidified 5% CO2 atmosphere at 37°C. The medium was changed every 48h, until cells were split at 90% of confluence. All experiments were conducted using cells at two passages of subculture to minimize alterations in phenotypic drift associated with increased subculturing.

2.1.2. PRP preparation

PRP was prepared from whole blood using the double centrifuge method reported by Tognoloni et al. (2023) [26]. Blood was collected from two healthy horses by jugular venipuncture in acid citrate-dextrose (ACD) solution. Blood underwent two centrifugation steps, the first at 200× g for 20 min at 25◦C and the second at 1800× g for 10 min at 25◦C. The platelet pellet was then re-suspended in a 1 ml volume of platelet-poor plasma to obtain a final platelet concentration of 1×10^6 platelets/µL; platelets counts were determined with a hemocytometer (EosBIO, Cervarese Santa Croce, Italy). The leukocyte concentration in the PRP preparations was notably low: 0.421 ± 0.1 x 10^3/µL in the *in vitro* study and 0.39 ± 0.29 x 10^3/µL in the *in vivo* study.

2.1.3. Cell viability analysis

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay based on the conversion of MTT into a purple-colored formazan products by viable cells. Briefly, cells at density of $10x10^3$ cells/well were cultured in 96-well plates for 24 hours in medium supplemented with 10% FBS. After a washing step in PBS, chondrocyte cells were exposed to 0.25, 0.5, 1, 2 and 4 mg/ml TA (Kenacort, Bristol Mayers Squibb) for 48 hours in presence or in absence of PRP. Control cells were cultured in complete medium alone. After treatments, medium MTT solution (0.5 mg/mL) was added. After 2 hours of incubation, the reaction was stopped by adding DMSO which acts also for solubilizing formazan crystals. The absorbance was measured at 570 nm using a multiplate spectrophotometer (Infinite® 200 Pro-Tecan). The experiments, conducted in triplicate, yielded mean values and standard deviation (SD) from four independent trials. Viability was expressed a percentage of ratio between optical density (OD) of treated cells and OD of control cells.

2.2. In vivo study

2.2.1. Cases Selection

Patients included in this study were stabled at the Jockey Club of Turkey, Ankara Hippodrome, Turkey. The inclusion criteria were Thoroughbred racehorses actively in race and training, age between 2 and 5 years-old and

at least 2 of the following criteria: unilateral or bilateral MCP joint effusion, pain on passive flexion of the MCP joint, lameness localized to the MCP joint by clinical examination with or without intra-articular diagnostic analgesia. Additionally, radiographic findings consisting with OA of the MCP joint were part of the inclusion criteria. Horses were evaluated by two equine veterinarians, each with over 15 years of experience in the racing industry. All horses underwent a clinical and radiographic examination of the MCP joints. The static examination consisted in the evaluation of joint effusion and the pain at passive flexion. In addition, the evaluation of painful response to passive flexion and joint effusion were graded using a four-point scale ranging from none to severe (0=none, 1=mild, 2=moderate, 3=severe). The dynamic examination included observing the horses trot in a straight line on hard surfaces. Lameness was graded from 0 to 5 using the modified AAEP grading scale [27]. The radiographic examination varied among the cases; however, at least the latero-medial, dorso15°proximal-palmarodistal, dorso45°proximolateral-palmarodistomedial oblique, dorso45°proximomedial-palmarodistolateral oblique, flexed lateromedial and flexed dorsopalmar projections of the MCP joint were available [28]. Radiographs were assessed by the jockey club veterinarians and the presence of the following radiographic findings were recorded: periarticular osteophytes, capsular enthesophytes, subchondral bone sclerosis/lysis of the proximal phalanx and/or the metacarpal condyle and loss of joint space [28]. Exclusion criteria were horses with bilateral lameness and lameness graded as 4 and 5 on the AAEP grading scale, any type of fracture of the proximal phalanx and of the distal condyle of the metacarpus and any horse that was treated with any IA injection or other systematic anti-inflammatory therapy within 4 weeks before the inclusion in the study.

2.2.2. Treatments

The horses included in the study were randomly divided into two groups. The randomization was performed with Coin-flip randomization technique. The group TA received one single intra-articular injection of 4 mg (2 mg/ml) of TA (Sinakort-A, Ibrahim Etem) in the affected MCP joint. The group TA+PRP received one single intra-articular injection of 4 mg (2 mg/ml) of TA (Sinakort-A, Ibrahim Etem) followed by one single intra-articular PRP injection one week later. Each joint received 1 ml of a platelet concentration of 1×10^6 PLT/μL. All horses returned progressively to full training within 2-4 weeks following the last treatment. They underwent 24 hours of box rest and avoided high speed exercise for at least 1 week. Follow-up clinical examinations were performed by the same clinicians for all time points. Effusion score and passive flexion score, and lameness evaluations were recorded at 1 week (T1) and 2 weeks (T2) after last treatment. In addition, the clinical outcome was evaluated recording the weeks between T2 and the return of the horse to the Jockey Club Hospital with the same complaint (i.e.: MCP joint disease) including joint effusion, pain at flexion or lameness. Adverse effects after treatments were monitored to check for any occurrence.

2.3. Statistical analyses

The statistical analyses were performed using the statistical software JASP (version 0.18.1, Jasp Team, Amsterdam, The Nederland). The quantitative data are expressed as mean ± SD or median and range, as

appropriate; nominal data are expressed as prevalence and percentage. For numerical data (age, weeks of the clinical outcome), homoscedasticity of the variables was tested for normality using Shapiro-Wilk test and homogenicity of the variance with Levene test. Cell viability of the in vitro study was evaluated using one-way analysis of variance (ANOVA) and *post-hoc* Tukey test for multiple comparisons. For the in vivo study, descriptive statistics was applied for age, sex, limb affected (right forelimb or left forelimb), lameness score (baseline, T1, T2), effusion score (baseline, T1, T2), flexion score (baseline, T1, T2) and clinical outcome (weeks). Differences in age and weeks of the clinical outcome between group TA and group TA+PRP were tested using unpaired Student't test or Mann-Whitney U test, as appropriate. Differences in sex and limb affected between the two groups were tested with Chi-squared test. Two-way Kruskal-Wallis test with Dunn's *post-hoc* correction for multiple comparisons were used to test for differences between the groups and time points (baseline, T1 and T2) for effusion score, passive flexion score and lameness score. A p-value < 0.05 was considered significant. A p-values less than 0.05 were considered as significant.

3. Results

3.1 In vitro study

3.1.1. Effect of PRP on chondrocyte culture

Cell viability assessed by (MTT) test after 24 hours of treatment with different concentrations of TA with or without PRP are shown in figure 1. Exclusive use of TA led to a roughly 50% decrease in cell viability, beginning at the minimum dosage of 0.25 mg/ml ($p < 0.05$) compared to the control and maintaining a gradual decline as the concentration of TA increased to 4 mg/ml dosage vs. control ($p < 0.01$). In contrast, the combined use of TA with PRP demonstrated a protective effect on cellular viability, reaching average values around 130-140% for all the dosages and showing marked differences compared to TA treatment.

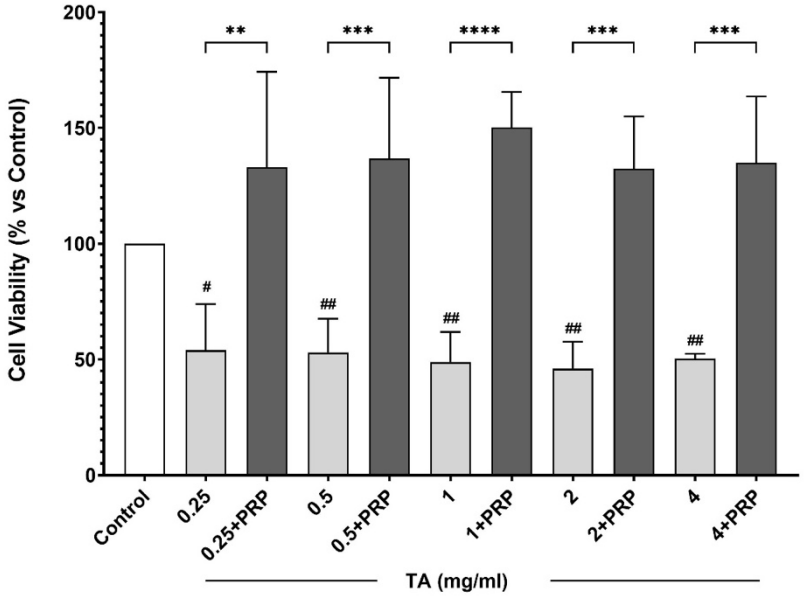

Figure 1. Cell viability after the addition of platelet-rich plasma (PRP) to the triamcinolone acetonide (TA) after 24 hours at the treatment dose 0, 0.25, 0.50, 1.0, 2.0 and 4.0 mg/ml, as compared with triamcinolone acetonide alone. Data are the mean ± SD of four independent experiments performed in triplicates. **p<0.01; ***p<0.001; ****p<0.0001; # p<0.05; # #p<0.001 vs. control (CTRL)

3.2 In Vivo study

A total number of 53 MCP joint from 32 horses were included in this study, ranging between 2 and 5 years with a mean ± SD age of 2.7 ± 0.9 years. There were 13 females and 19 intact males. Thirty-one joints (right forelimb = 17; left forelimb = 14) from 18 horses were in group TA and twenty-two joints (right forelimb = 12; left forelimb = 10) from 14 horses were in group TA+PRP. In the group TA, from a total of 18 horses, 13 were bilateral and 5 unilateral affected, and in the group TA+PRP, from a total of 14 horses, 8 were bilateral and 6 unilateral affected. There were no differences in age ($p = 0.06$), sex ($p = 0.33$), limb affected ($p = 0.98$) between the group TA and group TA+PRP.

In group TA, 24 out of 31 MCP joints showed effusion; a total of 29 joints had positive responses to the passive flexion and 6 forelimbs were lame in the TA group. In group TA+PRP, 19 out of 22 MCP joint showed effusion; a total of 21 joint had a positive response to the passive flexion and 8 forelimbs were lame. The details of effusion score, passive flexion score and lameness score at each time point in the two groups are summarized in Table 1.

Table 1. Median and range of effusion score, passive flexion score, lameness score, weeks of the follow-up and frequencies of adverse effects obtained in group TA and group TA+PRP.

Groups	*Baseline*	*T1*	*T2*	*p-value**
	Effusion score (median; range)			
Group TA (n=31)	1; 0 – 3	0; 0 – 1[a]	0; 0 – 2	<.001
Group TA+PRP (n=22)	2; 0 – 3	0; 0 – 1[a]	1; 0 – 1[b]	<.001
p-value#	1.00	1.00	1.00	
	Passive flexion score (median; range)			
Group TA (n=31)	2; 0 – 3	0; 0 – 2[a]	**1; 0 – 2[b,c]**	< 0.03
Group TA+PRP (n=22)	2; 0 – 3	0; 0 – 2[a]	**0; 0 – 1[b]**	<.001
p-value#	1.00	1.00	**<.001**	
	Lameness score (median; range)			
Group TA (n=31)	0; 0 – 2	0; 0 – 1	0; 0 – 1	1.00
Group TA+PRP (n=22)	0; 0 – 3	0; 0 – 1[a]	0; 0 – 1[b]	0.002
p-value#	0.36	1.00	1.00	
	Weeks of follow-up (median; range)			
Group TA (n=31)	**4; 4 – 5**			na
Group TA+PRP (n=22)	**7; 6 – 8**			na
p-value#	**<0.001**			
	Adverse Effects (prevalence; %)			
	No		***Yes***	
Group TA (n=31)	31 (100%)		0 (0%)	
Group TA+PRP (n=22)	22 (100%)		0 (0%)	
p-value#	na			

* p-value of multiple comparison for time; # p-value of the multiple comparison for time. Bold defined significant differences between group TA and group TA+PRP; [a] defined significant difference between T1 and baseline ($p<0.05$); [b] defined significant difference between T2 and baseline ($p<0.05$); [c] defined significant difference between T1 and T2 ($p<0.05$); [d] defined significant difference between T1 of Group TA and T2 of Group TA+PRP ($p<0.05$); na = not applicable.

There were no significant differences in the effusion score between the groups at each time point ($p = 0.77$). For both groups, the effusion score at T1 ($p < 0.001$) was significantly lower than that at baseline; for group TA+PRP, T2 also was significantly lower than that at baseline ($p < 0.001$). There was no significant difference

between T1 and T2 ($p = 0.1$). There were significant differences in the flexion scores between the groups ($p = 0.04$) and the time points ($p < 0.001$). There was a significant reduction in the passive flexion score in both groups between baseline and T1 ($p < 0.001$) and T2 ($p < 0.001$); however, the passive flexion score in the group TA+PRP at T2 was significantly lower compared to that of the group TA ($p<.001$). There were no significant differences in the lameness score between the groups at each time point ($p = 0.73$), but a significant interaction between group*time was found. Only in group TA+PRP, the lameness score significantly decreased at T1 ($p = 0.002$) and T2 ($p = 0.002$), compared to the baseline. Finally, there was a significant difference ($p < 0.001$) in the outcome weeks between the two groups. Horses in which the MCP joint/s was/were treated with TA (group TA) were re-admitted early (median: 4 weeks; range: 4-5 weeks) to the Jockey Club Hospital compared to horses in which the MCP joint/s was/were treated with TA and PRP (group TA+PRP) (median: 7 weeks; range: 6-8 weeks). The causes of re-admission to the hospital are depicted in Figure 2. In the group TA+PRP, the cause of readmission was effusion in 18 (82%) joints, pain on flexion was noted in 4 joints (18%) and lameness was observed in 1 out of 22 horses (5%). In the TA group, the cause of readmission was effusion in 24 (77%) joints, pain on flexion in 14 (45)% and lameness was observed in 6 horses (19%).

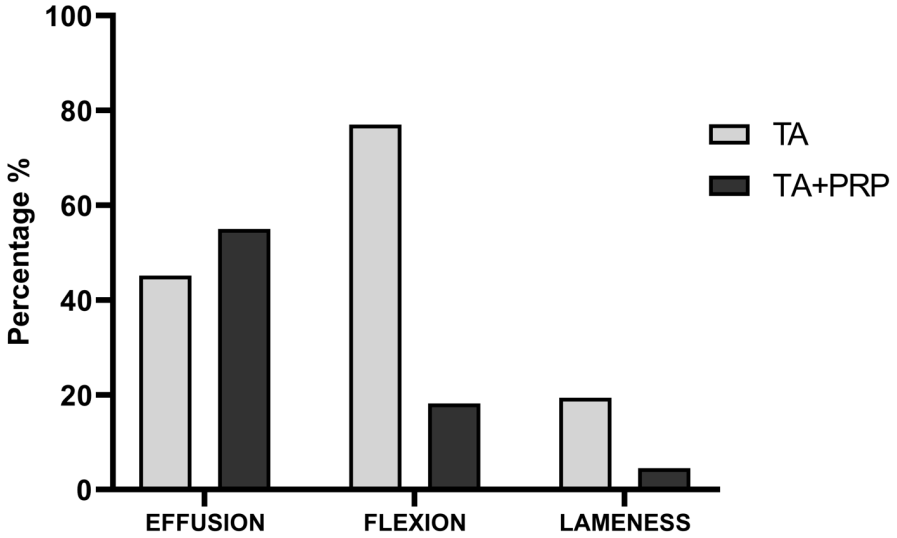

Figure 2. Reasons for re-admission to the hospital following the treatments in the TA and TA+PRP groups. The chart demonstrates the frequency of recurring clinical signs observed during readmission to the hospital, revealing variations in, effusion, flexion and lameness complaints between the TA and TA+PRP groups.

4. Discussion

Intra-articular corticosteroids are often used as a first-line treatment for OA affecting equine athletes because they can improve range of motion, and effusion of the MCP joints and relief pain [3]. However, the use of IA corticosteroid is a questionable issue, because they can result in changes of chondrocyte metabolism, cellular toxicity, mitochondrial dysfunction, reactive oxygen species increase and cell death [29,30]. In this study, the effect of combining TA and PRP has been investigated and compared to the effects of TA alone, *in vivo* and *in vitro*. Firstly, the potential detrimental effects of TA on equine chondrocytes have been demonstrated in vitro. Indeed, we observed a significant decrease in the viability of equine chondrocytes induced by TA (0.25mg/ml-4 mg/ml), consistent with those demonstrated by other author exposing human chondrocytes to similar [31] and higher concentrations [32,33]. Similar effects have been also observed rabbit chondrocytes [10,34,35] and canine chondrocytes [29,36].

In contrast, the combined use of TA and PRP led to an increase in cell viability, indicating a clear protective effect of PRP on the cytotoxicity exerted by TA in vitro. These findings support the results obtained on human chondrocytes exposed to other corticosteroids [19,21]. Additionally, several studies have reported the cytoprotective effects of PRP against the toxicity of various drugs on different fibroblast cell types, including chondrocytes [23,37–40]. For example, PRP increased the cell viability and decreased apoptosis of human rotator cuff tear cells exposed to TA [18,41]. By speculating on our results, it is plausible to hypothesize that PRP can protect chondrocytes by counteracting the pro-oxidant effects of TA. Indeed, it has been reported that TA significantly increases the levels of oxidized glutathione, leading to oxidative stress in human chondrocytes [42]. In contrast, PRP appears to enhance the antioxidant cellular response via the NRF2 pathway [26,44].

The results of our in vivo study supported the role of the IA administration of TA+ PRP as able to improve the clinical signs in horses with positive flexion test of the MCP joint and/or lameness due to its anti-inflammatory activity [45], as well as the synergistic effect of PRP when combined with other drugs [45]. Moreover, these effects could be also related to the ability of PRP in promoting chondrocyte proliferation and cartilage matrix secretion and in stimulating cartilage repair [46,47]. It has been reported that PRP exerts beneficial effects on joint cartilage, synovium, tendon and overall healing processes [48–50]. Specifically, anabolic effects of PRP have been observed in porcine chondrocyte cultures, highlighting its regenerative potential in cartilage tissue [51,52].

Synovitis, trauma or insult can lead to activation of the mechanoreceptors [53,54] which in turn can stimulate an inflammatory response with the release of pro-inflammatory cytokines and degradative enzymes (IL-1B and TNF-alpha and MMPs). This further increase the amount of joint swelling and further activating nociceptors and perpetuating pain. All these events result additional osteochondral damage and cartilage degeneration in OA [54]. Acute synovitis is considered the most common problem in equine high-motion joint, as the MCP joint is, contributing to the degradative mechanism of the articular cartilage [55]. For these reasons, it was not surprising that the effusion score in this study group improved after both treatments. Both groups received TA, which is a potent anti-inflammatory drug inhibiting the inflammatory process at all levels [56].

The effusion score reduced at T1 compared to the baseline and remained significantly lower at T2 in group TA+PRP. In this study, the use of PRP after the TA did not result in a significant reduction of the synovitis compared to group TA. On the other hand, decreased range of motion and pain on passive flexion, with or without synovial effusion, are thought to indicate an essential underlying condition, which is likely OA [57].

A painful response to passive flexion of the joint and lameness are well known clinical signs of pain in horses and human being [58,59]. In our clinical study, a significant reduction in the passive flexion score was demonstrated in both groups. However, in contrast to group TA, the group TA+PRP maintained a lower flexion score compared to the baseline, for a longer time. The group TA+PRP had a longer effect in maintaining a lower flexion scores after 2 weeks (T2), while group TA returned towards the baseline score at T2. Indirectly, this difference demonstrated a shorter analgesic effect in horses treated only with TA compared to that treated by TA followed by PRP. The lameness score was another clinical variable considered. Similarly, despite the low number of horses presenting lameness, a significant decrease in the lameness score was recorded in group TA+PRP at T1 and T2, which was not the case of group TA. These findings may explain the favorable effect of TA+PRP group on lameness score as PRP products were shown to be effective in relieving clinical signs of OA [59,60].

In support to the beneficial use of PRP after TA, authors would highlight those horses treated with TA alone returned to the hospital in a shorter time (4.4 weeks on average), compared with horses treated with TA and PRP (7.1 weeks on average). Regarding T1 and T2 differences between the groups, it is crucial to note that in the TA group, the assessments at T1 and T2 evaluated the effects of TA alone. In contrast, in the TA + PRP group, T1 and T2 assessments were spaced based on the timing of both TA and PRP administrations. Therefore, the difference in the follow-up weeks may be affected by a slightly different treatment protocols between the two groups. However, this may be attributed to the therapeutic synergy between PRP and TA, offering a promising approach to reducing the adverse effects associated with corticosteroid use in joint treatments [21,61]. The short duration of improvement observed in this study may be related to several contributing factors influencing the recurrence of clinical signs. In the literature, the most common doses of TA administered by equine practitioners range from 5 to 10 mg, with a therapeutic duration rarely exceeding 4 to 6 weeks [62-65]. In our study, a dose of 4 mg per joint was used, and the readmission of the horses in the TA group at 4.4-weeks aligns with previous studies [62-65]. Other factors include training strategies, the overall management of the horses, as well as the experience and quality of the staff and riders. Another important point might be the training track surfaces. Thus, horses may be predisposed to recurrence of clinical signs if trained at high intensity on surfaces to which they are not accustomed [65,66]. Additionally, horses with higher athletic demands may experience greater stress on their joints, leading to faster recurrence of issues despite treatment.

Moreover, the fact that the flexion score of the horses in the group TA+PRP did not worse in the weeks following the treatment and that the owners of these horses did not complain in training showed that PRP application had a positive effect on pain and longer suppression of the clinical signs. In human studies, it has been demonstrated that PRP improves joint and tissue function, relieves pain and results in favorable clinical

517 outcomes [67,68]. However, there are many studies have shown that PRP is a more suitable method for use in
518 humans than horses. Interestingly, in our study, no adverse effects of IA administration of PRP were recorded,
519 even though it has been reported that this treatment may induce a reaction or transient synovitis [13]. The side
520 effects of the IA administration of PRP are likely to involve the preparation and standardization protocols of
521 PRP and in particular to the leucocyte concentration [69,70]. Overall, the authors suggest that the PRP has a
522 safe profile when used IA and might have a pivotal role in the disease progression due to its ability to protect
523 chondrocytes by reducing the adverse effect of TA.
524 There are some limitations in this study. The first limitation is lack of the use of diagnostic analgesia to
525 determine if the presence of lameness in some horses was due to pain in the MCP joint. From the clinical
526 perspective, it is fair to suggest that the distal limb flexion test is sensitive to examining the MCP joint, but it
527 may be less significant for tissues distal to the joint. Two researchers suggest that the MCP joint is the primary
528 contributor to a positive flexion test [71,72], but clinical signs and radiographic evidence should also be
529 evaluated for a thorough evaluation. For this reason, to avoid bias, more than one criteria was used as inclusion
530 criteria in this study group. The second limitation is that synovial samples were not analysed to assess changes
531 in synovial biomarkers. As a result, this hypothesis remained to be validated experimentally and is the aim of
532 our next study. Third limitation is that in vitro, PRP was added concomitantly with TA-treated chondrocytes,
533 whereas in vivo, PRP was administered one week after TA. This timing also may have influenced the clinical
534 results. However, the in vivo study was conducted on horses with OA symptoms, while the in vitro study used
535 healthy chondrocytes treated with TA. Additionally, tracking the effects of TA for one week on chondrocytes
536 in vitro is challenging, as we observed cytotoxicity even at lower doses. Administering TA prior to PRP
537 allowed us to verify if PRP treatment is more effective in joints with reduced inflammation.
538 Finally, horses with similar pathologies may exhibit individual response to treatment. The therapeutic effects
539 may also be influenced by the horses' working discipline and post-treatment exercise protocols.

540 **5. Conclusions**

541 There are many research going on in the literature use of corticosteroids and PRP, yet in equids use of combined
542 TA and PRP has limited study. The studies evaluating the cytotoxic effects of TA on equine chondrocytes are
543 also limited and controversial. This study is the first to investigate in vitro the potential harmful effects of this
544 corticosteroid on equine cartilage cells and the possible protective effect of PRP when administered together
545 with this drug. The results of the in vivo study suggest a promising strategy to alleviate any adverse effect on
546 chondrocyte viability after the corticosteroid administration, highlighting the potential for mid-term pain relief
547 and reducing lameness through a strategically timed PRP injection. Indirectly, these results could indicate that
548 PRP could elicit a proliferative effect on chondrocytes, even though low, despite the presence of TA. Further
549 clinical trials are crucial for a comprehensive evaluation of the therapeutic potential and safety profile
550 associated with the integration of PRP with triamcinolone in the treatment of osteoarthritis in equine athletes.
551 Multiple PRP injections are likely to lead to better clinical outcome than a single injections. Comparing
552 multiple PRP injections after a single dose of TA versus a single dose of TA alone may reveal changes in

553 outcome [73–75]. However, this need further investigations and also might help a better understanding of the
554 beneficial effect of PRP injections in the long term.

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556 **References**

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SYNOVIAL BIOMARKER CHANGES IN OSTEOARTHRITIC HORSES TREATED WITH PLATELET-RICH PLASMA AND TRIAMCINOLONE ACETONIDE.

Abstract

Joint disease, particularly osteoarthritis (OA), is a leading cause of lameness and performance decline in equine athletes. This study aimed to assess the effects of intra-articular injections of platelet-rich plasma (PRP) alone and in combination with triamcinolone acetonide (TA) on the levels of specific OA biomarkers in the synovial fluid (SF) of the fetlock joint. Fourteen horses presenting with unilateral lameness, joint effusion, and pain on flexion were enrolled and divided into two treatment groups: PRP-alone (n=7) and TA+PRP (n=7). The PRP-alone group received a single intra-articular injection of PRP, while the TA+PRP group received a PRP injection one week following TA administration. Synovial fluid samples were collected before and after treatment to measure concentrations of eight biomarkers: fibrinogen, haptoglobin, total protein, MMP-2, MMP-9, IL-1β, IL-6, and HIF-1α. Within the TA+PRP group, significant differences were observed in fibrinogen, haptoglobin, and total protein levels at each time point. TA injection initially decreased these biomarkers, but subsequent PRP administration restored their levels, except for total protein, which continued to decrease gradually. After PRP injection, a significant difference in total protein concentration was observed between the two groups, with a gradual decline in total protein levels in the TA+PRP group. These findings suggest that PRP alone does not significantly alter biomarker expression, supporting its safety as an intra-articular treatment without corticosteroid preconditioning. However, the combination of TA and PRP appeared to have a synergistic effect, potentially promoting controlled inflammation within the joint. Further studies with larger sample sizes and additional time points are recommended to better understand the efficacy of these treatments on synovial biomarkers in OA management.

1. Introduction

Lameness from osteoarthritis (OA) is a leading cause of reduced performance and early retirement in horses, causing significant economic losses due to its high prevalence in racehorses [1–3].The diagnosis of joint disease is routinely based on physical lameness examination, diagnostic imaging methods and diagnostic arthroscopy and synovial fluid (SF) analyses[4]. Analysis of synovial fluid can aid in finding candidate biomarkers by revealing abnormal metabolic processes to reduce the pathophysiological mechanism of OA, monitor changes in joint tissue metabolism during the progression of OA and also monitor the response to therapy and observe curative effects [5,6]. SF is an alternative approach for determining the effects of therapeutic agents in vivo [7]. However, still have to be fully validated to determine how alterations in biomarkers specifically relate to structural or functional outcomes [8]. Analyses of SF in case of joint disease, show increasing concentrations of proteins compared to physiological conditions [9]. Joint damage induces

834 the production of cytokines such as-1β, IL-6 which also trigger the acute phase response and production of
835 acute-phase proteins (APPs) [10,11].

836 IL-6 production is triggered by the release of IL-1β and is a secondary mediator associated with inflammatory
837 cells, IL-6 is believed to be one of the major factors in joint destruction, being a pleiotropic proinflammatory
838 cytokine which is markedly upregulated at times of tissue inflammation.[12] . The APPs are believed to play
839 major roles in several aspects of the systemic reaction to inflammation and overall regulation of different stages
840 of inflammation [13]. Moderate positive APPs, such as haptoglobin (HP) and fibrinogen (FIB), also play crucial
841 roles, though their increase during inflammation tends to be less pronounced compared to SAA [14]. HP, in
842 particular, has been investigated in various body fluids, including peritoneal fluid in horses with abdominal
843 pain and in serum during experimentally induced arthritis [15,16]. Recent studies have pointed to the potential
844 role of Hp as a marker of inflammation in equine synovial fluid, supporting its relevance in assessing
845 inflammatory conditions in horses [17]. Fibrinogen, in contrast, an acute phase protein that is always present
846 in blood samples, increases by 1-2 times with an inflammatory stimulus, and whose changes lag behind the
847 resolution of inflammation or infection [18]. It has been a long time since clinical treatments of OA focused on
848 improving joint pain symptoms rather than on the decline of the disease progression [19]. Despite the use of
849 many medications, both locally and systemically as well as topically, the management and treatment of equine
850 osteoarthritis remains a challenge [20].

851 Currently, the mainstay of intra-articular therapy focuses on alleviating the signs of disease through temporary
852 inflammation reduction, often achieved via corticosteroid (CS) injections [21]. Although corticosteroids have
853 a strong analgesic effect, they are also associated with multiple adverse effect such as promote cartilage
854 degradation, and inhibitory effects on collagen synthesis by chondrocytes [22]. For these reasons, new
855 approaches to the treatment of OA in horses are necessary. Cell therapy, such as platelet-rich plasma, has lately
856 emerged as a promising treatment option for various disorders, including osteoarthritis [23], and the field of
857 equine regenerative medicine is drawing increasing attention in the scientific community for its treatment
858 strategies for joint pathologies [24].

859 The aim of this study is to determine the effect of intra-articular injection of platelet-rich plasma (PRP) alone
860 and combined with triamcinolone acetonide (TA) into the osteoarthritic (OA) fetlock on the changes in the
861 levels of specific OA biomarkers synovial fluid (SF). For this purpose, eight biomarkers (Fibrinogen,
862 Haptoglobin, MMP-2, MMP-9, Total Protein, IL-1β, IL-6, and HIF-1α) were evaluated. We hypothesized, that
863 both PRP and TA+PRP treatments suppress inflammation and cartilage degradation.

864

865 **2. Material and Methods**

866 *2.1. Study Design*

867 14 thoroughbred racehorses were included in this study, ranging between 2 and 5 years, with a mean ± SD age
868 of 3.2 ± 1.3 years. The horses were evaluated by two equine veterinarians, each with over 15 years of

experience in the racing industry working for the Racehorse Hospital at the Jockey Club of Turkey. Cases were defined as horses having the following inclusion criteria; clinical evidence of osteoarthritis including effusion, pain on flexion and lameness localised to one limb/one fetlock joint only. Lameness, 2 to 3 grade was included in this study.

Exclusion criteria were any fracture and history of infection associated with the joint, and any horse that was treated with any IA injection or other systematic anti-inflammatory therapy within 4 weeks before the inclusion in the study. Passive flexion test and joint effusion was graded using a four-point scale ranging from normal to severe (0=none, 1=mild, 2=moderate, 3=severe). Lameness was assessed when the horses trot in a straight line on hard surfaces with digital flexion test (fetlock flexion test) and graded from 0 to 5 using the modified AAEP grading scale.

Radiography of the fetlock included five standard projections, and additional projections were taken in different cases. The standard radiographic views obtained were the following: lateromedial, dorso15°proximal-palmarodistal, dorso45°proximolateral-palmarodistomedial oblique, dorso45°proximomedial-palmarodistolateral oblique, flexed lateromedial and flexed dorsopalmar projections. The radiographs assessed including at least one of the following findings; periarticular osteophytes, capsular enthesophytes, loss of joint space, subchondral bone sclerosis/lysis of the proximal phalanx and/or the metacarpal condyle were assessed by the veterinary surgeons of the jockey club.

Intra-articular injections were performed by passing a needle percutaneously into the joint, and synovial fluids were aspirated prior to the injection of PRP or TA+PRP for each group, as shown in Figure 1.

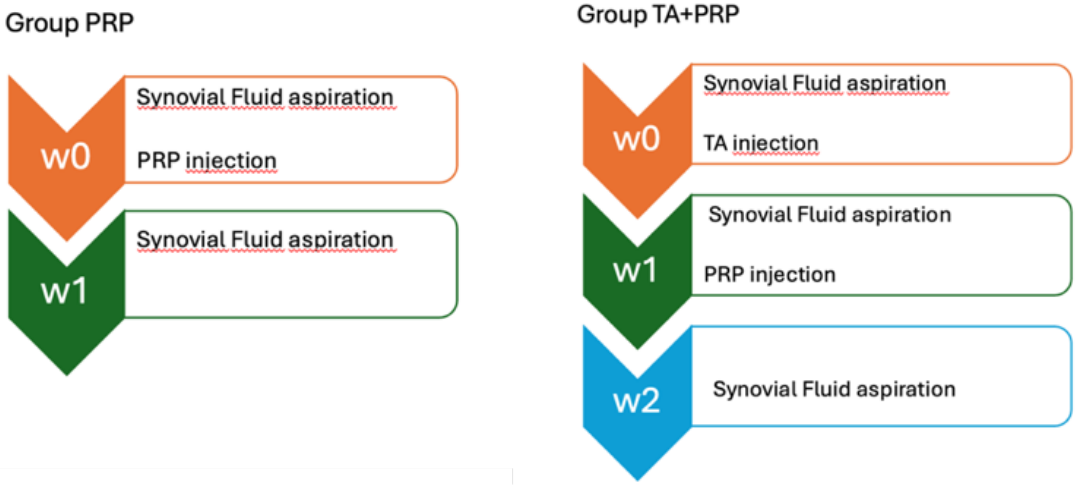

Figure 1. Timeline for PRP and TA+PRP treatment groups in synovial fluid aspiration and injection.

905 *2.2. Treatment*

906 The selected horses were randomly divided into two groups. The group PRP received one single intra-articular
907 injection of PRP in the affected fetlock joint. The group TA+PRP received one intra-articular injection of 4
908 mg/2ml of TA (SINAKORT-A, ml/40 mg), followed by a single intra-articular PRP injection one week later in
909 the affected joint. Each joint received 1 mL of a platelet concentration of 1×10^6 PLT/µL. After receiving the
910 intra-articular injections, all horses were instructed to have 24 hours of box rest. Following this rest period,
911 they were to engage in walking and trotting for a total of 20 minutes during the treatment period for both
912 groups. If the horses did not exhibit signs of lameness, they gradually progressed to the previous training
913 regimens.

914 *2.3. Synovial Fluid Sampling*

915 All fetlock joints were aseptically prepared. Synovial fluid samples were aspirated from the affected fetlock
916 joint using a 21 G 0.80 x 40 mm needle with lateral palmar approach. SF sampling was performed only as pre
917 and post-treatment for each group (PRP and TA+PRP) as shown in Figure 1. Fluid was centrifuged at 3200
918 rpm for 5 min at 5–10°C. The supernatant was aspirated, and the pellet was discarded. The supernatant was
919 stored at −80^{o} C until future analysis.

920 *2.4. Platelet-Rich Plasma preparation*

921 PRP was prepared from whole blood using the double centrifuge method reported by Tognoloni et al 2023.
922 After performing antisepsis, 85 ml of blood was collected from the horse by venipuncture of the jugular vein
923 using a 14-gauge (G), 2.10 mm x 45 mm orange catheter. The collected blood was drawn into two sterile 50
924 ml syringes and then transferred into 10 Vacutainer® tubes containing acid citrate-dextrose (ACD) solution.
925 PLT pellet was then resuspended in a volume of platelet-poor plasma to obtain a final platelet concentration
926 of 1×10^6 PLT/µL; PLT, counts were determined with a hemacytometer. The leukocyte concentration in the
927 PRP preparations was notably low: 0.38 ± 0.16 x 10^3/µL.

928 *2.5. APPs measurement*

929 APP concentrations in synovial fluid samples were measured using the Equinostic EVA1 diode array
930 spectrophotometer after being thawed at room temperature (RT) prior to sampling in all samples. Fibrinogen
931 and haptoglobin concentrations were determined via a turbidimetric ‘latex-enhanced’ method and an
932 immunoturbidometric method, respectively. The measurement of synovial fibrinogen, and haptoglobin
933 concentrations as well as the calibration of the equipment, were all performed according to the manufacturer’s
934 guidelines.

935

936 *2.6. Gelatin zymography for MMP-2 and MMP-9 assays*

937

938 Gelatin zymography analysis was performed to evaluate the MMPs present in the synovial fluid. The test is
939 based on the ability of MMPs to degrade the gelatin contained in the substrate in which they are incubated and
940 separated, as MMP-2 and MMP-9 are two gelatinases. The zymography was conducted under non-reducing
941 conditions; the samples were diluted in Loading Buffer containing 0.125 M Tris-HCl pH 6.8, 4% glycerol, 1%
942 SDS, and 0.125M Bromophenol Blue. DTT was not added, and the samples were incubated for 10 minutes at
943 room temperature. Following incubation, the samples were loaded onto the gel, and electrophoresis was
944 performed. After that, a triple wash was performed, each for 30 minutes, in a 2.5% Triton X-100 solution to
945 remove the SDS and allow the renaturation of the MMPs. Subsequently, the gel was transferred into the assay
946 buffer consisting of 50mM Tris-HCl (pH 7.5), 5mM $CaCl_2$, 0.2M NaCl, and 0.02% Brij-35, and incubated at
947 37°C for 20 hours. After the 20-hour period, the gel was first stained with 0.25% Coomassie Brilliant Blue R-
948 250 (Sigma-Aldrich) for 1 hour and then destained for 30 minutes with a destaining solution composed of 30%
949 methanol, 10% acetic acid, and bidistilled water. Proteolysis was observed through the formation of a white
950 area on a blue background. Images were acquired every 5, 10, 15 and 30 minutes of destaining using the GS-
951 800 Imaging System Scanner (Bio-Rad). The intensity of the bands related to the gelatinase activity was
952 quantified through densitometric analysis using the software Quantity One 4.5.0 (Bio-Rad).

953

954 *2.7. Protein quantification*

955

956 The total protein concentration in synovial fluid was measured using the Bradford assay (Bradford Protein
957 Assay Kit). It's based on the Coomassie Brilliant Blue G-250 dye binding to proteins, which causes a colour
958 change from reddish-brown to blue. The absorbance and 595 nm proportional to the protein concentration was
959 measured by using a plate spectrophotometer (Infinite® 200 Pro-Tecan, Mennedorf, Switzerland). BSA was
960 used as an external standard.

961 *2.8. IL-1ß, IL-6 and HIF-1α measuremet*

962 Synovial fluid samples were thawed at room temperature (RT) prior to ELISA. Commercially available horse
963 IL-1ß, IL-6 and HIF-1α ELISA kits were used for the in vitro quantitative determination in synovial fluid. All
964 three kits were sandwich enzyme-linked immunosorbent assay (sELISA) and were processed according to the
965 manufacturer´s instructions. The ELISA kits for the determination of IL-1ß, IL-6 and HIF-1α were validated
966 for use on equine SF using standard parallel and serial dilutions. Validation assays generated consistent results
967 in intra-assay and inter-assay comparisons. The intra-assay coefficient of variation (CV) was < 8% and the
968 inter-assay coefficient was <10% for IL-1ß, IL-6 and HIF-1α. Each sample was measured in duplicate.

969

2.9. Data Analyses

The study involved two groups of racehorses: the PRP group and the TA+PRP group. Three statistical analyses were conducted to assess treatment effects on biomarkers, according to the nature of the data. The quantitative data are expressed as mean ± SD or median and range as appropriate. The Shapiro-Wilk test was performed to check the normality of the data. The data resulted in a normal distribution was assessed for homoscedasticity and variance homogeneity by using Levene's test. Data with deviation from the normal distribution was statistically tested using a non-parametric test (i.e., Kruskal-Wallis). ANOVA was used to compare pre- and post-treatment levels of synovial biomarkers between PRP group and TA+PRP group. And also used for analyzing pre- and post-treatment changes in synovial biomarkers within the group TA+PRP. The post-PRP treatments were compared between the PRP and TA+PRP groups using independent sample T-tests or Mann-Whitney U tests, depending on data distribution and variance homogeneity. All analyses were performed using JASP software (version 0.18.1), and a p-value < 0.05 was considered statistically significant for all tests.

3. Results

All synovial fluid samples were collected within 1 year from 14 horses (n = 14). A total of 14 MCPJs from 14 thoroughbred racehorses were included in this study, ranging between 2 and 5 years, with a mean ± SD age of 3.2 ± 1.3 years. There were 7 females and 7 males. Seven joints (right forelimb = 2; left forelimb = 5) from 7 horses were in group PRP, and 7 (right forelimb = 3; left forelimb = 4) from 7 horses were in group TA+PRP. There were no differences in age (p = 0.53), sex (p = 0.28), or limb affected (p = 1.0) between group PRP and group TA+PRP. Of the 14 cases, eight horses (57%) were lame in the left forelimb, and six horses (42%) were lame in the right forelimb. In total, 11 of the horses (%79) were grade 2/5 lame, and 3 horses (%21) were grade 3/5 lame.

3.1. Fibrinogen and Haptoglobin Concentration

Fibrinogen levels in the PRP group remained constant before (1.2±2.1 g/l)) and after the treatment (1.1±0.7 g/l) without showing significant changes. One-way ANOVA revealed a significant change in fibrinogen levels over time in the TA+PRP group (Figure 2A). Pairwise comparison showed initially a significant decrease in fibrinogen from pre-TA treatment (0.6±0.3 g/l) to post-TA treatment (0.3±0.2 g/l) (p-value = 0.04) and a subsequent increase post-TA+PRP administration (0.9±0.2 g/l) (post-TA vs. post-TA+PRP p-value = 0.002). Levels of fibrinogen post-TA+PRP administration were also significantly higher when compared to the pre-TA levels (p-value = 0.04). Mann-Whitney test indicated significant differences when comparing fibrinogen levels between the PRP and TA+PRP groups after the administration of PRP and TA, respectively, with the PRP group displaying higher fibrinogen levels (post-PRP vs. post-TA p-value = 0.03) as shown in Figure 2D.

Haptoglobin levels in the PRP group did not significantly differ before (2±2.7 g/l) and after the treatment (1±0.7 g/l). ANOVA test, however, showed significant changes in the TA+PRP group (Figure 2B): multiple

comparison found haptoglobin levels slightly decreasing (without statistical significance) from before TA treatment (0.8±0.4 g/l) to after TA treatment (0.5±0.2 g/l) and then increasing significantly after PRP administration (1.1±0.5 g/l) (post-TA vs. post-TA+PRP p-value = 0.02), exceeding pre-TA levels (pre-TA vs post-TA+PRP p-value = 0.001). No statistically significant change between groups was found.

3.2. *TP concentration*

For total protein, PRP group did not show significant differences between the pre-PRP (12±8.3 mg/ml) and the post-PRP levels (14.7±6.5 mg/ml). Within the TA+PRP group, concentrations of total protein (TP) were constantly decreasing throughout the duration of the study (Figure 2C), from the pre-TA levels (15.6±7.4 mg/ml) to post-TA (7.3±5.5 mg/ml) and to post-TA+PRP levels (4± 2 mg/ml). Multiple comparison showed statistical significance between the levels of the pre-TA phase and the post-TA+PRP phase (p-value = 0.01). Mann-Whitney test between PRP and TA+PRP groups showed that TP levels in the PRP group post-PRP administration were significantly higher than in TA+PRP group post-TA+PRP administrations (p-value = 0.01) as shown in Figure 2E.

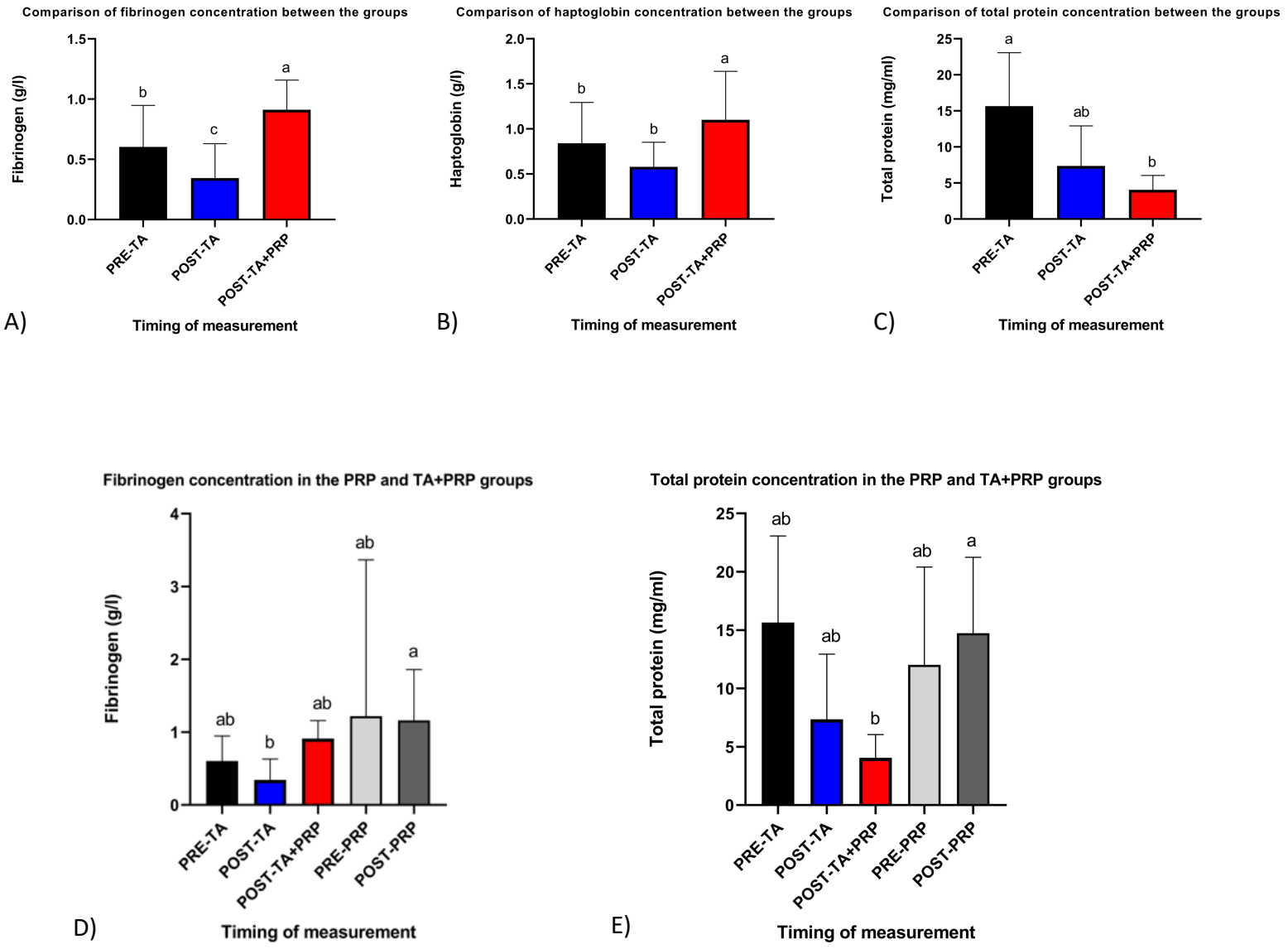

Figure 2. Bar plots showing the mean ± SD of the concentration levels of three different protein biomarkers in SF. Figures 2A, 2B and 2C display the trends of fibrinogen, haptoglobin and total protein in the TA+PRP group at each time point: before TA treatment, after TA treatment and after PRP treatment. Figures 2D and 2E compare fibrinogen and total protein concentration between the TA+PRP group and the PRP group in their different treatment time. Bars with different letters indicate the presence of statistically significant differences (p-value < 0.05).

3.3. MMP-2 and MMP-9 expression

In the statistical tests performed, no significant difference was found within and between the groups for the MMP-2 and MMP-9 biomarkers. The MMP-2 levels detected in the PRP group were 0.8±0.4, pre-PRP and 0.8±0.1, post-PRP, while in the TA+PRP group where 0.6±0.2 pre-TA, 0.9±0.6 post-TA and 0.9±0.3 post-TA+PRP. The MMP-9 levels detected in the PRP group were 0.5±0.1, pre-PRP and 0.4±0.1 post-PRP, while in the TA+PRP group where 0.5±0.6 pre-TA, 0.4±0.2 post-TA and 0.3±0.1 post-TA+PRP.

3.4. Zymography MMP-2 and MMP-9

The zymography analysis demonstrated distinct enzymatic activities for both MMP-2 and MMP-9 in the SF samples (Figure 3). Clear bands were observed at approximately 72 kDa and 92 kDa, corresponding to the pro-forms of MMP-2 and MMP-9, respectively. Additionally, faint bands at around 62 kDa for MMP-2 and 82 kDa for MMP-9 indicated the presence of active forms of these enzymes. The zymography indicates that MMP-2 may be more active or abundant in certain samples, which resulted in a constant level before and after treatments. In contrast, MMP-9 activity modulated over the treatment period.

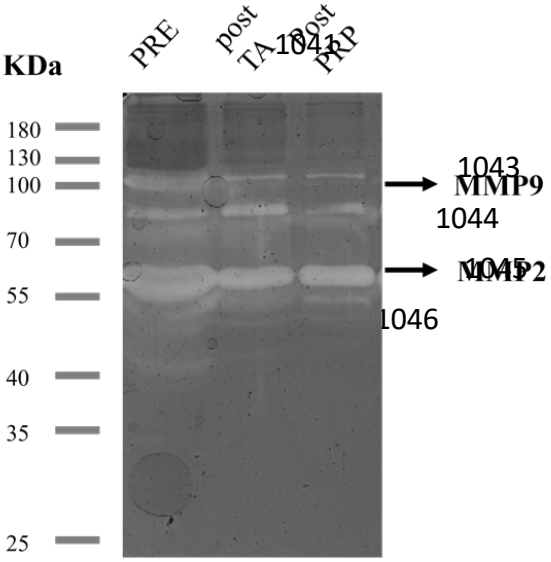

Figure 3. Zymographic analysis of MMP-2 and MMP-9 activity following the TA and PRP administrations. This figure demonstrates zymography results illustrating the activity levels of MMP-2 and MMP-9 in synovial fluid samples from horses treated with TA and platelet-rich plasma PRP. Bands indicate enzymatic activity, with brighter bands representing areas where the enzymes actively degraded the gel substrate.

3.5. IL-1β, IL-6, and HIF-1α expression levels in synovial fluid

In this investigation, we used ELISA test to measure the expression of inflammatory cytokines and hypoxia-related biomarkers in synovial fluid, such as IL-1β, IL-6, and HIF-1α. These indicators were chosen for their possible involvement in inflammatory processes and tissue response under hypoxic circumstances. Our research found no significant differences in IL-1β, IL-6, or HIF-1α expression levels among treatment groups or time points. This lack of detectable changes might mean that the expression of these biomarkers in synovial fluid is unaffected by the therapies used or that ELISA's sensitivity under our study conditions was insufficient to detect minimal fluctuations.

4. Discussion

In the present study, five synovial biomarkers revealed variable degrees of susceptibility to OA and intra-articular therapeutic approaches. We aimed to see if PRP and TA+PRP treatments could regulate the inflammatory or degenerative processes associated with OA by measuring changes in biomarker

concentrations. The findings show that whereas PRP alone did not significantly modify biomarker levels, the combination of TA and PRP resulted in significant changes indicating a potential synergistic effect in controlling OA-associated inflammation.

Fibrinogen, essential plasma protein for blood clothing and is found in the inflamed tissues including the joints [25,26]. Corticosteroids are known as their potential anti-inflammatory effect. In this study, in the TA+PRP group, after the administration of TA into the joint, the authors observed a significant reduction in fibrinogen concentration. These findings agree with the studies that showed a reduction of fibrinogen levels in the active inflammation, as well as in the affected joint following corticosteroid use [25,27]. However, PRP administration following the TA injection, the fibrinogen concentration was significantly increased. Some studies showed that PRP reduce the fibrinogen levels, in contrast to our study [26]. PRP naturally contains various plasma proteins, whose main functions are carrier, immunity, blood clothing as well as that activate fibrinogen to form fibrin scaffolds, induce chondrocyte proliferation and differentiation, and promote cartilage damage repair[28,29]. Surprisingly, in the PRP group, there was no significant difference at each time point. This brings the question about the TA+PRP group the level of fibrinogen increase following the PRP injection, while in the PRP group, it does not. This is most likely because fibrinogen has a multifaceted role in tissue injury and inflammation [30]. Since fibrinogen has common and ancient domains that play pivotal roles in the transition from inflammation to tissue repair, this might explain the increase observed with PRP followed by preconditioned TA [31].

Haptoglobin (Hp) is part of the acute phase response to inflammation, which is considered a biomarker in clinical practice. Hp was identified in the synovial fluid of horses with osteoarthritis (OA), demonstrating the ability to serve as a biomarker for joint disease an has been linked to OA severity, particularly knee OA [32]. The presence of Hp in joints observed, supported by the studies, indicating that Hp is produced locally in inflamed joints [17]. Previous research, such as that conducted by Stevens et al., demonstrated that chondrocytes in bovine cartilage might produce haptoglobin [33]. Similarly, Smeets et al. and Rosenkranz et al. found Hp expression in arthritic tissues in rats and synovial fluid from juvenile idiopathic arthritis in humans, respectively [34,35]. This cross-species reliability in Hp's activity highlights its likely significance in OA diagnostics and as an inflammatory marker in joint disease. Within the PRP group, there was no significant difference in Hp concentration over time, while in the TA+PRP group, there were significant increases between each time point. Hp concentration was also found in healthy equine joints as 0.004 ± 0.004 (g/dL) [36]. In this research the concentration of haptoglobin was 0.98 ± 1.04 (g/L). This might indicate haptoglobin serves as a OA biomarker in accordance also with the abovementioned studies. Interestingly, haptoglobin and fibrinogen showed similar trends in our study, with both markers declining following the TA administration post-treatment. The temporary elevation in fibrinogen and haptoglobin following PRP injection might be attributed to local synthesis by inflamed joint tissues, potentially as an acute response to inflammation. Moreover, PRP rich in leukocytes have been shown to cause a significantly greater acute inflammatory response [37,38]. However, this excessive acute inflammatory response was not seen in this research, most likely due to our preparation was poor in leukocytes.

Synovial fluid TP content has been reported to be higher in OA joints than in normal joints [39]. In our study, the TA+PRP group showed a significant reduction in TP levels at each time points ($p = 0.003$). In contrast, the PRP-only group showed an increase in TP concentration post-treatment. Statistically significant differences were observed post-PRP treatment in both PRP-only and TA+PRP groups ($p = 0.001$). Chen et al. found a significant decrease in TP concentration after PRP injection [32]; however, in our study, this decrease was noted in the TA+PRP group, not after direct PRP injection alone. The TP increase in the PRP-only group may be related to the fact that PRP products naturally contain total proteins [6,29] and PRP can also induce an acute inflammatory response in the joint, thus changes in total protein were observed in response to intra-articular PRP injection. This changes aligns with findings from Moraes et al. and Textor et al. [40,41].

Nevertheless, this TP increase appears to have no lasting clinical effect, as no adverse reactions to PRP administration were observed. This further supports that PRP remains a safe therapeutic option for intra-synovial administration [28].

Matrix metalloproteinases are enzymes capable of matrix digestion; they are normal constituents of the matrix but are present in an inactive form. In the pathogenesis of the joint disease, the activation of MMP-9 is associated with an increase in MMP-2 expression [42]. MMP-9 is produced and released into SF by chondrocytes and synoviocytes in joint disease in horses but not in clinically normal joints [43]. In our study, there were expressions of both MMP-2 and MMP-9. Similar to findings that found in this study supported by Gaudi et al. [44]. However, the expression of MMPs did not result in significant changes statistically.

PRP exposure did not result in significant changes in matrix metalloproteinases (MMPs) gene expression, specifically MMP-2 and MMP-9. Our finding aligns with previous research by Hur et al., which also reported that PRP did not significantly reduce MMP expression [45]. MMPs are known also released by platelets on a platelets dose-dependent basis, increasing the total concentration of MMPs [29]. Although we observed no statistically significant differences in MMP-2 and MMP-9 concentrations in the PRP group following treatment, a notable trend in MMP-9 levels showed a gradual decline in zymographic assessment in the TA+PRP group. This suggests that TA+PRP may have a moderating effect on MMP-9 activity, although further research is needed to understand the mechanisms involved. The decrease in MMPs by PRP was confirmed by various researchers, while others mention either no effect or a decrease in these parameters [46–48]. Given the small sample size in our study, future investigations with larger sample sizes are essential to elucidate the full spectrum of MMP activity in PRP treatments, especially in combination with corticosteroids like TA.

It was difficult to draw definitive conclusions regarding IL-6 and IL-1β cytokine levels or the effects of the treatments on these biomarkers due to their low concentrations. It is worth noting that synovial fluid (SF) provides a close reflection of individual joint status. However, SF aspiration involves greater risk than other sample types like urine or serum, and biomarker concentrations in SF may vary significantly based on the level of active joint inflammation [49]. This variability may result from dilution or “washout” effects, particularly in cases of high joint effusion [49]. However, 2 out of 14 horses, a significant increase in pro-inflammatory cytokines was observed, which subsequently decreased following TA and PRP treatments. Only these two

horses had an effusion score of 0, whereas the remaining horses had scores of 2 or higher (data not shown). This difference suggests that higher effusion scores may dilute synovial biomarkers, making them harder to detect. This observation aligns with previous studies indicating that excessive joint fluid can mask biomarker levels through dilution and altered clearance rates [7,50,51]

Interestingly, haptoglobin levels were also notably higher in these same two horses that showed increased pro-inflammatory cytokines. A possible relationship between inflammatory cytokines and haptoglobin levels is in agreement with the study that authors suggest an increase in haptoglobin could be influenced by IL-1β, which is known to stimulate its production [33,52]. Samut et al. demonstrated that IL-6 significantly enhances haptoglobin expression and activity produced in response to IL-1β activity [53]. This suggests that the elevated haptoglobin levels in these horses' synovial fluid may partly reflect upstream increases in IL-1β and IL-6, which might indicate haptoglobin's potential role as a biomarker of inflammation in OA [17]. For instance, it is well known that increased activity and amounts of MMPs contribute to the metabolic imbalance in the articular cartilage in most OA patients [54] Haptoglobin might act as a nonspecific inhibitor of MMP-2 and MMP-9 [55] which constitute the gelatinase subgroup and are capable of catabolising the macromolecules in the extracellular matrix [56]. Therefore, it is reasonable to deduce that the increased concentration of haptoglobin in the SF of OA patients probably represents feedback from the enhanced MMP activity to protect the macromolecules in the extracellular matrix from being broken down excessively [52].

Conclusion

PRP, when applied alone without TA, does not cause significant changes in biomarkers, nor does it induce any inflammation, thereby demonstrating a safe profile within the joint. Sequential use of TA and PRP, however, has shown improved effects. This suggests that PRP produces a more controlled inflammatory response when administered in a joint where inflammation is reduced. Further research with a larger sample size and additional time points is needed to examine the long-term effects of these two treatments on synovial biomarkers in OA.

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GENERAL DISCUSSION

Osteoarthritis (OA) is a common disease in equine patients, causing up to 60% of all lameness cases. In current equine practice, the diagnosis is commonly based on clinical signs, such as joint effusion, lameness, and

synovial fluid parameters, combined with diagnostic imaging evaluation. The treatment of OA frequently remains uncertain whether a particular therapy is having its effect by simply relieving the symptomatic pain associated with the joint disease, or whether it has a positive effect on the joint environment. Today, multiple treatments on the market to reduce symptoms of OA for equine patients such as orthobiologics as well as conventional treatment options like intra-articular corticosteroid or hyaluronic acid, used separately or in combination. The PhD thesis evaluated corticosteroids (triamcinolone acetonide) and orthobiologics (Platelet-rich Plasma) use in fetlock joint OA and their effectiveness in vitro and in vivo studies. In vitro, TA showed adverse effects on chondrocytes, with a significant decrease in viability, as observed in the other studies. However, the combined use of TA and PRP increased cell viability, indicating a protective effect of PRP against cytotoxicity. PRP has also been reported to protect fibroblast cell types, including chondrocytes, against drug toxicity. Treatment with intra-articular PRP following to TA, showed promising improvement in clinical effect. Additionally, it is found that fibrinogen and haptoglobin levels were affected by treatment, especially in the TA+PRP group. Fibrinogen levels decreased after TA treatment and increased post-PRP administration, suggesting a reparative response. Haptoglobin levels increased post-TA+PRP administration, suggesting the combined treatment influences acute-phase proteins associated with inflammation in osteoarthritic joints. Total protein concentrations decreased, suggesting a sustained anti-inflammatory effect. MMP-9 expression were decreased by the TA+PRP treatment, that might indiciate combining these to treatment possible decreasing cartilage degradation.

This PhD thesis had some limitations. The first limitation was that treatment protocols were different between groups. In vitro, PRP was added concomitantly with TA-treated chondrocytes, whereas in vivo, PRP was administered one week after TA. This timing also may have influenced the clinical results. However, the in vivo study was conducted on horses with OA symptoms, while the in vitro study used healthy chondrocytes treated with TA. Additionally, following the effects of TA for one week on chondrocytes in vitro is challenging, as we observed cytotoxicity even at lower doses. The second limitation, horses with similar pathologies may exhibit individual response to treatment. The therapeutic effects may also be influenced by the horses' working discipline and post-treatment exercise protocols.
The third limitation, blinded and placebo-controlled study not performed. In the horse, there has been a lack of good randomized controlled trials investigating the efficacy of articular therapeutics, most likely because they are difficult and expensive to perform. However, it is important to realize this lack of a placebo-controlled group and other objective measures may lead to erroneous conclusions, hence potentially treatment of many horses with ineffective medications over a long time period. Thus, the horses in the control group are at risk of suffering from joint-related pain throughout the study period. Besides that, training day loss in the racing industry and its caused economic burden.

The fourth limitation states that the limited number of horses used in the study could impact the assessment of the therapy's efficacy and any observed changes in synovial biomarkers. A limited sample size may limit the study's power in statistics, making it more difficult to identify meaningful variations in treatment effects and restricting the ways that the results may be applied. Individual response differences can have a more significant

impact on the results of studies with fewer horses, thus overestimating or underestimating the effectiveness of the treatment. Because individual biological variability may obscure the effects of treatment, a limited sample size for synovial biomarkers may make it more difficult to detect modest biochemical changes linked to inflammation or healing.

Overall, this is the first study to assess the combined effects of triamcinolone acetonide (TA) and platelet-rich plasma (PRP) on synovial biomarkers and equine cartilage cells, providing information regarding its possible therapeutic use in equine osteoarthritis (OA). While PRP alone had no influence on biomarker levels, it showed its safety profile when administered alone in the joint. The combination of TA and PRP significantly controlled inflammation-related biomarkers, particularly fibrinogen and haptoglobin, indicating a viable strategy for treating OA-affected joints. Although MMP-2 levels remained stable, the steady decline of MMP-9 suggests TA+PRP as a promising method for longer effects on pain and lameness reduction. More clinical trials with numerous PRP injections and longer follow-up are needed to better understand the long-term advantages and safety of combining TA and PRP in OA therapy for horse athletes.