CLINICAL SIGNS, SYNOVIAL FLUID CYTOLOGY AND GROWTH FACTOR CONCENTRATIONS AFTER INTRA-ARTICULAR USE OF A PLATELET-RICH PRODUCT IN HORSES WITH OSTEOARTHRITIS

By

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Submitted in partial fulfilment of the requirements for the degree of MMedVet (Surgery)(Equine) in the Department of Companion Animal Clinical Studies in the Faculty of Veterinary Science, University of Pretoria

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PROJECT TITLE	**Clinical signs, synovial fluid cytology and growth factor concentrations after intra-articular PRP in horses with osteoarthritis**
PROJECT NUMBER	**V011-15**
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STUDENT NUMBER (where applicable)	**22145649**
DISSERTATION/THESIS SUBMITTED FOR	**MMedVet**

ANIMAL SPECIES	**Horses (Equine)**	
NUMBER OF ANIMALS	**10**	
Approval period to use animals for research/testing purposes		**March 2015- March 2016**
SUPERVISOR	**Dr. HJ Marais**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date	23 February 2015
CHAIRMAN: UP Animal Ethics Committee	Signature	

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LIST OF ABBREVIATIONS

AAEP	American Association of Equine Practitioners
ACPCs	Articular cartilage progenitor cells
ACS	Autologous conditioned serum
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
AP-1	Activating protein-1
APP	Acute phase proteins
bFGF	Basic fibroblastic growth factor
BK	Bradykinin
BMPs	Bone morphogenetic proteins
BMP-2	Bone morphogenetic protein-2
BMP-7	Bone morphogenetic protein-7
BMSCs	Bone-marrow stroma cells
CaPPS	Calcium pentosan polysulfate
CBC	Complete blood count
CS	Chondroitin sulfate
COMP	Cartilage oligomeric protein
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CT	Computed tomography
DMOAD	Disease-modifying osteoarthritis drug

ECM	Extracellular matrix
HA	Hyaluronic acid/Hyaluronan
IGF-1	Insulin-like growth factor-1
IL-1	Interleukin-1
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
IL-1ra	Interleukin-1 receptor antagonist
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IRAP	Interleukin-1 receptor antagonist protein
KS	Keratin sulfate
LAP	Latency-associated peptide
MMPs	Matrix metalloproteinases
MPA	Methylprednisolone acetate
MRI	Magnetic resonance imaging
MSCs	Mesenchymal stem cells
NaPPS	Sodium pentosan polysulfate
NCC	Nucleated cell count
NF-κβ	Nuclear factor-κβ
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
PAW	Platelet-activation white blood cell

PDGF-BB	Platelet-derived growth factor-BB
PG	Proteoglycan
PGE	Prostaglandin-E
PGE_2	Prostaglandin-E_2
PPS	Pentosan polysulfate
PRP	Platelet-rich plasma
PRPr	Platelet-rich product
PSGAG	Polysulfated glycosaminoglycan
RBCs	Red blood cells
SAA	Serum amyloid A
SMOAD	Symptom-modifying osteoarthritis drug
TA	Triamcinolone acetonide
TP	Total protein
TGF-β1	Transforming growth factor-B1
TIMP	Tissue inhibitors of metalloproteinases
TNF-α	Tumour necrosis factor-α
VEGF	Vascular endothelial growth factor
WBC	White blood cells

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SUMMARY

CLINICAL SIGNS, SYNOVIAL FLUID CYTOLOGY AND GROWTH FACTOR CONCENTRATIONS AFTER INTRA-ARTICULAR USE OF A PLATELET-RICH PRODUCT IN HORSES WITH OSTEOARTHRITIS

By

YOLANDI SMIT

Supervisor: Prof. A. Goddard
Co-supervisor: Dr. H.J. Marais
Department: Companion Animal Clinical Studies
Degree: MMedVet(Surgery)(Equine)

Background: Osteoarthritis (OA) is a common cause of lameness in equines, resulting in poor performance. Platelet-rich plasma (PRP) can deliver a collection of bioactive molecules that play important roles in conditions such as OA.

Objectives: To (1) investigate the clinical and synovial changes, (2) determine serum amyloid A (SAA) and total protein (TP) concentrations on serum and synovial fluid, and (3) determine synovial fluid platelet-derived growth factor-BB (PDGF-BB) and transforming growth factor-B1 (TGF-β1) in normal and osteoarthritic joints after intra-articular injection with a platelet-rich product (PRPr).

Study design: Experimental.

Methods: Horses (n = 5) with naturally occurring OA, affecting either antebrachiocarpal, middle carpal or metacarpophalangeal joints, and normal horses (n = 5). A PRPr was prepared from all horses using a gravity filtration system. Clinical evaluation, synovial fluid analysis, and determination of serum and synovial SAA and TP concentrations were performed on day 0 (prior to PRPr treatment), day 1, day 2, day 5, day 21 and day 56 post-treatment. Synovial fluid growth factor concentrations (PDGF-BB and TGF-β1) were determined on day 1 and day 5. PRPr composition was also analysed.

Results: The gravity filtration system produced a moderately concentrated PRPr. The synovial effusion score was statistically significant between the control and OA group on day 0 ($p < 0.05$) with a higher score in the OA group. However, within the control group the synovial effusion score was significantly elevated on day 1 and 2 compared to day 0 ($p < 0.05$). For both groups the synovial fluid nucleated cell count, predominantly intact neutrophils, were significantly increased on day 1 and day 2 ($p < 0.001$ for both), with no significant difference between groups. The mean PDGF-BB and TGF-β1 concentrations were high for both groups but significantly lower in the OA group ($p < 0.01$ and $p < 0.001$, respectively) on day 1 compared to normal joints. Concentrations for PDGF-BB remained similar on day 5, compared to day 1, with no significant difference between groups.

Conclusions: Intra-articular treatment with PRPr resulted in a transient synovial inflammatory reaction of short duration. Synovial fluid growth factor concentrations were elevated after intra-articular injection of PRPr in OA joints.

INTRODUCTION

BACKGROUND

Osteoarthritis (OA) is one of the most common causes of lameness in equines, resulting in poor performance and economic loss (Sutton et al., 2009). It is a chronic degenerative joint disorder that is multifactorial in origin and characterized by progressive deterioration of the articular cartilage, subchondral bone alterations, synovitis and capsulitis (Alcaraz et al., 2010; Mcllwraith et al., 2012). Synovitis and capsulitis play a significant role in the contribution to the OA process, as it may lead to an increase in production of matrix metalloproteinases (MMPs), aggrecanases, prostaglandins (PG), free radicals, interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α) (Mcllwraith and Van Sickle, 1981).

Formerly, the pharmacological approach towards OA was confined to symptomatic treatments (i.e. corticosteroids and hyaluronic acid (HA)), of which the goal was to diminish functional impairments and pain severity (Mcllwraith et al., 2012). Over the past decade, regenerative medicine has been considered as one of the most promising therapies of OA (Filardo et al., 2011; Monteiro et al., 2015). Intra-articular biological therapies that have been described include autologous conditioned serum (ACS), stem cells and platelet-rich products (PRPr) (Abellanet and Prades, 2009; Fortier, 2009; Frisbie et al., 2007; McIlwraith et al., 2011; Textor and Tablin, 2013a).

Platelet-rich plasma (PRP) is defined as an autologous biological product produced from whole blood that contains a higher platelet concentration compared to baseline blood values. Upon platelet activation, growth factors are released from α granules at the site of injury (Textor and Tablin, 2013a). Growth factors are biologically active polypeptides with anabolic and anti-catabolic effects that may regulate neo-chondrogenesis, as well as chondrocyte metabolism and differentiation, potentially improving cartilage repair (Fortier et al., 2011; Sun et al., 2010; Van Den Berg et al., 2001; Xie et al., 2014). Growth factors, such as PDGF and TGF are normally low or not detectible in healthy joints. Textor et al. (2013) found no detectible levels of PDGF and approximately 600-700 pg/mL TGF- β in healthy joints prior to PRP administration in their study evaluating growth factor concentrations within synovial fluid pre- and post PRP administration. According to Van der Kraan (2017) the concentration

of TGF- β differ greatly between healthy and OA joints, being low in healthy joints and high in OA, leading to the activation of different signaling pathways in joint cells. Thus supporting the finding that low levels of TGF- β is present in healthy joints. Investigating the duration that PDGF and TGF-β are detectable within synovial fluid after PRP administration, it is found that PDGf has a biological half-life of 2.5 hours, however the release of PDGF is constant and elevated levels can be sustained for at least seven days (Roh et al., 2016). Roh et al. (2016) stated that synovial fluid TGF- β concentration can be rapidly induced and a maximum concentration can be detected within one hour after PRP administration.

Platelet-rich plasma was first recognized as an effective agent for bone and tissue repair within the field of human dentistry and oral maxillofacial surgery, followed by evidence of improving skin graft wound healing in the field of plastic surgery (Castillo et al., 2011). In recent years, the musculoskeletal effects of PRP have been the main focus in human sports medicine and orthopaedics (Sheth et al., 2012; Xie et al., 2014). Intra-articular use of PRP has been widely reported in human sports medicine and positive outcomes have been observed in cases of osteochondral lesions and early OA (Filardo et al., 2011; Guadilla et al., 2011; Kon et al., 2011; Mei-Dan et al., 2012; Sampson et al., 2010; Sanchez et al., 2008). Intra-articular PRP has also been used successfully in humans and experimental animals for the treatment of synovitis, cartilage defects, meniscal injury, intra-articular fractures and OA (Ishida et al., 2007; Liu et al., 2011; Mei-Dan et al., 2012; Saito et al., 2009; Wei et al., 2012). The use of PRP in horses has evolved from the treatment of tendon and ligament lesions to intra-articular therapy, where it is reported to relieve pain and reduce effusion (Carmona et al., 2007). Carmone et al. (2007) study in 2007 described the use of a platelet concentrate intra-articular in horses with osteoarthritis. The study had a small number of horses (4 horses) and included no healthy controls. Strict selection criteria were applied for inclusion of cases; however, the periods between follow-up examinations should have been longer (i.e. one year instead of 2 months). Moreover, a repeat of radiographs at one-year follow-up would have lead to a better evaluation of platelet concentrate.

However, studies to assess the response of diseased joints to intra-articular PRP injection, specifically the levels of growth factor concentrations in synovial fluid, as well as the changes of serum amyloid A (SAA) and total protein (TP) concentrations in synovial fluid and blood, are limited (Carmona, 2007; Textor, 2013a, b).

HYPOTHESIS

a) Horses that exhibit OA will show an improvement in clinical signs after a single intra-articular injection of a PRPr.

b) Intra-articular injection of a PRPr will induce a transient but statistically significant synovial cellular response; this response will differ between horses exhibiting OA and control horses.

c) Synovial fluid growth factor concentrations of platelet-derived growth factor-BB (PDGF-BB) and transforming growth factor-β1 (TGF-β1) will differ between horses exhibiting OA and control horses.

d) Serum and synovial fluid SAA and TP will differ between horses exhibiting OA and control horses.

OBJECTIVES

a) To evaluate the cellular composition of a PRPr end product.

b) To investigate the systemic and synovial cellular response after a single intra-articular injection of a PRPr in control and OA equine joints.

c) To evaluate the extent and duration of clinical signs after a single intra-articular injection of a PRPr in control and OA equine joints.

d) To evaluate synovial growth factor concentrations of PDGF-BB and TGF-β1 in control and OA equine joints after a single intra-articular injection of PRPr.

e) To evaluate serum and synovial SAA and TP levels in control and OA equine joints after a single intra-articular injection of PRPr.

f) To serve as a baseline for future studies evaluating a single intra-articular injection of a PRPr in horses exhibiting OA.

BENEFITS ARISING FROM THE PROJECT

a) Equine OA is an economically important disease in South Africa and globally, and early recognition and treatment using intra-articular injection of a PRPr may have an economic impact on the equine industry.
b) The research conducted serves as partial fulfilment of the principal investigators' MMedVet (Surgery)(Equine) degree.

LITERATURE REVIEW

OSTEOARTHRITIS

The joint is a structure that plays a key role in the musculoskeletal system, and needs to meet several requirements to allow for adequate locomotion. Joints have to be as robust as the bony elements in the musculoskeletal system, it has to exhibit smooth and frictionless motion at the articulation surfaces of the bones, and act as a digital cushion to absorb, mitigate and dampen the vibrations generated by the impact during locomotion (VanWeeren, 2016). All the aforementioned requirements have to be incorporated into this single structure, and it serves to be a challenge.

Osteoarthritis is a chronic degenerative joint disorder that is multifactorial in origin and characterized by destruction of the articular cartilage, subchondral bone alterations, synovitis and capsulitis (Alcaraz et al., 2010; Mcllwraith et al., 2012). A schematic representation of the development of OA can be found in McIIwraith (2016c).

The pathogenesis of OA has been classified into two fundamental mechanisms. The first mechanism involves the placement of normal loading on abnormal cartilage. In this case the articular cartilage is fundamentally defective, displaying abnormal biomechanical properties from i.e. ageing, osteochondrosis or secondary to synovitis and capsulitis, leading to failure when placed under normal loading conditions. The second mechanism involves the placement of abnormal loading on normal cartilage. This can result in remodelling and micro fractures/ necrosis in the subchondral bone, loss of stability within a joint due to, for example, fractures or ligamentous tears, and joint incongruity from intra-articular fractures and developmental defects. Subchondral bone disease can lead to secondary damage to the normal cartilage, either from the loss of support or from the release of inflammatory cytokines. Subchondral sclerosis can lead to a decrease in shock absorption capacity leading to physical damage to the articular cartilage. The abnormal loading sustained from loss of joint stability and soft tissue injuries will overwhelm the normal metabolic repair mechanisms in the normal cartilage, ultimately leading to its failure (McIlwraith, 2016c).

OSTEOARTHRITIS IN THE HORSE

Osteoarthritis is believed to be one of the most common joint disorders in the horse (Sutton et al., 2009). Surveys estimate that up to 60% of lameness is related to OA (Mcllwraith et al., 2012) and that this condition is frequently associated with lameness, poor performance and early retirement in many equine sports (Sutton et al., 2009).

Osteoarthritis (previously known as equine degenerative arthritis) was first reported in 1938 and received its first clinical attention by the American Association of Equine Practitioners (AAEP) in 1966 where its relationship with lameness and 'use trauma' became a central etiological concept (Callender and Kelser, 1983). In 1975, articular cartilage lesions were considered the integral criteria of OA but it was also acknowledged that it may not be the centrally important cause of clinical disease (Mcllwraith et al., 2012).

Today, equine OA can be considered a group of disorders characterized by a common end stage, which is progressive deterioration of the articular cartilage accompanied by changes in the bone and soft tissue of the joint (Mcllwraith et al., 2012). It is also now recognized that the OA process can be triggered by disease of the synovial membrane, fibrous joint capsule, subchondral bone, ligaments or articular cartilage, or a combination of the above mentioned (Mcllwraith, 2005).

ANABOLIC/CATABOLIC BALANCE OF JOINT CARTILAGE

In normal, healthy cartilage there is a balance between anabolic and catabolic activities that play a key role in the maintenance, integrity and repair of molecular damage that is sustained during daily work.

Articular cartilage is avascular, aneural and consists largely of an extracellular matrix (ECM) and a small cellular component (1% to 12%). The major components of the extracellular matrix of articular cartilage are collagen, proteoglycans and water. Collagen and proteoglycans comprises approximately 50% and 35% on a dry weight basis. Minor fractions are minerals (3%), lipids (1%) and miscellaneous components (1%). The cellular component is relatively small at approximately 1% to 12% (Todhunter, 1996). Cartilage can be classically divided into four layers, namely the superficial zone, the middle zone, the deep zone and the calcified layer (Aydelotte and Kuettner, 1988).

Collagen type II is the predominant collagen type in healthy cartilage; this is however replaced by collagen type X in clinically affected cartilage of mature horses with OA. Collagen type X is associated with enhanced mineralization that can interfere with normal collagen homeostasis, resulting in ECM dysregulation.

Proteoglycans are a group of composite molecules featuring a protein and a sugar (Fig. 1). In cartilage, the core protein is attached to side chains, which are predominantly sulphated glycosaminoglycans that forms part of the lectican family, which includes aggrecan, versican, neurocan and brevican of which aggrecan is the most abundant form and widely researched. Proteoglycan is connected to collagen either directly or via hyaluronan, which is also known as hyaluronic acid (HA).

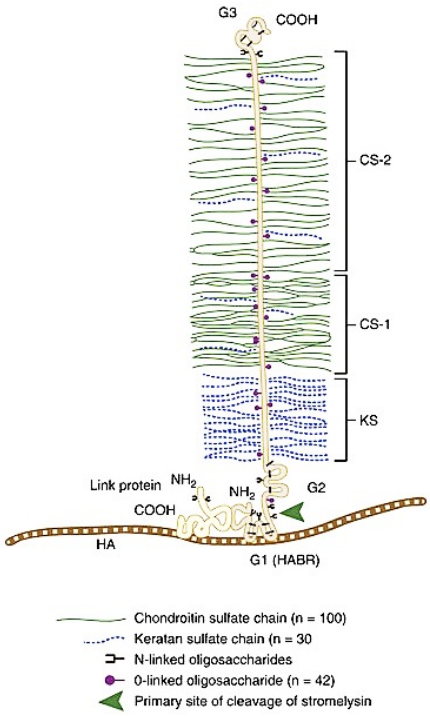

Figure 1: Schematic presentation of an aggrecan molecule, consisting of a core protein (COOH-NH_2) that is bound to hyaluronic acid (HA) by a link protein. The core protein has a large number of side chains consisting of sulfated glycosaminoglycans. These are keratin sulphate (KS) and chondroitin sulphate (CS) side chains (VanWeeren, 2016).

The cellular component traditionally was believed to consist solely of chondrocytes, but recent research has shown the presence of articular cartilage progenitor cells (ACPCs) in the horse (McCarthy et al., 2012). Chondrocytes found in normal articular cartilage is the sole source of production of all the components of the ECM.

Articular cartilage has an extremely high ECM to cell ratio and the ECM has an exceptionally low turnover rate in mature animals, resulting in a very limited capacity for repair, making it the most likely tissue of a joint to fail (VanWeeren, 2016).

Articular cartilage can through its homeostatic mechanisms (anabolic/catabolic, cytokine/growth factor balance) retain a high degree of integrity and functional characteristics over prolonged periods. However, traumatic events, repetitive high loading of joints or presence of inferior tissue quality will results in pathological deterioration and development of OA (VanWeeren, 2016). In the latter scenario, the delicate homeostatic balance is interrupted, leading to the release of enzymes, cytokine-initiated release of MMPs, aggrecanases and prostaglandin-E_2 (PGE_2) from chondrocytes in response to IL-1 (McIlwraith, 2016c).

The main inflammatory cytokines involved in OA are IL-1 and TNF-α. Interleukin-1 is the principal cytokine responsible for articular cartilage degeneration, compared to TNF-α which is more responsible for the clinical morbidity and pain (McIlwraith, 2016c). It is known that IL-1 induces PG depletion in the articular cartilage, either through increasing the rate of degradation or by decreasing the synthesis in close association with MMPs, aggrecanases and PGE_2 from chondrocytes. A study by Frisbie et al. (2002) best demonstrated the role of IL-1 on the pathogenesis of cartilage degradation in the horse. Using IL-1 receptor antagonist (IL-1ra) gene therapy, the study demonstrated that IL-1 could be inhibited at cellular level, resulting in seizing of articular cartilage degeneration in experimentally induced OA models. Interleukin-1 has been shown to induce chondrocytes to secrete collagenase, a MMP capable of collagen cleavage, and stromelysin, a MMP capable of degrading proteoglycan subunits, link protein and type II collagen (Platt and Bayliss, 1994). Increased levels of TNF-α have also been found in the synovial membranes in horses with OA, however, its role in OA is less clear (Trumble et al., 2001). The literature reports that IL-1 is a pivotal cytokine in early and late stages of OA, and that TNF-α is primarily involved only in the initial stages of OA (Goldring, 1999).

Inflammatory cytokines also trigger the release of matrix metalloproteinases (MMPs). Matrix metalloproteinases are a group of enzymes involved in ECM degradation. In healthy articular cartilage, expression of MMPs provides routine tissue remodelling and turnover. However, in OA where there is an upregulation of proinflammatory cytokines (particularly IL-1β) which activates the signalling pathways that involve nuclear factor-κβ (NF-κβ), as well as activating protein-1 (AP-1) transcriptase, leading to the upregulation of MMPs expression and resulting in ECM degradation (McIlwraith, 2016c). An increase in MMPs concentration has been demonstrated in the synovial membrane and articular cartilage for equine joint disease (Trumble et al., 2001). Aggrecans that form part of the PG molecule within the ECM is

degraded principally by a proteolytic process driven by aggrecanases-1 and -2 (also known as ADAMTS-4 and -5)(ADAMTS: a disintegrin and metalloproteinase with thrombospondin motifs) leading to further cartilage degradation. ADAMTS-4 is specifically found to be regulated by both IL-1 and TNF-α (Bondeson et al., 2006) but both ADAMTS-4 and -5 can be expressed by synovial cells. In OA, PGE_2 is considered the main mediator of inflammation and pain. Interleukin-1β (IL-1β) and TNF-α can stimulate the release of PGE_2 from chondrocytes by the production of two enzymes, namely cyclooxygenase-2 (COX-2) and prostaglandin-E synthase (McIlwraith, 2016c). Prostaglandins result in vascular vasodilation, enhancement of pain perception, PG depletion from cartilage (by both degradation and inhibition of synthesis) and bone demineralization within joints.

Included in the above mention catabolic factors is oxygen-derived free radicals such as superoxide anion, hydroxyl radicals, hydrogen peroxide and nitric oxide (NO) can lead to the cleavage of HA and PG, and degradation of collagen within the ECM. Nitric oxide may also lead to the up regulation of NF-κβ pathway. Studies have reported that NO can be produced by osteoarthritic chondrocytes (Abramason, 2008) and that excess NO can accumulate within a joint when there is overexpression of inducible nitric oxide synthase (iNOS) by the osteoarthritic chondrocytes (secondary to mechanical factors and inflammatory cytokines, i.e. IL-1β and TNF-α) leading to chondrocyte death and ECM degradation (Melchiorri et al., 1998; Chubinskaya and Wimmer, 2013). The catabolic factors mentioned above are currently not investigated in this study but are of importance to understand the homeostasis mechanisms in articular cartilage.

Anabolic factors maintaining cartilage homeostasis include transforming growth factor-β (TGF- β), platelet derived growth factor (PDGF), bone morphogenetic proteins (BMPs), insulin-like growth factor-1 (IGF-1) and basic fibroblastic growth factor (bFGF) of which TGF-β and PDGF are the most widely researched. Transforming growth factor-β stimulates collagen type II and aggrecan expression, as well as the down regulation of matrix-degradating enzymes, thus counteracting the effects of IL-1 induced cartilage matrix degradation (Mueller and Tuan, 2011). The effect of TGF-β decreases with age and may be responsible for the increase in cartilage degradation observed in older horses. Platelet derived growth factor is known to play a critical role in cell proliferation, chemotaxis, cell differentiation and angiogenesis, all required for modulation of inflammation and repair (Castillo et al., 2011). The main anabolic factors focused on in the current study are TGF-β and PDGF-BB, which are the two most researched anabolic factors.

Bone morphogenetic proteins (BMPs) are signalling proteins that form part of the TGF-β family, and are responsible for the developmental and normal physiological functions of cartilage. There are different forms of BMPs, i.e. BMP-2 and BMP-7. Bone morphogenetic protein-2 (BMP-2) expression is increased in OA chondrocytes by the stimulation of IL-1β and TNF-α leading to cartilage degeneration. In contrast, bone morphogenetic protein-7 (BMP-7) is down regulated in OA chondrocytes and has a positive effect on matrix biosynthesis. Thus BMP-7, has stronger anti-degradation properties than BMP-2 which has been proven in horses (Carpenter et al., 2010). The anabolic effects of insulin-like growth factor-1 (IGF-1) are responsible for the down regulation of the catabolic effects of IL-1β by upregulating the IL-1 decoy receptor, IL-1RII. This results in the inhibition of matrix degeneration but also stimulates ECM production. Therefore, IGF-1 may play a crucial role in cartilage homeostasis (Wang et al., 2003). Basic fibroblastic growth factor (bFGF) has been extensively studied in OA, and its distinct anabolic effects have been demonstrated on the biosynthesis of the ECM (Berenbaum, 2013).

PATHOLOGICAL MANIFESTATION OF OSTEOARTHRITIS

CLINICAL EXAMINATION

Osteoarthritis is classically diagnosed based on clinical signs and diagnostic imaging findings. Osteoarthritis manifests as a lameness of varying degrees, synovial effusion, periarticular swelling and perception of pain in joints. Synovial effusion is the result of an increase in vascular permeability with a decrease in lymphatic drainage secondary to inflammation within the joint, which can progress to a synovitis and capsulitis.

DIAGNOSTIC IMAGING

Radiography is frequently used for assessment of the structural changes of OA. It has the advantages of availability, convenience, relative safety and affordability. However, this technique still lacks sensitivity and is of limited value in identifying horses with incipient or focal lesions (Ross and Dyson, 2011). Radiology does have some merit in characterizing changes in bone that accompany chronic OA and can be useful in adding confidence to the diagnosis. Radiological signs of OA include periarticular osteophytes, joint-space narrowing, subchondral bone sclerosis or lysis and the presence of osteochondral fragments (Frisbie, 2012).

Computed tomography (CT) and magnetic resonance imaging (MRI) produces a multiplanar image without superimposition of structures, thereby allowing the diagnosis of certain types

of joint injuries that might not be visible with standard imaging modalities, such as digital radiography. Both are expensive and needs to be performed predominantly under general anaesthesia, they can also difficulty in availability, as MRI is currently not available in South Africa and CT only at specialists' facilities. Computed tomography offers superior bone detail and will far exceed MRI in the detection of fine bone proliferation and lysis of the periarticular margins, joint capsule and other soft tissue attachments (McIlwraith, 2016c). Contrast CT arthrography can be used for the identification of articular cartilage defects that communicates with the articular surface, but also areas of cartilage thinning. Magnetic resonance imaging has the capacity to provide a non-invasive, high-resolution, three-dimensional image of all joint components. It is the only imaging modality that can identify fluid in bone (bone oedema), in conjunction with superior soft tissue and articular cartilage detail. Magnetic resonance imaging can demonstrate surface abnormalities of articular cartilage, but also defects, fissures and pathology within the articular cartilage not communicating with the articular surface (McIlwraith, 2016c).

SYNOVIAL FLUID AND SERUM BIOMARKERS

In a normal joint the synovial fluid is composed of TP concentration less than 25 g/L and a nucleated cell count (NCC) less than 1.0 $\times 10^9$/L, consisting of less than 10% neutrophils (Frisbie, 2012; Steel, 2008). Osteoarthritic joints have a NCC either within the normal reference interval or slightly above the upper limit of the normal reference interval, depending on the degree of active synovitis present, with values usually less than 5.0 $\times 10^9$/L and consisting predominantly of mononuclear cells (>85%). The TP concentration is usually within the reference interval (<20 g/L) or slightly above the upper limit of the reference interval (<35 g/L) (Steel, 2008).

Synovial fluid in OA joints will exhibit a decrease in viscosity, which is attributed to a decrease in HA concentration, and depolymerisation or shortening of hyaluronan. A correlation between the TP concentration in relation to the degree of synovitis has been reported and is closely correlated to the degree of articular cartilage damage as seen using arthroscopy (Frisbie, 2012).

Serum amyloid A is an acute-phase protein (APP) that will increase in response to acute inflammation, better known as the acute-phase reaction (Jacobsen et al., 2006b). Serum amyloid A is synthesized primarily in the liver. With joint disease, the inflammatory mediators present (IL-1β and TNF-α) will stimulate the hepatocytes to shift the cellular gene

expression towards the production of acute-phase proteins at the expense of albumin synthesis (systemic acute phase reaction), resulting in an increase in serum and synovial concentration of APPs (May et al., 1992). Acute phase proteins can thus be used to detect the early onset of inflammation within tissues and monitor the progression thereof (Hulten et al., 2002).

Jacobson et al. (2006b) found an increase in serum and synovial fluid SAA concentrations in horses with experimentally induced OA. The increased synovial SAA concentration found in OA joints was of lower magnitude compared to that of infectious arthritis. This finding was attributed to the chronicity of OA. The study also proposed that SAA might be synthesized locally within the joint in horses, similar to what has been reported in human studies. In addition, synovial SAA concentrations were not influenced by repeated arthrocentesis in their study; however, synovial TP concentration did increase significantly.

In humans, synovial SAA concentration is a more sensitive marker of joint disease compared to radiographic examination, and was found to be a better indicator of disease activity and prognosis compared to cartilage breakdown products (Jacobsen et al., 2006b). Serum amyloid A concentrations in the synovial fluid decreases during stages of clinical improvement and increases with clinical deterioration of joint disease (Jacobsen et al., 2006b).

Due to the limited capacity of articular cartilage to regenerate, cartilage preservation based on the maintenance of anabolic and catabolic homeostasis is paramount to joint function. Insulin-like growth factor-1 forms part of the anabolic cascade and is known to promote articular cartilage repair. A study has found lower IGF-1 concentrations in foals with osteochondrosis and horses with juvenile proximal interphalangeal joint OA, and concluded that IGF-1 levels are affected by systemic factors such as age, sex and diet (Lejeune et al., 2007). The study consisted of 30 foals evaluated for a period of 15 to 28 months under standardized conditions, of which 46.7% were healthy and 53.3% were pathological. Thus, a nearly equal distribution between sample size. The sample size also represented 9.3% of the births in Belgium for that year. The sample size was also nearly equally distributed between fillies (n=14) and colts (n=16). Growth factors often act at very low concentrations (i.e. nanomolar and picomolar quantities) and, thus, may not reflect their actions at various tissue depths within the cartilage. Therefore, growth factors are unlikely to be of use as indirect biomarkers in OA, but can be used to evaluate the efficacy of intra-articular therapies, such as PRP or ACS, which aim to replenish the intra-articular anabolic factors (Textor et al., 2013b).

As previously mentioned, IL-1β is the pivotal cytokine in articular cartilage degeneration. Synovial fluid concentrations of IL-1 in OA joints are quite variable and are often not significantly higher compared to control joints (McNutty et al., 2013). This can be attributed to the labile protein molecules that act in a paracrine fashion at nanometer distances within tissue and synovial fluid, and thus may not reflect the local tissue concentrations or actions (Frisbie, 2016c). Interleukin-6 (IL-6) has shown some promise as an indirect marker of inflammatory activity and has been shown to correlate with pain and physical function in human OA (Shimura et al., 2013; Stannus, 2013). In horses, IL-6 has been investigated in synovial fluid but not serum, and it was shown that IL-1β might induce IL-6 production leading to elevated levels upon onset of inflammation and lameness. Bertone et al. (2001) found that IL-6 was an excellent predictor of joint disease in a study evaluating OA in horses, however more research is required.

PRINCIPLES OF THERAPY FOR OSTEOARTHRITIS

NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

There are two main goals for medical treatment of OA in the horse. Firstly, reducing the pain and secondly, reducing or minimizing the progression of joint deterioration (Frisbie, 2010a). The ideal therapeutic agent should be an agent that both relieve the symptoms of lameness (symptom-modifying OA drug (SMOAD)) as well as producing disease-modifying effects (disease-modifying OA drug (DMOAD)).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are agents that inhibit the cyclooxygenase (COX) enzyme system that is responsible for the conversion of arachidonic acid to prostaglandins (especially prostaglandin-E (PGE)) and thromboxane. It has been the mainstay treatment of joint disease for decades, however due to renal and gastrointestinal side effects associated with NSAIDs their use for long-term treatment of joint disease are limited. All NSAIDs can inhibit the COX activity to some degree. Research has identified two isoenzymes for the COX pathways, namely cyclooxygenase-1 (COX-1) which is responsible for the normal physiological function (homeostasis) of the gastrointestinal and renal systems and COX-2 which is mainly associated with inflammatory events (McIlwraith, 2016d). The main focus to date is to use COX-2 selective NSAIDs for the symptomatic treatment of OA.

CORTICOSTEROIDS

Intra-articular corticosteroid treatment in equine joint disease has long been controversial in terms of the risk-to-benefit ratio of this class of therapy (Mcllwraith et al., 2012). There is a concern that certain corticosteroids can result in accelerated cartilage degeneration, due to the reported negative effect of corticosteroids on chondrocyte metabolism (Caron, 2005). However, most of these studies were done using one specific corticosteroid, namely methylprednisolone acetate (MPA). Corticosteroids are potent anti-inflammatory agents as they inhibit the inflammatory process at all levels (Fig. 5). Corticosteroids do not only reduce capillary dilation; margination, migration and accumulation of inflammatory cells, but also inhibit the synthesis and release of major inflammatory mediators (i.e. IL-1 and TNF-α), and act on the prostaglandin cascade in joint disease (Laufer et al., 2002). Different corticosteroid products vary in their risk-to-benefit ratio. Betamethasone esters (Celestone Soluspan®) has been proven to have no deleterious effect on articular cartilage, triamcinolone acetonide (TA) (Kenalog-10®) on the other hand has chondroprotective properties and can promote cartilage health (McIlwraith, 2016a). In contrast, MPA (Depo-Medrol®) has been consistently shown to have deleterious effects on articular cartilage.

HYALURONAN

Hyaluronan forms the backbone of the aggregating proteoglycan molecule in synovial fluid and articular cartilage. In normal synovial fluid, HA is thought to be between 0.5 to 3.0 million Dalton molecular weight, with a concentration of between 0.33 and 1.5 g/L, and is made by synoviocytes for the synovial fluid and by chondrocytes for the articular cartilage (Tulamo et al., 1994). Exogenous HA has a very short half-life when administered intra-articularly (hours), and an even shorter half-life when administered intravenously (minutes) (Fraser et al., 1993; Fraser et al., 1981). Hyaluronan is considered a SMOAD in the human medicine (Berenbaum et al., 2012). Intravenous use of HA in horses has been reported to decrease lameness and improve synovial membrane histology (specifically vascularity and cellular infiltration) and synovial fluid parameters (decrease in PGE_2 and TP) (Kawcak et al., 1997).

POLYSULFATED GLYCOSAMINOGLYCAN

Polysulfated glycosaminoglycan (PSGAG) belongs to the family of polysulfated polysaccharides, which include PSGAG (Adequan®), pentosal polysulfate and CS. Polysulfated glycosaminoglycan are considered to be DMOADs and have been used when

cartilage damage is suspected, in order to prevent, retard or reverse the morphologic cartilaginous lesions of OA and cartilage degeneration. The main ingredient of therapeutic PSGAG and CS consists of an extract of bovine lung and trachea modified by sulfated esterification, and is registered for both intra-articular and intramuscular use in horses (McIlwraith, 2016b). A significant decrease in synovial effusion, reduction in the vascularity and fibrosis of the synovium and cartilage fibrillation has been shown with intra-articular treatment of PSGAG compared to HA and a saline control (Frisbie et al., 2009).

PENTOSAN POLYSULFATE

Sodium pentosan polysulfate (NaPPS) and calcium pentosan polysulfate (CaPPS) are semisynthetic products derived from beech trees. It can be administered intramuscularly, subcutaneously or orally. Evidence show that pentosane polysulfate (PPS) can act on multiple pathways in the pathogenic process of OA (DMOADs), such as preservation of PG, inhibition of proteolytic enzymes responsible for PG and collagen degeneration and increasing production of tissue inhibitors of metalloproteinases -3 (TIMP-3) by synoviocytes and chondrocytes (Mcllwraith, 2016e).

BIOLOGICAL THERAPIES

Over the past decade, biological therapies have transformed the therapeutic approach of joint disease in humans and horses. Currently there is sensible proof-of-principle that some biological therapies are effective in joint disease, but further research is required to improve and optimize biological therapies available.

Autologous conditioned serum

Autologous conditioned serum is also known as IRAP (interleukin-1 receptor antagonist protein) and IRAP II. The principle method of action of IRAP is to inhibit production of IL-1 through the inhibition of synthesis, however evidence have shown that IL-1 receptor antagonist protein is not the only protein in the composition of IRAP, and that there is also upregulation of IGF-1, TGF-β, TNF-α and IL-1β (Frisbie, 2016a). The clinical use of ACS in equine patients suffering from joint disease remains anecdotal (Frisbie, 2016a). A survey revealed that up to 54% of equine practitioners make use of ACS, predominantly in joints non-responsive to intra-articular corticosteroid therapy (Ferris et al., 2009). A study evaluating the use of ACS in experimental equine OA found a significant improvement in lameness, improved synovial membrane parameters and a significant decrease in articular cartilage fibrillation, thus indicating a DMOADs effect of ACS (Frisbie et al., 2007a).

Stem cell therapy

Stem cells have the ability to self-replicate and differentiate into specific tissue types. In orthopaedic medicine mesenchymal stem cells (MSCs) are of main interest (Frisbie, 2016b). The use of stem cells for the treatment of orthopaedic diseases has increased dramatically since 2003. There is strong evidence in the literature that MSCs have both anti-inflammatory and immunomodulatory properties. Mesenchymal stem cells have been shown to decrease PGE_2 levels in synovial fluid compared to placebo controls in horses, acting as a SMOAD (Frisbie et al., 2009). In rabbits with anterior cruciate ligament transection surgery, MSCs have been shown to prevent cartilage degeneration, osteophyte formation and subchondral bone sclerosis compared to control animals, thus exhibiting a DMOAD (Singh et al., 2014). For intra-articular application of stem cells an autologous bone marrow-derived culture is used, and a total dosage of 50 million MSCs has been proven to have the most reliable outcome in human clinical studies (Vangsness et al., 2014). The issue of a reaction to intra-articular administration of MSCs has been investigated. Intra-articular inflammatory reactions following the administration of 15 million MSCs (autologous, allogeneic, or xenogeneic) into normal fetlock joints of horses revealed an increase of synovial NCC of greater than 40 000 cells/μL and an increase in TP greater than 40 g/L (Pigott et al., 2013). Intra-articular MSCs have been associated with a joint 'flare' in 9% of clinical cases. Further research is required on the use of MSCs in horses in terms of joint disease.

Platelet-rich plasma

Platelet-rich plasma was first recognized as an effective agent for bone and tissue repair within the field of human dentistry and oral maxillofacial surgery in 1998 (Marx et al., 1998), followed by evidence of improving skin graft wound healing in the field of plastic surgery. Platelet-rich plasma is classically described as a volume of plasma that has a platelet count above baseline (Zhu et al., 2013). In general, PRP is defined as a concentration of platelets that is significantly greater than the baseline count in peripheral blood (Frisbie, 2016a). Platelets are small, anucleated structures in the peripheral blood and play an important role in haemostasis (Grozovsky et al., 2010). The normal platelet count in peripheral blood of horses range between 80–350 $\times 10^9$/L (Grozovsky et al., 2010).

DeLong et al. (2012) found that a significant improvement in musculoskeletal disorders (i.e. tendonitis), could be seen with platelet preparation concentrations 2–6 times above the baseline value. However, research has shown that platelet concentrations greater than 6 times

above the baseline value have been associated with deleterious effects on bone healing, suggesting that a higher concentration of platelets are not necessarily beneficial (Gruber et al., 2002; Weibrich et al., 2004).

The generic term "PRP" has recently expanded to include a variety of final products. In an attempt to more precisely delineate these products based on their leukocyte and fibrin content, they have been called pure PRP, leukocyte-rich PRP, pure platelet-rich fibrin and leukocyte- and platelet-rich fibrin. The terms "platelet-rich concentrate" and "platelet concentrate" are also used interchangeably for PRP (Zhu et al., 2013).

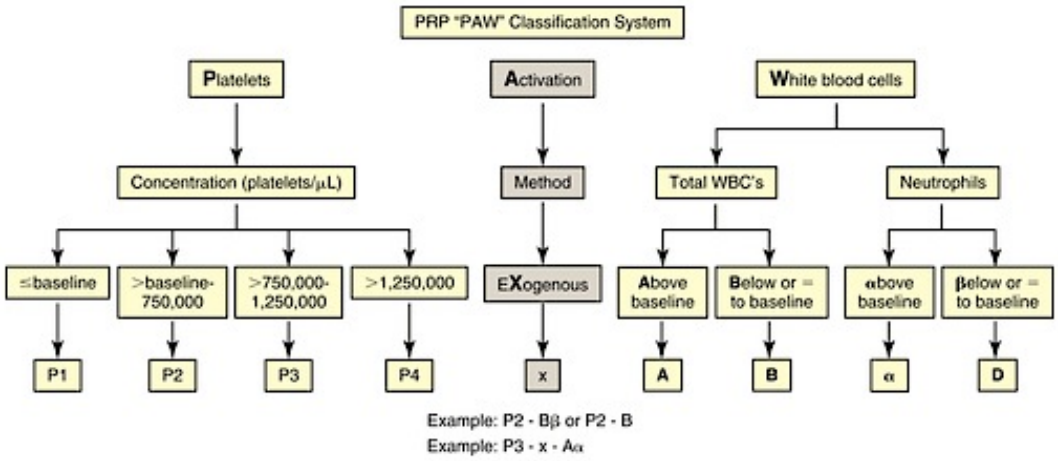

Figure 2: Schematic representation of the platelet-activation white blood cell (PAW) classification system for platelet-rich plasma products (DeLong et al., 2012).

Knowledge of the preparation technique and composition of a PRP product is paramount to its clinical use. Three basic factors are of importance here namely the concentration of platelets, the presence of platelet activators and the concentration of leukocytes in the preparation. The role of each of these basic factors as well as optimization has not been well defined. In 2012, DeLong et al. proposed a classification system for PRP that outlines the above, called PAW (platelet-activation white blood cell) system (Fig.2).

Platelets

It is important to understand the physical composition of the platelet to relate its function in PRP (Fig.3). Platelets are derived from the division or fragmentation of megakaryocytes, and

are cellular fragments devoid of a nucleus, with a relatively short half-life of 1 week (Frisbie, 2016a).

Platelets contain α granules, which upon activation will degranulate and release proteins. There are over 200 proteins contained within platelets, and PRP should thus be considered a "soup" of therapeutic factors rather than a single entity. This milieu of bioactive molecules contains both good and bad factors, resulting in a ratio of factors that will ultimately dictate the response in any given situation. Platelets do synthesize some proteins for up to 2 days after activation, these can be proinflammatory or modulatory cytokines (i.e. IL-1, TNF-α and IL-6 etc.); this however has not yet been conclusively demonstrated for growth factors (Textor, 2011).

A significant proportion of the milieu of bioactive molecules consists of growth factors. The predominant growth factors cited currently in platelets are PDGF-BB, TGF-β, and vascular endothelial growth factor (VEGF).

Platelet-derived growth factor-BB and TGF-β are known to play a critical role in cell proliferation, chemotaxis, cell differentiation and angiogenesis, all required for modulation of inflammation and repair (Castillo et al., 2011). Transforming growth factor-β favours specifically the chemo attraction of monocytes and macrophages, and a combined attraction and proliferation of fibroblasts (Arguelles et al., 2006). Platelet-rich plasma may therefore be used to deliver high concentrations of growth factors to injured tissue sites, thus improving the quality of native tissue repair.

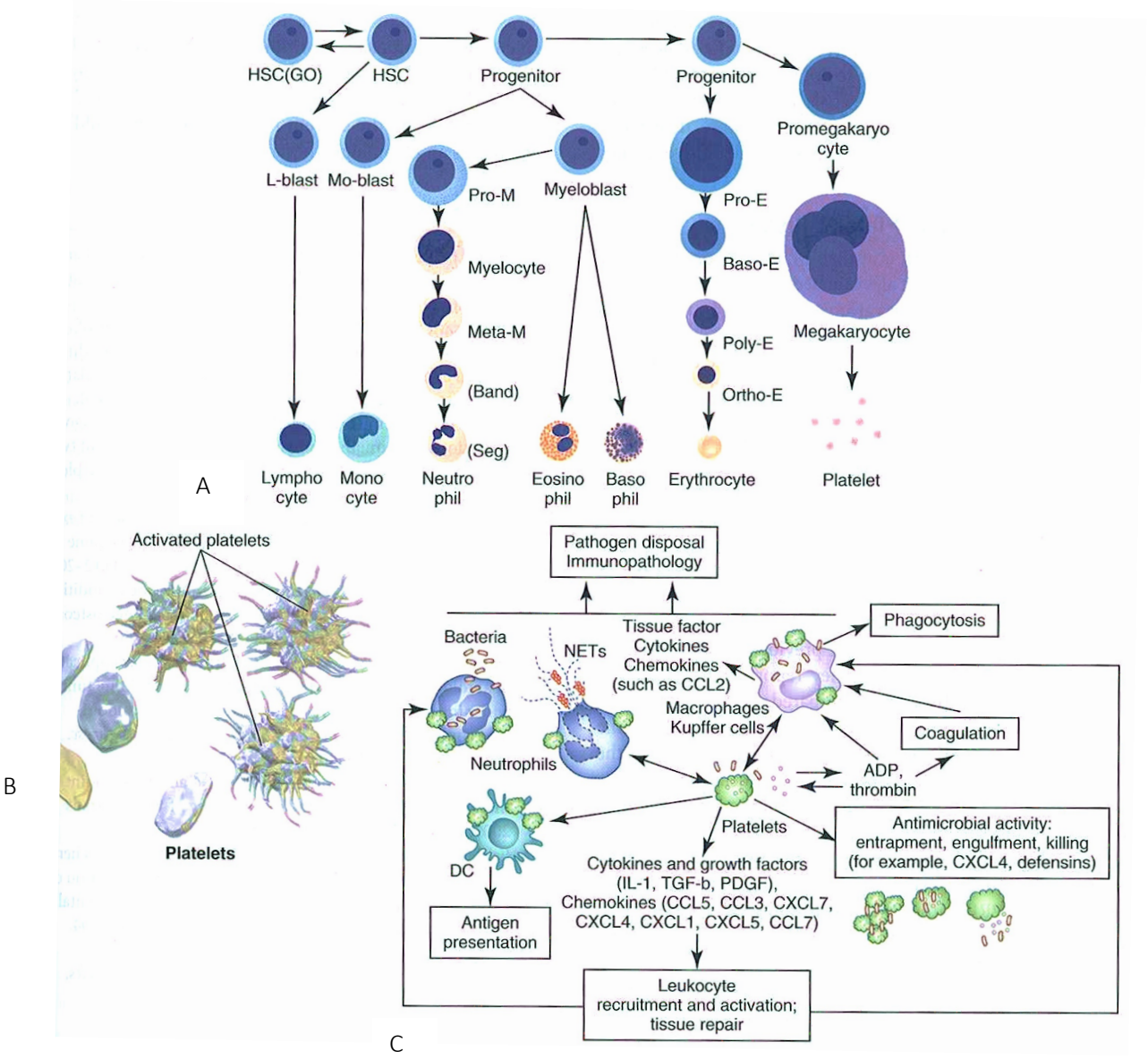

Figure 3: (A) Schematic representation of blood cell and platelets, outlining the origin of platelets. Baso-E, Basophillic erythroblast; HSC, hematopoietic stem cell; L-blast, lymphoblast, lymphocyte; Meta-M, metamyelocyte, neutrophil, eosinophil, basophil; Mo-blast, monoblast, monocyte, myeloblast; Ortho-E, orthochromatic erythroblast, erythrocyte, promegakaryocyte, megakaryocyte, platelet; poly-E, polychromatic erythroblast; Pro-E, proerythroblast; Progenitor, progenitor cell; Pro-M, promyelocyte, myelocyte. (B) Activated platelets. (C) Platelets and leukocytes as partners in innate immunity (Frisbie, 2016a).

Transforming growth factor-β is released from platelets in its active form and is widely considered a promoter for chondrocyte anabolism *in vitro* (enhancing matrix production, cell proliferation, osteochondrogenic differentiation), thus intra-articular administration is expected to improve bone formation *in vivo*. It decreases type I collagen gene expression, which simultaneously upregulates type II collagen and aggrecan gene expression.

Transforming growth factor-β cooperates with bFGF to induce the migration and supplementation of bone-marrow stromal cells (BMSCs) toward the site of injury and also stimulate cell homing, proliferation and chondrogenic differentiation; however, sustained release of TGF-β is required in the entire process of differentiation into cartilage. In addition, TGF-β facilitates cell proliferation by changing cellular morphologic features; therefore, both the quantitative and qualitative components of PRP are effective in mimicking the natural processes. By initiating and facilitating the formation of cartilage, PRP may be a potential candidate for inducing chondrogenesis, and sustaining chondrocyte phenotype *in vivo* and *in vitro* (Zhu et al., 2013).

Transforming growth factor-β has various effects in the pathogenesis of OA, both pro-and anti-inflammatory. Evidence reveal that TGF-β can play a role in human OA, as synovial fluid concentrations of TGF-β were found to be elevated during synovitis (Sutton et al., 2009). This growth factor may reduce the proliferation of synoviocytes, and induce a more mature cell phenotype with cells that are slower growing, produce more collagen and produce fewer proteases to degrade cartilage, therefore offering a protective roll (Sutton et al., 2009). Evidence of this protective effect has been provided by the observation that cartilage damage is associated with decreased levels of the TGF-β isoform, TGF-β3. Transforming growth factor-β1 and TGF-β2 have been shown to deactivate macrophages that may be present in the synovial fluid, thereby removing their function to undergo respiratory burst. Increased production of PG and greater concentrations of this cartilage matrix component have been found in murine articular joint cartilage after an intra-articular TGF-β injection. A study on rabbit articular chondrocytes showed that TGF-β reduced IL-1β stimulated production of the MMPs and collagenase by lowering mRNA levels. It was also shown that the cytokine inhibited IL-1β receptor expression, suggesting that TGF-β has the ability to suppress some of the degenerative effects of IL-1β on articular cartilage (Sutton et al., 2009).

In contrast, TGF-β1 has also been reported to induce various pro-inflammatory changes such as synovial fibroplasia, synovitis, cartilage destruction and excess subchondral bone formation in humans. Moreover, up-regulation of TGF-β has been discovered in developing human osteophytes, suggesting a role of ossification in OA. TGF- β may also induce MMPs in human OA cartilage samples, resulting in cartilage degeneration. Various inflammatory mediators have been shown to induce TGF-β1 release, such as IL-1β in bovine articular chondrocytes and proteases. This all leads to further evidence to the pro-inflammatory role of TGF-β1 in OA (Sutton et al., 2009).

Platelet-derived growth factor is primarily found and stored in the α granules of platelets. It is chemotactic to macrophages and fibroblasts and enhances fibronectin and glycosaminoglycan deposition, as well as increases cell activity early in the healing response of articular cartilage (Wasterlain, 2012). Platelet-derived growth factor also plays a role in the mitigation of mesenchymal stem cells, which plays a crucial role in cartilage regeneration (Provost, 2012).

White blood cells

The concentration of leukocytes in PRP that is acceptable has been debated numerous times in literature, but evidence supports that a low number of leukocytes are more suitable (Frisbie, 2016a). Leukocytes may play a valuable antimicrobial role in PRP treatment. The concentrated presence of leukocytes within a leukocyte rich-PRP may create a local environment of increased immunomodulatory capability, which may aid in the prevention or control of infection at the injection site (Zimmerman et al., 2001).

Evidence also suggests that leukocytes may enhance PRP growth factor concentrations, either through release of growth factors from leukocytes within the PRP or by acting as a stimulus for the release of growth factors from platelets (Zimmerman et al., 2001). Zimmerman et al. (2001) found that leukocyte concentration in PRP accounted for a third to a half of the variance of the growth factor concentrations in PRP.

Leucocyte-rich PRP systems (GPS III®, Magellan®) demonstrated a significant increase in the concentrations of PDGF-αβ, PDGF-BB and VEGF, compared to leukocyte-poor PRP systems (Cascade®, Arthrex®). The significant positive correlation between leukocyte concentration and VEGF and PDGF-BB concentrations explains some of this observed difference in growth factor concentration between the leukocyte-rich and leukocyte-poor systems. Thus, the leukocyte concentration may account for an increase in growth factor concentrations in leukocyte-rich PRP and may be a reason to rather use leuckocyte-rich PRP to optimize growth factor concentrations (Zimmerman et al., 2001).

Conversely, leukocytes, specifically neutrophils, may release pro-inflammatory mediators such as IL-1, TNF-α, IL-6, MMP 8 and 9 that could result in an increased local inflammatory environment and may contribute to further cartilage degeneration (McLellan and Plevin, 2011). Many researchers agree with this sentiment and recommend a maximum leukocyte concentration of 0.1–3.0 $\times 10^9$/L to prevent inflammatory cytokine accumulation (McLellan and Plevin, 2011). One study evaluated the anabolic and catabolic activities of high-platelet

and low-platelet systems on equine articular cartilage *in vitro* and concluded that leukocyte-rich systems should be avoided (Frisbie, 2016a).

Platelet activation

Platelets are naturally activated (otherwise known as degranulation) by the clotting cascade, resulting in the release of growth factors from α granules. Approximately 70% of the stored growth factors are released within 10 minutes, and nearly 100% of the growth factors are released within 1 hour upon activation.

The timing and cumulative release of growth factors are determined by the activation method used. Activation refers to two key processes within the PRP preparation system. Firstly, platelet degranulation to release α granules containing growth factors and secondly, the cleavage of fibrinogen to initiate the matrix formation (Wasterlain et al., 2012).

Three methods of platelet activation have been reported in the literature. The first is endogenous activation; the second is through the addition of calcium chloride; and the third through the addition of thrombin (Frisbie, 2016a). Once platelets are activated, the PRP composition is often then referred to as the PRP releasate (Wasterlain et al., 2012).

The latest evidence shows that platelets are activated when PRP is administered intra-articularly, upon exposure to synovial fluid (Textor et al., 2013b). Platelet activation upon exposure to synovial fluid is most likely physical due to the high viscosity of the fluid which is likely to exert different shear forces on platelets. Platelet response to shear forces is not uniform and are reported to be less stimulated by high shear conditions (i.e. rapid, laminar blood flow), than compared to low shear or turbulent conditions (Yin et al., 2011). Thus, this is a valued option of platelet activation in PRP in equine private practice.

Calcium chloride results in a more acidic solution intra-articularly, that has been proven to result in pain and a burning sensation in humans (DeLong et al., 2012); however, this finding has not been documented in equine studies when calcium chloride was used as an platelet activator for intra-articular use of PRP (Textor and Tablin, 2013a).

Thrombin activation of PRP for intra-articular use has resulted in severe side effects in horses, specifically an increase in synovial fluid total protein and leukocyte concentration, increase in synovial effusion, periarticular effusion and heat, pain on flexion and lameness of the limb used (Textor and Tablin, 2013a). Although endogenous and calcium chloride

activation may also result in the above mentioned synovial and clinical findings, the magnitude of change is significantly less for these two activation methods. Thus, it is widely accepted in the literature to either use endogenous or calcium chloride activation methods, as they are considered safe (Frisbie, 2016a).

Platelet systems

Several commercial platelet separation systems are currently available, functioning either as a centrifugation of a gravitational system (Fig. 4, 5). Various human systems are also used in veterinary medicine (Hessel et al., 2015). Platelet-rich products produced by these systems differ significantly regarding the relative platelet count, platelet collection efficiency and repeatability, leukocyte count, erythrocyte count and anabolic growth factor concentration, all which will influence the cellular response elicited by the product (Hessel et al., 2015).

A study evaluating the platelet count, leukocyte count, PDGF-BB and TGF-β_1 of four commercially available PRP systems (i.e. Angel™[a], ACP™[b], E-PET(V-PET)™[c] and GPS™III[d]) and a manual double centrifugation system found that one method reduced total leucocyte counts to 9% of the baseline value (ACP™), while the others had a mean fold increase varying from 116 to 663% of the baseline. In addition, the differential leucocyte counts differed between the products and the various systems had significantly different mean growth factor enrichments (184–1255% for PDGF-BB and 93–560% for TGF-β_1) (Hessel et al., 2015).

Thus, different preparation methods or volumes of administration for the PRP preparation will most likely yield different results (Frisbie, 2016a). The Arthrex ACP™ double syringe system requires 10 mL of blood to produce 2-5 mL of PRP with a platelet concentration of 300-500 $\times 10^9$/L, however requires centrifugation for 5 minutes at 1500 rpm. This means that the sample will need to be prepared at a laboratory/off site. The V-PET™ system requires 60 mL of blood to produce 5 mL of PRP with a platelet concentration of 396-1089 $\times 10^9$/L, and the process is performed using gravity, thus it can be performed at the stable.

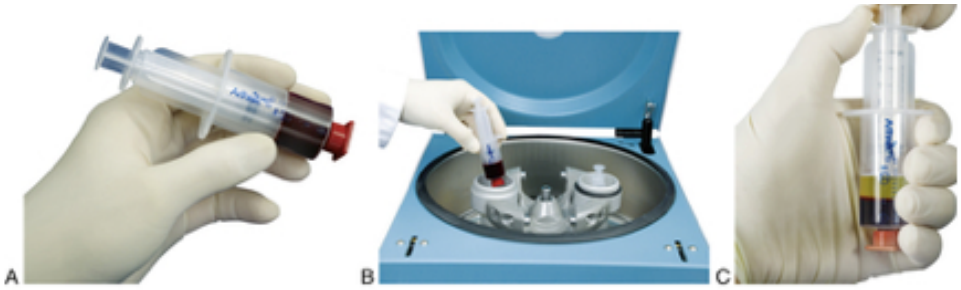

Figure 4: (A) Arthrex ACP™ double syringe system. Blood is collected into the syringe, (B) centrifuged for 5 minutes where the platelet-containing plasma (ACP) (C) is separated from the red blood cells (RBCs) (Frisbie, 2016a).

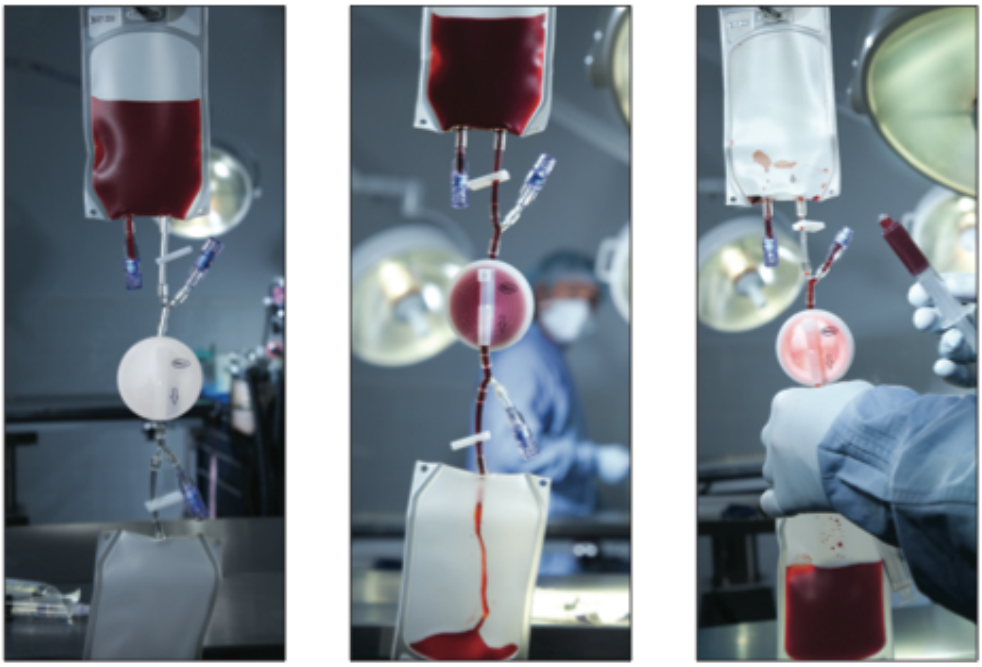

Figure 5: V-PET™ gravitational filtration based system. With clamps closed, whole blood is injected into the top bag port, and mixed with sterile water. The clamps are then opened, allowing the blood to flow through a filter into the bottom waste collection bag. Platelets are retained within the filter. Once the blood has passed completely through the filter and into the bottom bag, the clamps are closed, isolating the filter. 2% Saline (NaCl) solution is used to recover the platelets from the filter, by attaching an empty, collection syringe to the port B and flushing with the saline-filled syringe from port C.

Intra-articular PRP in humans

Two reviews have been published on the treatment of knee OA with PRP in humans (Khoshbin et al., 2013). The first review included a total of 653 patients and 727 knees, four studies were randomized control and two were prospective cohort studies. The PRP volume used for administration was 3-8 mL, injected at a frequency of 1-3 weeks for two to four injections in total. The PRP systems were different in terms of the number of centrifugations performed, the platelet- and leukocyte counts, and the use of an activator. In five of the six studies, PRP was also compared to HA, and in the sixth study, to saline. The results found a significant improvement of functional outcome with PRP treatment for a minimum of 24 weeks. There were few adverse effects reported, which included pain at the site of injection or synovial effusion.

The second review documented 1543 patients (Chang et al., 2014). The review reported a significant improvement of function after PRP therapy in knee pathology and that PRP was superior to HA with a longer duration of action. A 9.59% incidence of adverse effects was recorded, which was not significantly different compared to HA. The review could not identify significant differences in effectiveness of PRP products based on centrifugation methods or activation methods. However, it has been shown that a single centrifugation technique was not as compelling as the double-centrifugation technique. The review recommended the use of PRP in cases of mild OA as opposed to severe cases, with a minimum of three treatments (Chang et al., 2014).

Intra-articular PRP in horses

There is very little published work available on the intra-articular use of PRP in horses. Textor and Tablin (2013a) evaluated one commercial product (V-PET™) in normal horses using various platelet activation methods prior to administration of PRP intra-articularly. The study indicated that the use of calcium chloride as an activator, or the lack of an activator, yielded the fewest clinical adverse reactions (i.e. synovial effusion, periarticular heat or swelling, and response to limb flexion), the highest growth factor concentrations (i.e. PDGF-BB and TGF-β1) and the lowest endogenous leukocyte release into the synovial fluid. The study also concluded that intra-articular PRP may induce a mild to moderate inflammatory response in the synovial fluid of normal joints, which can last approximately one day. The use of thrombin platelet activation lead to an increase in IL-1 and TNF levels within the synovial fluid and is not advised to be used as an activator due to severe clinical adverse reactions.

To date there has been no published research, only anecdotal reports, describing the use of PRP in horses with joint disease. Bertone et al. (2014) studied the use of an autologous protein solution (APS) product in 40 horses exhibiting OA. The end product consisted of a 12-fold increase in the leukocyte count, 1.6-fold increase in the platelet count, as well as >3-fold increase of various proteins. The study found a reduction in lameness at 14 days after treatment, and satisfactory improvement based on reported client-assessed parameters at 12 and 52 weeks. The use of APS in milder cases of OA has been shown to be more effective compared to more severe cases of OA (Bertone et al., 2014).

MATERIALS AND METHODS

MODEL SYSTEM

Experimental study

EXPERIMENTAL DESIGN

ANIMALS

Five mature horses with OA and synovial effusion, affecting either the antebrachiocarpal, middle carpal or metacarpophalangeal joints were studied. Osteoarthritis was graded radiographically on a scale of 0 to 3 (0 = no abnormality detectable, 1 = mild abnormalities detectable, 2 = moderate abnormalities detectable and 3 = severe abnormalities detectable) (Frisbie et al., 2007). Five healthy mature horses with no radiographic and clinical abnormalities of the antebrachiocarpal, middle carpal or metacarpophalangeal joints were studied. Horses were excluded from the study if they had intra-articular injections or arthroscopic surgeries within 60 days of the study, received medication or oral supplementation for joint disease within 28 days of the study, or if there was evidence of a fracture, active infection, or a history of chronic infection associated with the joint. Horses were housed in small paddocks at the Onderstepoort Veterinary Academic Hospital for the duration of the study. The animals used were part of research herds belonging to the Institution.

EXPERIMENTAL PROCEDURE

DATA CAPTURE

Preparation of the platelet-rich product

An autologous PRPr was prepared using a gravity filtration system, Veterinary Platelet Enhancement Therapy (V-PET™)[c], in accordance with the manufacturer's instructions. Fifty-five mL of whole blood was collected from each horse into a 60 mL syringe containing 5 mL acid-citrate-dextrose A (ACD-A) solution. The anti-coagulated blood was injected into the primary bag (top bag) containing 9 mL sterile water using port A (Fig. 6). After gentle mixing of the solution, the system was suspended with the primary bag at the top and the solution allowed to pass through a filter to the secondary bag at the bottom using gravity. Platelets were preserved in the filter and flushed back, using a 2% saline (NaCl) solution in a syringe connected to port C, into a sterile recovery syringe connected to port B. Using sterile

techniques throughout, 1 mL of the PRPr was retained for determination of platelet-, leukocyte and erythrocyte counts. The remaining 4 mL was used for the PRPr treatment.

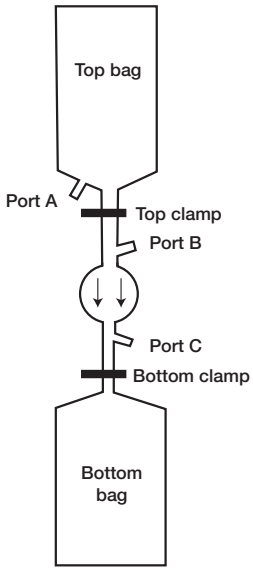

Figure 6: Veterinary Platelet Enhancement Therapy (V-PET™)[c] system. With clamps closed, whole blood is injected into the top bag port, and mixed with sterile water. The clamps are then opened, allowing the blood to flow through a filter into the bottom waste collection bag. Platelets are retained within the filter. Once the blood has passed completely through the filter and into the bottom bag, the clamps are closed, isolating the filter. 2% Saline (NaCl) solution is used to recover the platelets from the filter, by attaching an empty, collection syringe to the port B and flushing with the saline-filled syringe from port C.

*

Sterile water in the primary bag is used to promote hypotonic swelling of the platelets to assist in their preservation in the filter. As blood flows through the (V-PET™)[c] system, platelets are selectively captured on the surface of the filter through a complex interaction of size exclusion and adsorption, while the majority of erythrocytes pass through. The platelet concentrate is then recovered by back-flushing the filter with a 2% saline (NaCl) solution.

Treatment, sampling, and clinical evaluation

The clinical evaluation of the horses was performed by Y.S. and H.J.M., and arthrocenteses were performed by Y.S. Data were collected at 6 time points: days 0 (immediately prior to PRPr intra-articular injection), 1, 2, 5, 21, and 56 post-treatment. One week prior to the commencement of the study all the horses underwent clinical, radiographic and lameness examinations. On clinical examination the temperature, pulse, and respiration rates were recorded (normal reference intervals were considered 37.5-38.5 °C, 28-44 beats/min, and 8-24 breaths/min, respectively).

Radiographic assessment included standard radiographic views of the joint being evaluated (Appendix 1). Views for the antebrachiocarpal- and middle carpal joint consisted of a latero-medial (LM), flexed LM, dorso-palmar (DPa), dorsal 45° lateral-palmaromedial oblique (D45°L-PaMO) and dorsal 45° medial-palmarolateral oblique (D45°M-PaLO). For the

metacarpophalangeal joint a LM, flexed LM, DPa, D45°L-PaMO, D45°M-PaLO were performed. Radiographs were assessed by Y.S. for periarticular osteophytes, joint-space narrowing, subchondral bone sclerosis or lysis and/or the presence of osteochondral fragmentation. An OA score was assigned accordingly (0-absent, 1-mild, 2-moderate and 3-severe).

Lameness evaluations were performed according to the AAEP (Ross, 2011). Subjective lameness grades were allocated from 0 to 5 (0-lameness not perceptible under any circumstances; 1-lameness is difficult to observe and is not consistently apparent regardless of circumstances (e.g. under saddle, circling, inclines, hard surface, etc.); 2-lameness is difficult to observe at a walk or when trotting in a straight line but consistently apparent under certain circumstances (e.g. weight-carrying, circling, inclines, hard surface, etc.); 3-lameness is consistently observable at a trot under all circumstances; 4-lameness is obvious at a walk; 5-lameness produces minimal weight bearing in motion and/or at rest or a complete inability to move). In addition, joints were evaluated for response to flexion at rest only (0-absent; 1-mild; 2-moderate; 3-severe), synovial effusion (0-absent; 1-mild; 2-moderate; 3-severe), and presence of periarticular signs, such as palpable swellings or heat (present or absent). At each time point the clinical and lameness examinations were repeated.

Blood was collected by jugular venipuncture for a complete blood count (CBC), TP- and SAA concentrations determination and PRPr preparation. For arthrocentesis the horses were sedated with romifidine (0.02 – 0.08 mg/kg intravenously) as required. Arthrocentesis sites were aseptically prepared, and each selected joint was sampled using a sterile technique. On day 0, 2 mL synovial fluid was aspirated from each joint using a 20 gauge, 1.5” needle, and 5 mL syringe prior to injection of 4 mL PRPr. For all the days post-injection 2 mL synovial fluid was collected. On days 1 and 5, 0.5 mL synovial fluid was retained for growth factor concentration analysis. A light bandage was applied to each joint used after arthrocentesis.

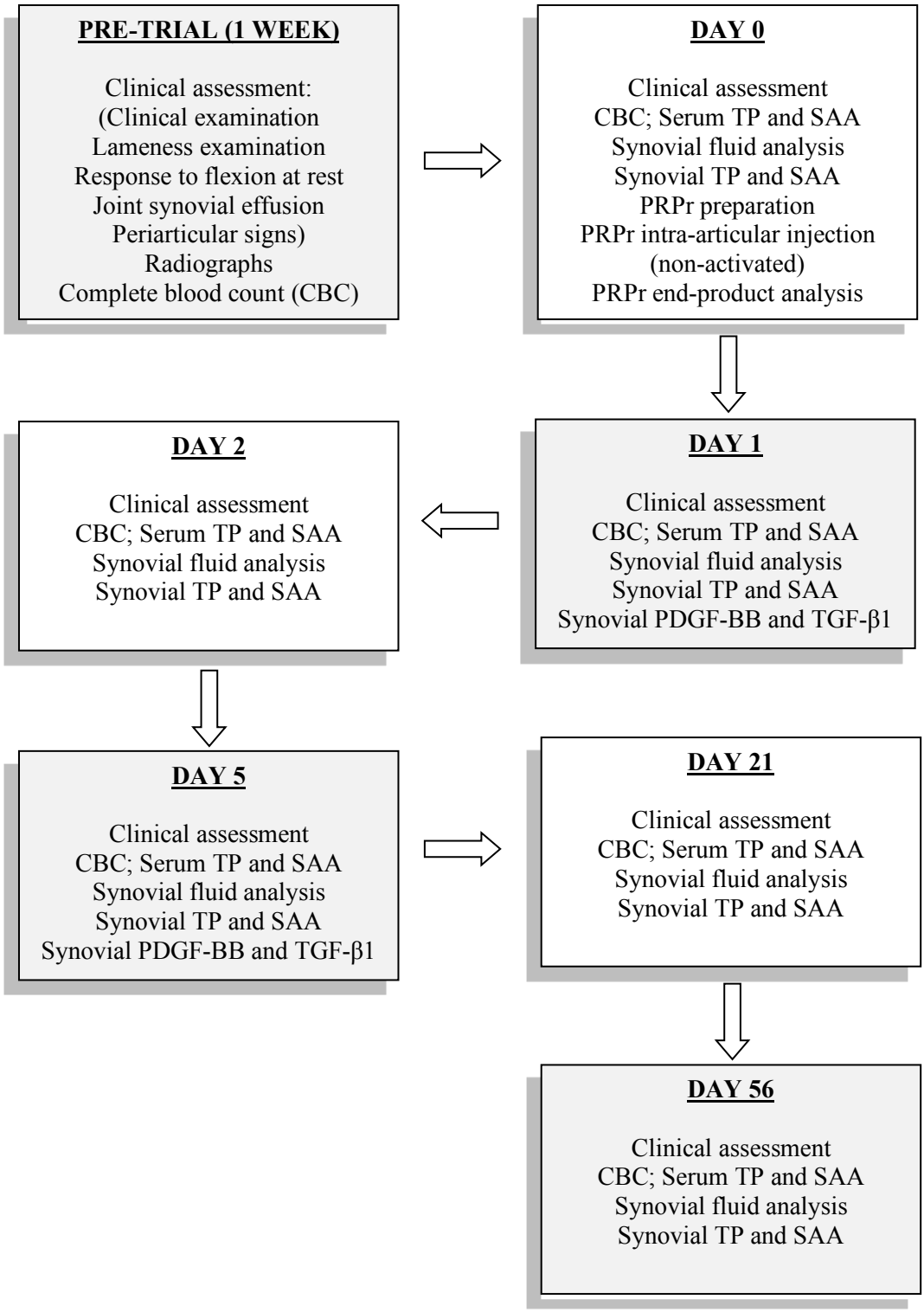

Figure 7: Schematic representation of treatment, sampling and clinical evaluation of the study.

SAMPLE HANDLING AND ANALYSIS

Blood for a CBC was collected into EDTA vacutainer tubes and for TP and SAA concentrations into serum vacutainer tubes. The serum sample was left to clot and then centrifuged at 2100 *g* for 8 minutes and aliquoted into different cryovials for storage at -80°C. Synovial fluid was immediately transferred to EDTA and serum vacutainer tubes for cytological assessment, as well as SAA- and TP concentration determinations. A 1 mL aliquot of the PRPr was transferred to a standard EDTA tube.

All samples were analysed by the Clinical Pathology laboratory, University of Pretoria, South Africa. A CBC was performed within 2 hours of collection on an automated analyser (ADVIA® 2120)[e], and data recorded included the total leukocyte count and differential leukocyte counts that were manually confirmed by an experienced laboratory technologist. A cell count was also performed on the PRPr, specifically to determine the platelet count.

Serum and synovial SAA- and TP concentrations were analysed as a batch using an automated chemistry analyser (Cobas Integra 400 Plus)[f]. Serum amyloid A concentrations were measured using an automated latex agglutination turbidimetric immunoassay (Eiken SAA)[g], previously validated for use in equines (Jacobsen et al., 2006a; Sanchez Teran et al., 2012). The NCC was determined on the synovial fluid using an automated analyser (Cell Dyne 3700)[h]. A direct smear for cytological evaluation was prepared, after which the remaining sample was centrifuged for 8 min at 1520 *g*. A smear was made of the sediment and the supernatant was stored at -80°C. An experienced clinical pathologist that was blinded from the group distribution evaluated a direct and concentrated smear of the synovial fluid. A differential leukocyte count was performed on all the fluid samples.

Concentrations for PDGF-BB and TGF-β1 in the synovial fluid samples were determined on days 1 and 5 using a sandwich enzyme-linked immunoassay technique that has been validated for use in equines (Human PDGF-BB and Human TGF-β1; Quantikine™)[i] (Textor, 2013a, b). Synovial fluid samples for growth factor analysis were kept on ice. The samples were centrifuged at 1000 *g* for 15 minutes at 4 °C within 30 minutes of collection, and at 10,000 *g* for 10 minutes at 2-8 °C within 2 hours of collection to ensure complete platelet removal, as recommended by the assay manufacturers. The supernatant was stored at -80°C for PDGF-BB and TGF-β1 concentrations to be determined as a batch.

STATISTICAL ANALYSIS

The data were recorded using Microsoft Excel sheet. Descriptive and comparative statistical analyses were performed using Stata 14 statistical software[j]. The data was assessed for normality using the Shapiro-Wilk test, the majority of data were consistent with a normal distribution. Day and group calculated means and standard deviations. Linear mixed models were used to compare the concentrations of the blood and synovial fluid variables between the OA and control group on each day as well as within each group compared to day 0, and for comparison of growth factor concentrations between day 1 and day 5. Bonferroni adjustment for multiple comparisons was used and statistical significance was set at $P<0.05$.

RESULTS

ANIMALS

Animal data are presented in Table 1. The control group consisted of five mature horses (5 mares; 5 Nooitgedacht) with a mean ± standard deviation (± SD) age = 4 ± 0 years. The OA group consisted of five mature horses (4 mares, 1 gelding; 4 Thoroughbred, and 1 Nooitgedacht) with a mean ± SD age = 10.8 ± 6.57 years. Osteoarthritis scale in the OA group was as follows: two horses = 1; two horses = 2; and one horse = 3 (Fig. 8-10).

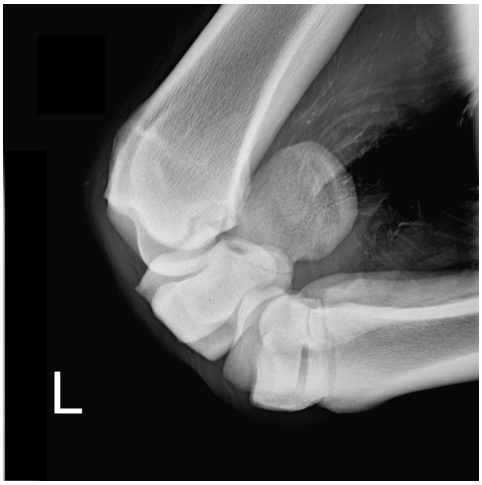

Figure 8: Osteoarthritis scale 1 = mild abnormalities detectable in the left antebrachiocarpal joint. Flexed lateromedial radiographic view of a left carpus. Dorsal is to the left. There is a small osteophyte formation on the dorsoproximal aspect of the intermediate carpal bone.

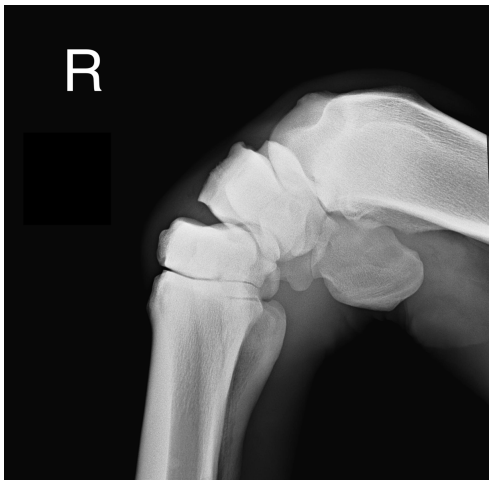

Figure 9: Osteoarthritis scale 2 = moderate abnormalities detectable in the right antebrachiocarpal joint. Flexed lateromedial radiographic view of a right carpus. Dorsal is to the left. There is a large osteophyte formation on the dorsoproximal aspect of the radiocarpal bone and a small osteophyte formation on the dorsodistal aspect of the radiocarpal bone.

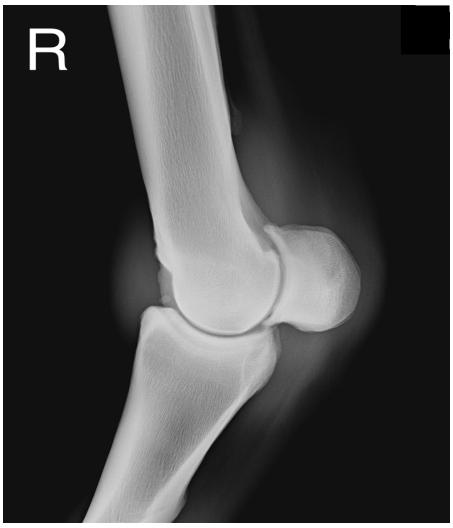

Figure 10: Osteoarthritis scale 3 = severe abnormalities detectable in the right metacarpophalangeal joint. Lateromedial view of right metacarpophalangeal joint. Marked dorsal and palmar soft tissue swelling present in the region of the metacarpophalangeal joint. A small, circular, well circumscribed fragment present on the dorso-proximal aspect of phalanx one. Moderate supracondylar lysis present in association with proximal sesamoid bone clubbing (sesamoiditis).

PLATELET-RICH PRODUCT

The V-PET™*c* was easy and rapid to prepare and 5 mL PRPr was retrieved from the system of each horse. The V-PET™*c* system successfully concentrated the platelets. Mean (± SD) platelet concentration was 621 (± 200) $\times 10^9$/L (range: 396-1089), which was a 4.7-fold increase compared to mean platelet concentration in the blood (Fig. 11). The mean (± SD) leukocyte count was 18.7 (± 4.5) $\times 10^9$/L (range: 12.9-26.1), which was a 2.1-fold increase compared to mean leukocyte count in the blood (Fig. 12).

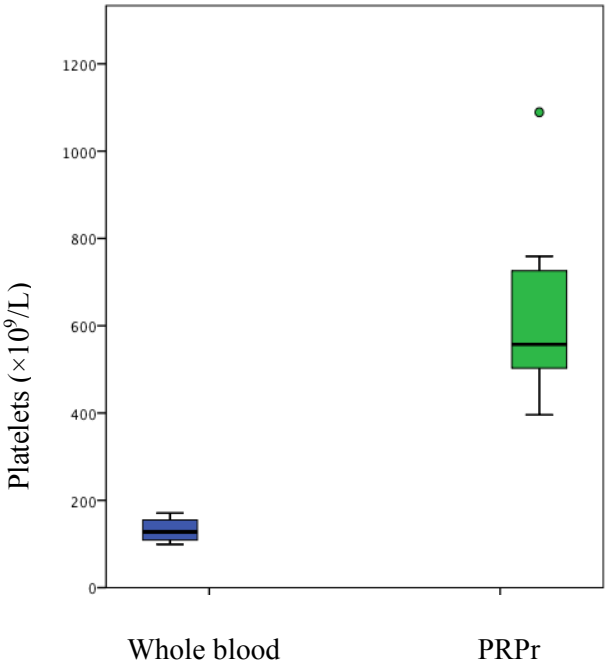

Figure 11: Platelet count of the PRPr produced by gravity filtration compared to the platelet count in the peripheral whole blood.

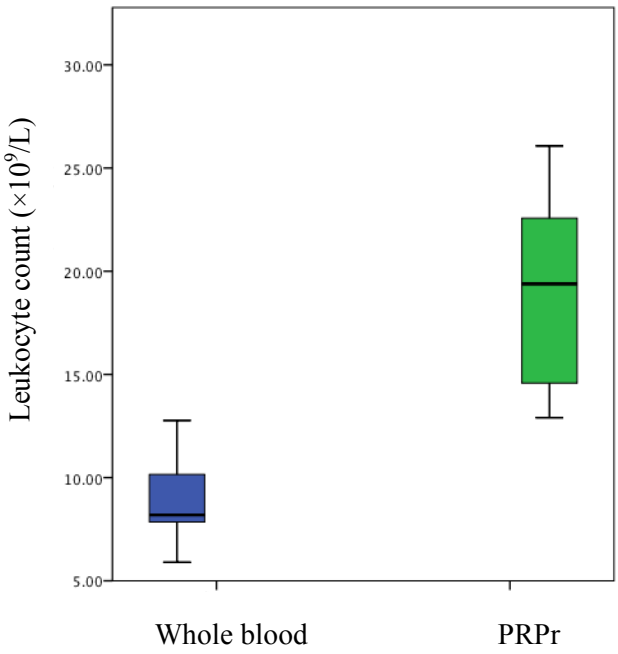

Figure 12: Leukocyte count of the platelet-rich product produced by gravity filtration compared to the leukocyte count in the peripheral whole blood.

CLINICAL EVALUATION OF JOINTS: SYNOVIAL EFFUSION AND FLEXION SCORES, AND PERIARTICULAR EFFECTS

Variables are presented in Table 1. The mean ± SD OA score for the OA group was 1.80 ± 0.83. The synovial effusion score was statistically significant between the control and OA group on day 0 ($p < 0.05$) with a higher score in the OA group. However, within the control group the synovial effusion score was significantly elevated on day 1 and 2 compared to day 0 ($p < 0.05$). The mean synovial effusion score for the OA group was lower on day 56 compared to day 0, however not statistically significant. No statistical significant findings for flexion score were found. Statistical analysis of periarticular signs was not performed due to data type.

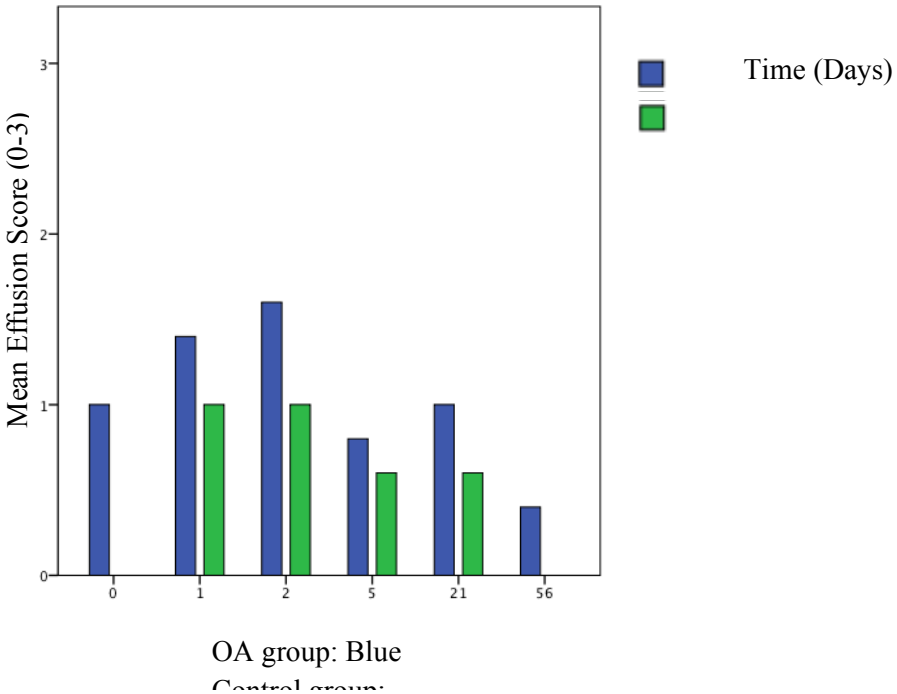

Figure 13: Mean effusion scores after intra-articular PRPr treatment

TABLE 1: Summary of clinical assessments for the control- and OA group (mean ± SD).

Group	Control	OA
OA scale (0-3)	0 ± 0	1.80 ± 0.83
Synovial effusion score (0-3)		
Day 0	0.00 ± 0.00[b]	1.00 ± 1.22[b]
Day 1	1.00 ± 0.00[a]	1.40 ± 0.89
Day 2	1.00 ± 0.00[a]	1.60 ± 0.54
Day 5	0.60 ± 0.54	0.80 ± 0.83
Day 21	0.60 ± 0.54	1.00 ± 0.70
Day 56	0.00 ± 0.00	0.40 ± 0.89
Flexion score (0-3)		
Day 0	0.00 ± 0.00	0.20 ± 0.44
Day 1	0.40 ± 0.54	0.40 ± 0.54
Day 2	0.00 ± 0.00	0.20 ± 0.44
Day 5	0.00 ± 0.00	0.20 ± 0.44
Day 21	0.00 ± 0.00	0.00 ± 0.00
Day 56	0.00 ± 0.00	0.00 ± 0.00
Periarticular signs (yes/no)		
Day 0	0/5	1/4
Day 1	5/0	3/2
Day 2	5/0	5/0
Day 5	3/2	2/3
Day 21	3/2	2/3
Day 56	0/5	0/5

[a]Statistically significant differences from baseline, within a given group ($p < 0.05$).

[b]Statistically significant differences between groups on a specific day ($p < 0.05$).

CYTOLOGIC AND CLINICAL CHEMISTRY CHANGES OF THE SYNOVIAL FLUID

Compared to day 0, the synovial NCC was significantly increased on day 1 and on day 2 in the control group (P<0.001 for both) and on day 1 in the OA group (P<0.001). There were no significant differences in synovial NCC between groups for day 1 or day 2 (Fig 14 and Table 2).

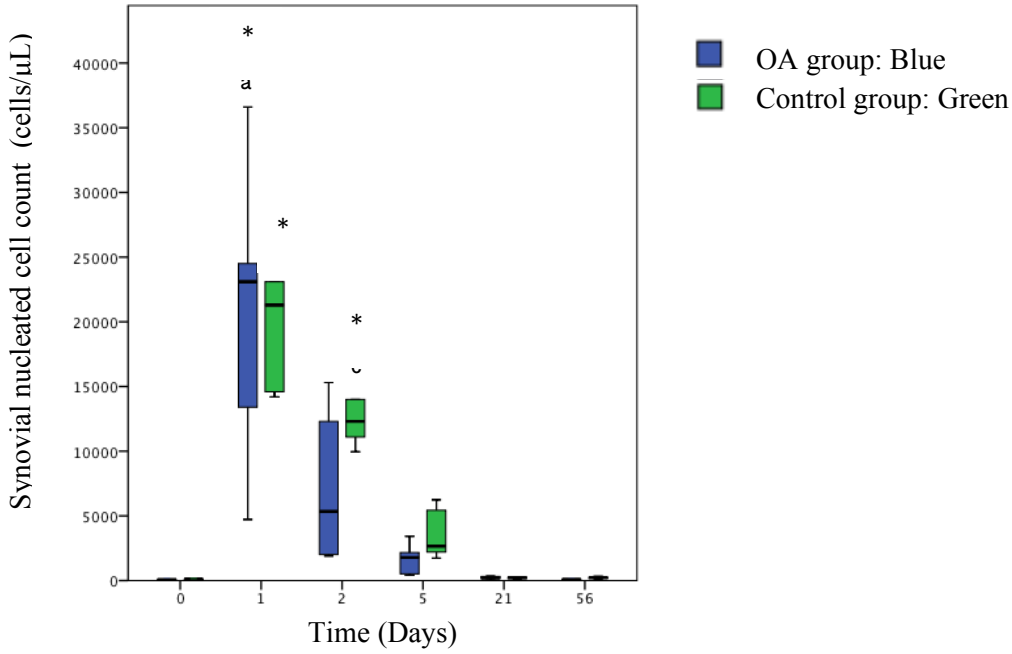

Figure 14: Total NCC in synovial fluid pre- and post intra-articular PRPr injection. On day 1 both groups were significantly different from their corresponding baseline value. On day 2 only the control group was significantly different from the baseline value. Asterisks denote statistically significant differences from baseline, within a given group (P<0.05).

The NCC consisted predominantly of mature intact neutrophils. Compared to day 0, the neutrophil count was significantly increased on day 1 and on day 2 in the control (P<0.001 for both) and OA (P<0.001 for both) groups, but returned to normal numbers by day 21. The neutrophil count was significantly higher in the control group on day 2 compared to the OA group (P<0.001) (Fig. 15).

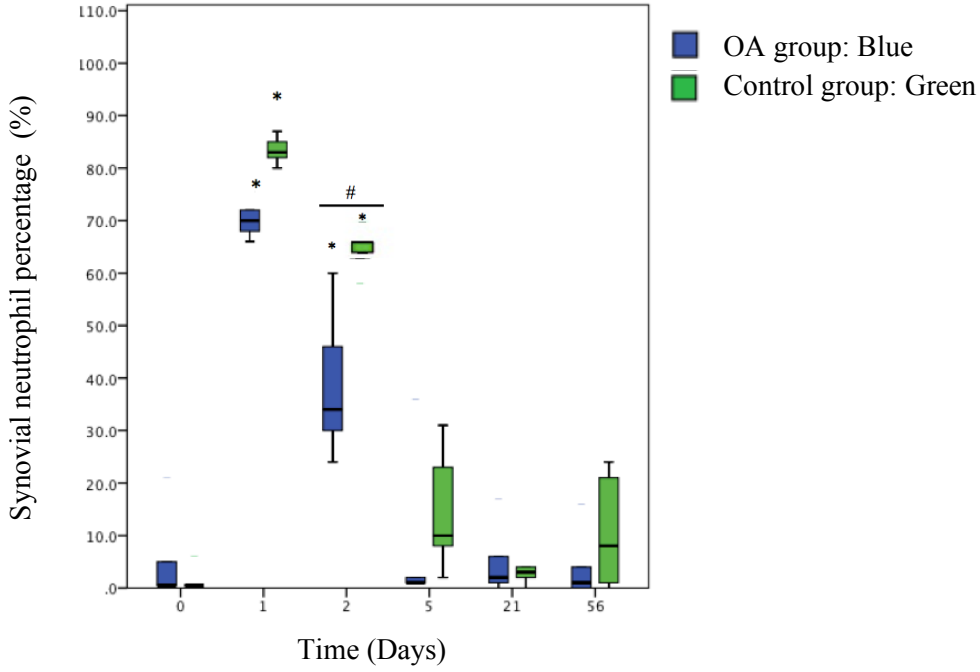

Figure 15: Percentage of neutrophils in synovial fluid pre- and post-intra-articular PRPr injection. Asterisks denote statistically significant differences from baseline, within a given group (P<0.05). Pound sign denote statistically significant differences between groups on a specific day (P<0.05).

GROWTH FACTOR CONCENTRATIONS IN SYNOVIAL FLUID

Variables are presented in Table 2. The mean concentrations of PDGF-BB (Fig. 16) and TGF-β1 (Fig. 17) were increased in both groups; however, concentrations were significantly lower in the OA group on day 1 ($p < 0.01$ and $p < 0.001$, respectively) compared to the control group. On day 5 the mean PDGF-BB remained unchanged in both groups, compared to day 1, with no significant differences between groups. However, the mean TGF-β1 concentration decreased in both groups on day 5 compared to day 1 with no significant difference between groups. There was a significant correlation between TGF-β1 and NCC in both the control (r=0.79; $p = 0.007$) and OA groups (r=0.83; $p = 0.003$), but PDGF-BB only had a significant correlation with NCC in the control group (r=0.80; $p = 0.006$). Only TGF-β1 showed a significant correlation with the synovial neutrophil percentage in both the control (r=0.94; $p < 0.001$) and OA groups (r=0.94; $p < 0.001$) (Table 2).

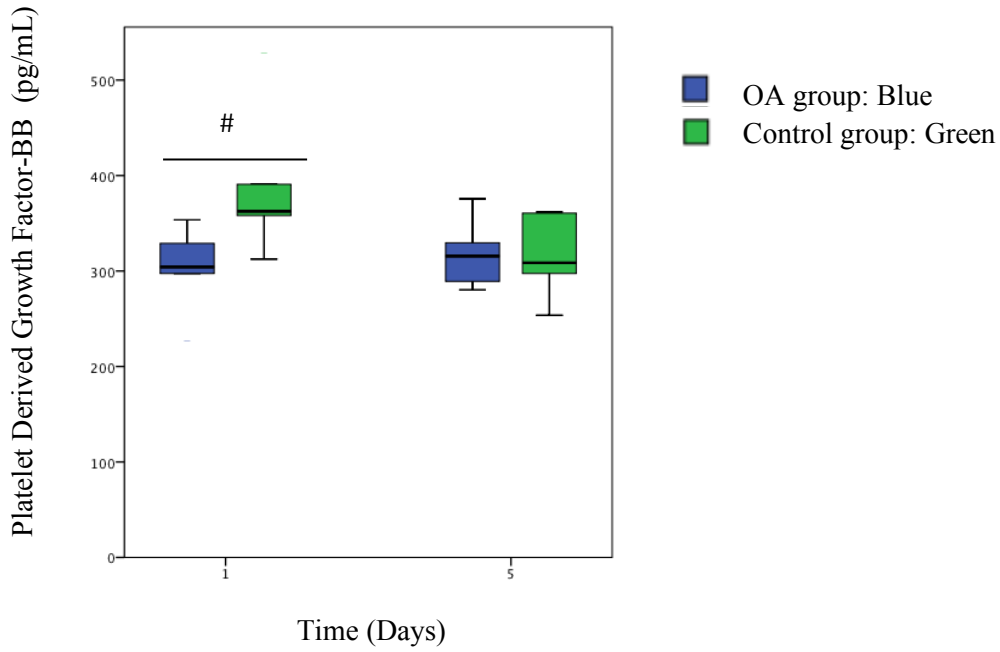

Figure 16: Mean synovial fluid PDGF-BB concentration after intra-articular PRPr injection. PDGF-BB was detected in both groups and was statistically significant between groups on day 1. Pound sign denote statistically significant differences between groups on a specific day ($P<0.05$).

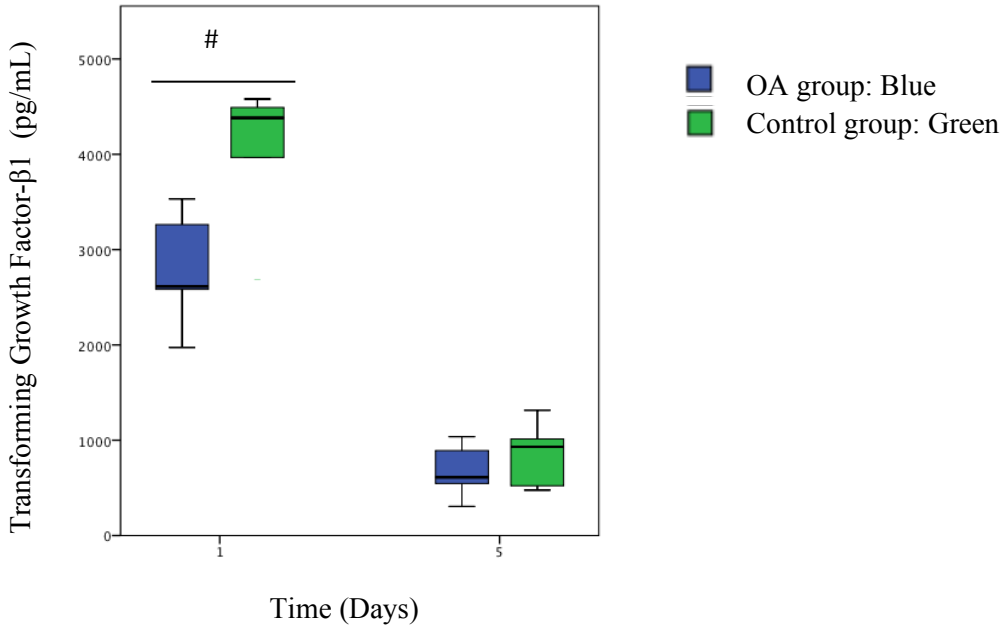

Figure 17: Mean synovial fluid TGF-β1 after intra-articular PRPr injection. TGF-β1 was detected in both groups and was statistically significant between groups on day 1. Pound sign denote statistically significant differences between groups on a specific day (P<0.05).

SYNOVIAL FLUID AND SERUM TP AND SAA CONCENTRATIONS

The mean synovial fluid TP concentration was not significantly different between groups on day 0. There were significant increases in the mean synovial fluid TP on day 1 and day 2 for the control (P<0.001 for both) and OA (P<0.001 and P<0.01, respectively) groups, compared to day 0. The mean synovial fluid TP concentration returned to normal for all horses by day 56 (Fig. 18).

The serum and synovial fluid SAA concentrations remained below the lower detection limit of quantification for both groups and were not compared statistically. The mean serum TP concentration was significantly different between groups only on day 1 (P<0.05), with control group increased compared to OA group.

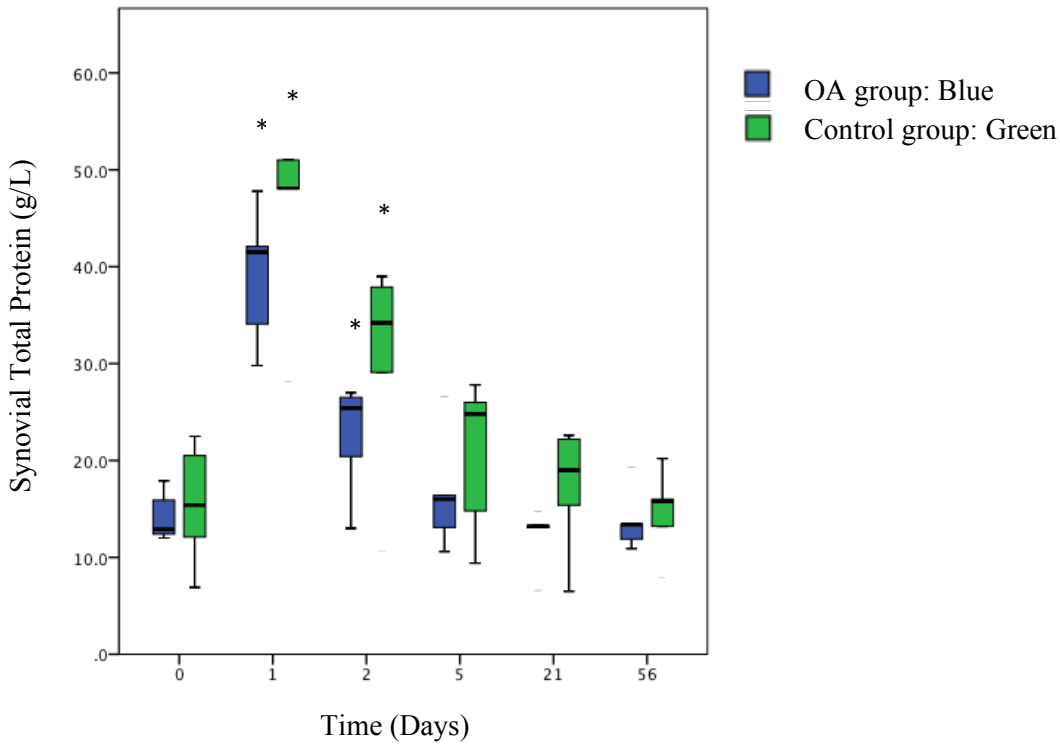

Figure 18: Mean synovial fluid TP concentrations pre- and post-intra-articular PRPr injection. At day 1 and day 2 both groups were significantly different from their corresponding baseline value. Asterisks denote statistically significant differences from baseline, within a given group (P<0.05).

TABLE 2: Summary of synovial fluid cytology, clinical chemistry and growth factor concentrations for the control- and OA groups.

	NCC (cells/μL)	Neutrophils (%)	TP (g/L)	SAA (mg/L)	PDGF-BB (pg/mL)	TGF-β1 (pg/mL)
Day 0						
Control	111.60 ± 39.89	1.60 ± 2.45	15.48 ± 6.31	0.00 ± 0.00	-	-
OA	87.40 ± 53.64	5.40 ± 8.50	14.22 ± 2.56	0.00 ± 0.00	-	-
Day 1						
Control	22940.00 ± 11103.28[a,c]	83.40 ± 2.70[a,d]	46.64 ± 11.11[a]	0.00 ± 0.00	390.47 ± 82.15[b,c]	4021.27 ± 783.00[b,c,d]
OA	20464.00 ± 12055.93[a,c]	71.20 ± 5.40[a,d]	39.06 ± 7.10[a]	0.00 ± 0.00	302.27 ± 47.44[b]	2792.48 ± 615.46[b,c,d]
Day 2						
Control	13212.00 ± 3413.46[a]	64.00 ± 4.00[a,b]	30.18 ± 11.55[a]	0.00 ± 0.00	-	-
OA	7372.00 ± 6122.10	38.80 ± 14.32[a,b]	22.46 ± 5.90[a]	0.00 ± 0.00	-	-
Day 5						
Control	3654.00 ± 2037.59[c]	14.80 ± 11.86[d]	20.56 ± 8.02	0.95 ± 2.14	316.42 ± 45.69[c]	851.73 ± 351.82[c,d]
OA	1660.40 ± 1250.72[c]	8.20 ± 15.54[d]	16.54 ± 6.09	0.00 ± 0.00	318.06 ± 37.82	678.35 ± 289.58[c,d]
Day 21						
Control	207.60 ± 49.90	2.60 ± 1.67	17.14 ± 6.61	0.00 ± 0.00	-	-
OA	213.00 ± 123.80	5.20 ± 6.97	12.18 ± 3.18	0.00 ± 0.00	-	-
Day 56						
Control	224.80 ± 71.94	10.80 ± 11.16	14.62 ± 4.51	0.00 ± 0.00	-	-
OA	95.60 ± 71.40	4.20 ± 6.79	13.78 ± 3.26	0.00 ± 0.00	-	-

[a]Statistically significant differences from baseline, within a given group ($p < 0.05$).

[b] Statistically significant differences between groups on a specific day ($p < 0.05$).

[c]Statistically significant correlation between NCC(cells/μL) and PDGF-BB/TGF-β1 ($p < 0.05$)

[d]Statistically significant correlation between Neutrophils (%) and PDGF-BB/TGF-β1 ($p < 0.05$)

DISCUSSION

Intra-articular PRPr administration resulted in high concentrations of synovial growth factors, PDGF-BB and TGF-β1, as well as a transient synovial inflammatory reaction in both groups and an increase in synovial effusion of the control group.

The control group was not age-matched to the OA group and was as a result of specific selection of joints that did not exhibit radiographic changes of OA for the control group. Osteoarthritis is a degenerative disease that is commonly seen in older aged horses, thus the control group of the same age distribution would be improbable. The small sample size and differences in age groups may have affected the outcome of the study.

A proprietary gravity filtration system was used in this study to prepare the PRPr (V-PET, Pall Corporation). The PRPr preparation system used has been evaluated before and was found to be safe in the treatment of equine joints (Textor & Tablin, 2013a). This system provides equine practitioners with a stable side preparation and immediate administration of a PRPr of known composition and quality. The preparation and processing of PRPr in the study was rapid and self-reliant. The PRPr was not activated, as exogenous platelet activation is not commonly used in equine practice, and platelets can be activated upon exposure to synovial fluid due to the shear forces experienced by platelets from the highly viscous fluid (Textor et al. 2013b; Yin et al. 2011). The physical interaction between hyaluronic acid in synovial fluid and the latency-associated peptide (LAP) of growth factor may be sufficient to break the non-covalent bonds linking the LAP to growth factor, thereby releasing the growth factor (Albro et al. 2012). Different methods of activating PRP probably affect the concentration of growth factors. PRPs are commonly activated by calcium chloride, thrombin, chitosan and batroxobin. Calcium chloride and thrombin activation are the two most common methods. However, in a previous study thrombin activated PRPr injected into joints was associated with effusion, pain on flexion and peri-articular heat, compared to resting-PRPr and $CaCl_2$-PRPr treated joints. Although these signs resolved each day with CaCl-PRP and resting-PRP, they were not resolved after 5 days with thrombin activation and the authors conclude that bovine thrombin should not be used for intra-articular injection. (Textor & Tablin 2013a).

The only significant clinical parameter found during the evaluation of joints were an increase in synovial effusion score of the control group on day 1 and day 2 compared to OA group, this can be seen in conjunction with a mild, short duration inflammatory response

demonstrable within the synovial fluid analysis findings by an increase in NCC and percentage neutrophils present. This finding is in alliance with another study by Textor & Tablin (2013a), in which they reported baseline physiological parameters within normal joints with and without different platelet activators and found a mild inflammatory response post intra-articular platelet-rich injection. Other studies in support of the above statement include Judy & Galuppo (2005) and Ovlisen et al. (2009), expressing that synovial fluid is normally low in cellularity and administration of whole blood intra-articularly is known to elicit an inflammatory response in joints. This has also been documented in intra-articular administration of MSCs with a prevalence of 9% in clinical cases. An intra-articular inflammatory reactions following the administration of 15 million MSCs (autologous, allogeneic, or xenogeneic) into normal fetlock joints of horses revealed an increase of synovial NCC of greater than 40 000 cells/μL and an increase in TP greater than 40 g/L (Pigott et al., 2013).

Growth factor concentrations, specifically PDGF and TGF-β, are normally low or undetectible in healthy joints (Textor et al. 2013; Van der Kraan. 2017). Textor et al. (2013) found no detectible levels of PDGF and approximately 600-700 pg/mL TGF- β in healthy joints prior to PRP administration in their study evaluating growth factor concentrations within synovial fluid pre- and post PRP administration. According to Van der Kraan (2017) the concentration of TGF- β differ greatly between healthy and OA joints, being low in healthy joints and high in OA, leading to the activation of different signaling pathways in joint cells. Thus, supporting the finding that low levels of TGF- β is present in healthy joints.

Investigating the duration that PDGF and TGF-β are detectable within synovial fluid after PRP administration, it is found that PDGf has a biological half-life of 2.5 hours, however the release of PDGF is constant and elevated levels can be sustained for at least seven days (Roh et al., 2016). Roh et al. (2016) stated that synovial fluid TGF- β concentration can be rapidly induced and a maximum concentration can be detected within one hour after PRP administration.

In our study, high concentrations of synovial PDGF-BB and TGF-β1 were present in both the control and OA horses on day 1 after intra-articular administration of PRPr. Concentrations within the synovial fluid for PDGF-BB and TGF-β1 were, however; significantly lower in the OA group compared to the control group on day 1. The lower growth factor concentrations in the OA group may be attributed to a decreased synovial fluid viscosity in joints exhibiting

OA and, thus, may affect normal platelet activation as mentioned earlier (Albro et al. 2012; Chen et al. 2012; Conrozier et al. 2012; Textor et al. 2013b). Once platelets are activated, an initial burst of growth factors release is followed by further sustained release, a 3- to 5-fold increase as compared with baseline. Furthermore, platelet activation increases levels of anti-inflammatory cytokines because of the presence of hepatocyte growth factor. Thus, external platelet activation could be considered prior to intra-articular administration of PRPr in OA joints.

Transforming growth factor beta was elevated on day 1 in both control- and OA groups, however it decreased rapidly in both groups by day 5. This finding has also been documented by Roh et al. (2016), that found that TGF-β1 is rapidly induced, and a maximum concentration was detected within one hour after PRP administration, irrespective of a single spin or double spin PRP preparation method. The double spin PRP preparation contained a higher platelet count (1145 $\times 10^9$/L) and nine times higher WBC concentration compared to single spin method, classifying it as a leukocyte-rich PRP preparation, similar to our study. The TGF-β1 concentration was found not to correlate with the platelet concentration of the PRP (Roh et al. 2016).

The persistently elevated PDGF-BB concentration in our study in both the control and OA groups is different from a previous report, and may be attributable to the slow, continues release of PDGF-BB (Textor et al. 2013). In one study, PDGF-BB release was constant and was sustained over seven days (Roh et al. 2016). The PDGF-BB concentrations of PRP are also correlated with platelet and leukocyte concentrations in the PRP (Roh et al. 2016). These results are consistent with the findings reported by Mazzucco et al. (2009), who reported on the individual dynamics of the growth factors. The study concluded that growth factor release depends exclusively on the type of growth factor, rather than on the preparation method. In addition, the study also demonstrated that TGF-β1 is promptly released within 24 hours of exogenous activation whereas PDGF-BB release is more dependent on the technique that is used.

For our study, various factors may have influenced the synovial fluid growth factor concentrations, as well as contributed to the differences seen between the control and OA groups, after intra-articular treatment with PRPr. As mentioned previously, decreased synovial fluid viscosity, as seen in OA, may have resulted in decreased in platelet activation. Moreover, the PRPr was considered to be leukocyte-rich and statistically significant

correlations were found between the synovial fluid NCC and TGF-β1 for both the control and OA groups, and PDGF-BB for the control group only. An important factor that should be considered in platelet concentrate technologies is not the quantity of platelets, but how platelets, leukocytes, activating factors and growth factors are interlinked in the final product. A strict quantitative approach does not define the biological signature and mechanism of action of the product, but rather the qualitative properties of the final product must be taken into consideration. A study by Dohan et al. (2012) comparing pure platelet rich plasma with leukocyte and platelet rich fibrin, found the latter product released growth factors slower and over a longer period of time, compared to pure platelet rich plasma. The same study also suggest that leukocyte populations have a strong influence on the release of some of the growth factors, particularly TGF-β1. Our presumption then that high synovial leukocytes, specifically neutrophils, contributed to the high growth factor concentrations present in the synovial fluid is in line with this study. Furthermore, evidence suggests that in leucocyte-rich PRP systems, leukocytes may enhance PRP growth factor concentrations, either through release of growth factors from leukocytes within the PRP or by acting as a stimulus for the release of growth factors from platelets (Zimmerman et al. 2001). A synovial inflammatory response consisting predominantly of intact mature neutrophils can also not be excluded as a source of synovial fluid growth factors.

Serum amyloid A is a major acute phase protein in the horse that is synthesized in response to acute infection or inflammation (Jacobsen et al. 2006b). In this study the serum and synovial concentrations of SAA in both groups remained undetectable and may be attributed to the chronicity of the condition within the OA group. This finding was similar to a previous report where SAA concentrations were evaluated in serum and synovial fluid samples from healthy horses and horses with infectious arthritis and OA (Jacobsen et al. 2006b). The latter study found SAA to be a good marker of infectious arthritis cases but OA cases had low concentrations of SAA in serum (0.48 mg/L) and synovial fluid (0.7 mg/L), suggesting decreased production of SAA due to the chronicity of the OA as it is synthesized only in response to moderate to severe active inflammation. In addition, repeated arthrocentesis also did not cause a detectable increase in serum and synovial SAA (Jacobsen et al. 2006b; Sanchez et al. 2008). The increased synovial TP concentration in our study, after intra-articular administration of PRPr, has been previously described and was ascribed to a mild acute inflammatory response elicited by intra-articular administration of PRPr, as well as repeated arthrocentesis. (Jacobsen et al. 2006b; Sanchez Teran et al. 2012).

CONCLUSIONS

Both normal and OA joints showed a moderate inflammatory response on cytological evaluation of the synovial fluid after intra-articular administration of PRPr, which returned to normal by day 21. Sufficiently high synovial concentrations of PDGF-BB and TGF-β1 were obtained in both groups without the use of an activator; however, significantly lower concentrations of PDGF-BB and TGF-β1 were seen on day 1 within the OA group. External activation of PRPr is probably required prior to administration into OA joints. Synovial growth factor concentrations may be influence by leukocyte-rich PRPr. Further research is needed to assess the cytokine concentrations of IL-1, IL-6 and TNF in synovial fluid after intra-articular PRP injection.

ETHICAL ANIMAL RESEARCH

The research was approved by the University of Pretoria's Animal Ethics Committee (protocol approval number V011-15). Animals used were part of research herds belonging to The University of Pretoria.

AUTHORS' DECLARATION OF INTERESTS

The V-PET™^{c} systems were sponsored. Authors declare no other conflicts of interest related to this product, nor are employees of, or consultants to, or benefit financially from Pall Corporation or its products.

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AUTHORSHIP

Authors made substantial contributions to study design, interpretation of data, drafting and revising of article. Y.S., A.G. and H.J.M. performed data collection. P.N.T. performed statistical analysis of data.

APPENDIX

APPENDIX 1: CLINICAL AND RADIOLOGICAL EXAMINATION DATA CAPTURE SHEET

CLINICAL AND RADIOLOGICAL EXAMINATION DATA CAPTURE SHEET					
Horse identification				**Date**	
Joint selected	**Carpus**	(1) Radiocarpal (2) Intercarpal		**Fetlock**	

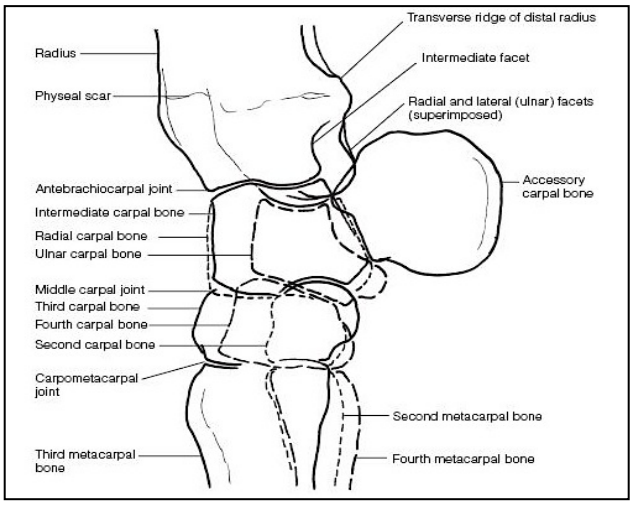
Clinical examination	**Temperature**		*37-38.5*
	Pulse		*28-44*
	Respiration		*8-24*

Radiographical examination: Carpus (Radiocarpal or Intercarpal joint) or Fetlock joint			
Peri-articular osteophytes			*0 = No abnormality detected*
Joint-space narrowing			*1 = Mild abnormality detected*
Subchondral bone sclerosis			*2 = Moderate abnormality detected*
Sunchondral bone lysis			*3 = Severe abnormality detected*
Osteochondral fragmentation			

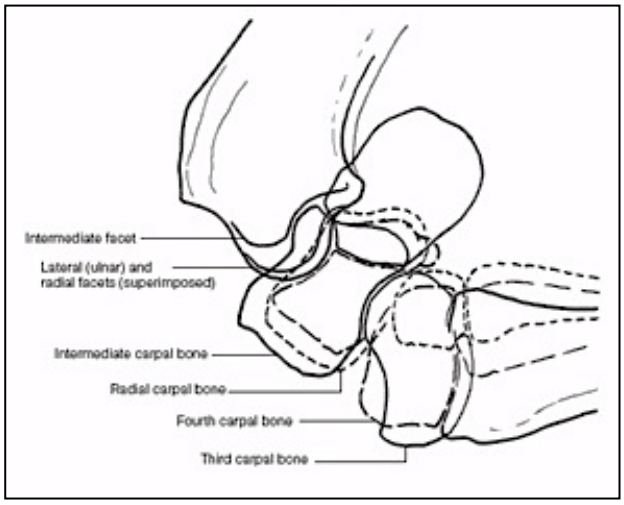
Schematic represenation of Radiographical examination: Carpus (Radiocarpal or Intercarpal Joint) or Fetlock joint

Carpus

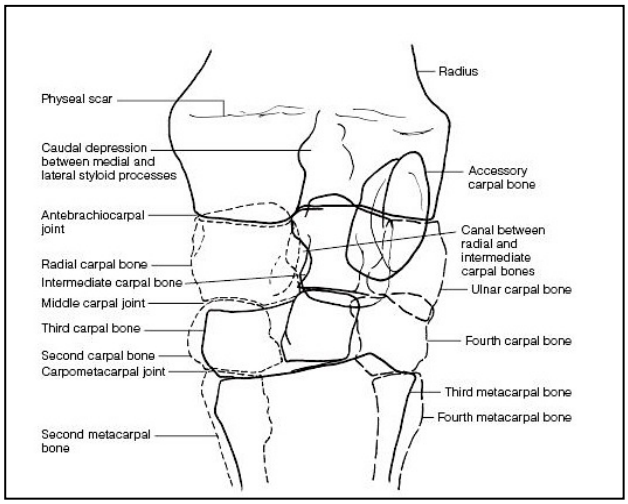
Lateromedial (LM)



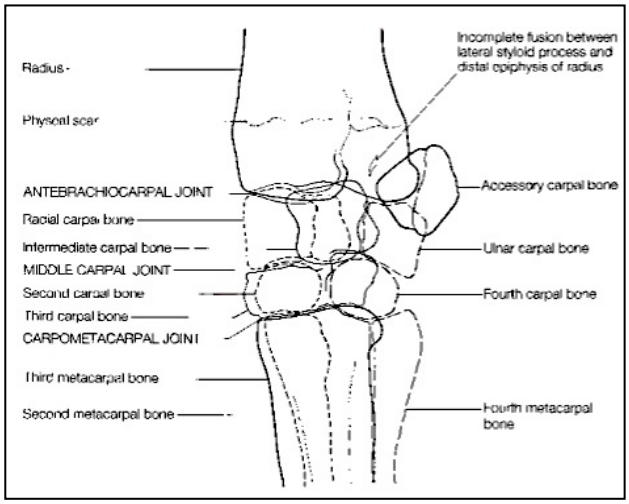
Flexed LM



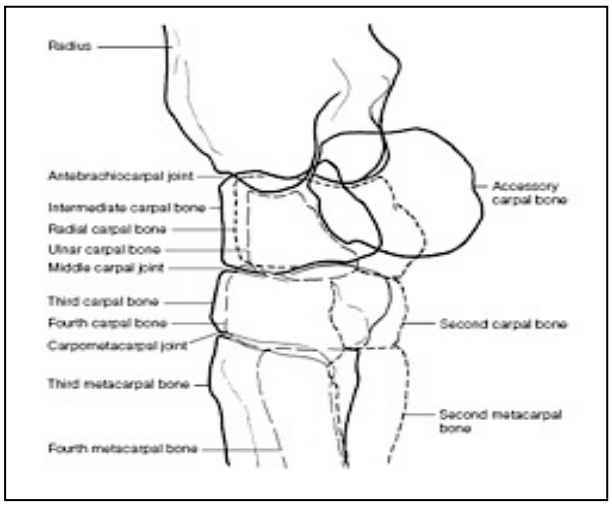
Dorsopalmar (DPa)



Dorsal 45° lateral-palmaromedial oblique (D45°L-PaMO)

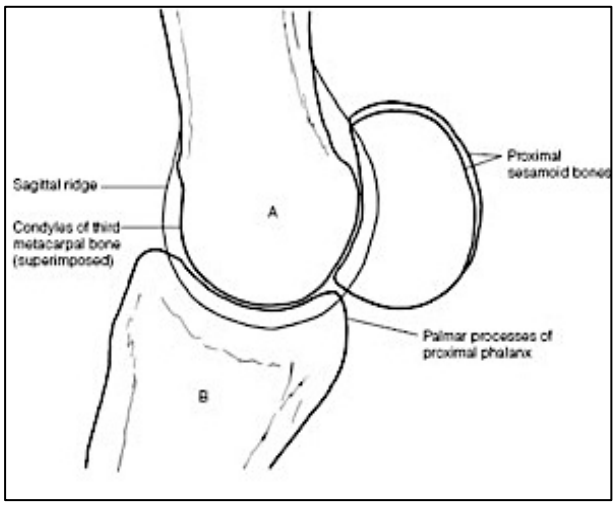


Dorsal 45° medial-palmarolateral oblique (D45°M-PaLO)

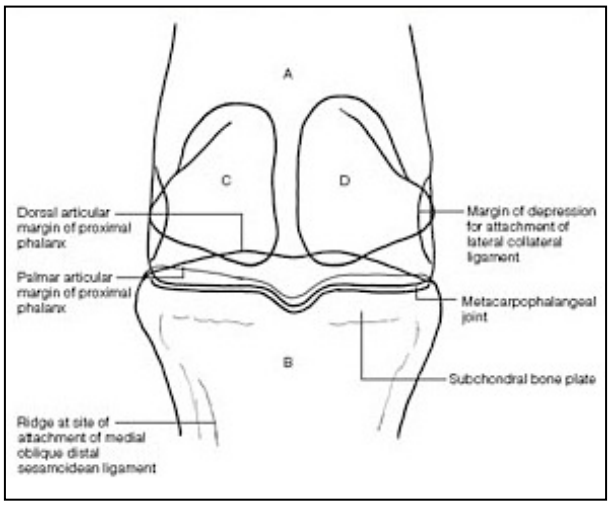


Fetlock

Lateromedial (LM) and Flexed LM

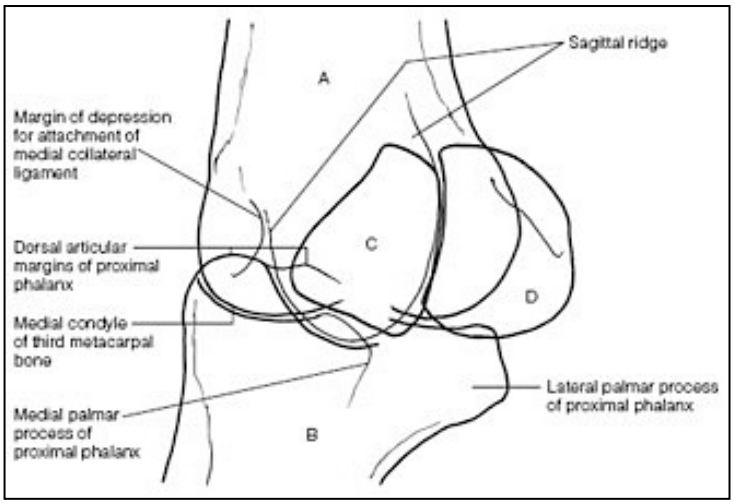


Dorsopalmar (DP)



Dorsal 45° lateral-palmaromedial oblique (D45°L-PaMO) and

Dorsal 45° medial-palmarolateral oblique (D45°M-PaLO)



Lameness (AAEP grading)		0,1,2,3,4,5 AAEP grading

Joint: Carpus / Fetlock					
Synovial Effusion		*0 = Absent* *1 = Mild* *2 = Moderate* *3 = Severe*	Response to flexion at rest		*0 = Absent* *1 = Mild* *2 = Moderate* *3 = Severe*

Peri-articular signs		*Yes / No*

Jugular venipuncture	EDTA	
	SERUM	

PRP	EDTA	

Synovial fluid	EDTA	
	SERUM	

APPENDIX 2: CLIENT CONSENT FORM

PRP has been extensively investigated in humans, research animals and dogs for the treatment of osteoarthritis. Currently PRP is being investigated in treatment of OA in horses.

PRP contains multiple growth factors, which have been proven to play a critical role in modulation of inflammation and repair. Thus, PRP may therefore be used to deliver high concentrations of growth factors to injured tissue sites, thus improving the quality of native tissue repair.

This study will evaluate the clinical signs, synovial fluid cytology and growth factor concentrations after intra-articular injection of PRP in horses with osteoarthritis.

At day -30 to -2 horses in the OA-affected group and the control group will be radiographed and clinically assessed (screening period).

At day -1 horses in the OA-affected group and the control group will be clinically re-assessed:

At day 0, the horses in the OA-affected group and the control group will receive a single intra-articular injection of PRP. Synovial fluid will be collected for analysis prior to the PRP injection. Blood will be collected by jugular venipuncture for a CBC analysis, SAA and TP analysis and PRP preparation.

Clinical assessment, CBC, serum SAA, serum TP, cytological analysis of synovial fluid, synovial fluid SAA and synovial fluid TP will be repeated at day 1, 2, 5, 21 and 56. Synovial fluid GFs (PDGF and TGF-β) analysis will be performed at day 1 and day 5.

Your horse will be admitted to the Equine Clinic, Onderstepoort Veterinary Academic Hospital for the period of data collection on Day 0 – Day 3. Thereafter the horse will be seen on an outpatient basis for further data collection on Day 5, 21, and 56.

Thank you for your willingness to allow your horse to participate in this study. Should you require any other information, do not hesitate to contact me:

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Department of Companion Animal Clinical Studies

Faculty of Veterinary Science

University of Pretoria

Tel: 012 529 8141

Email: yolandi.smit@up.ac.za

I, ……., hereby give permission that my horse, ……., may participate in this clinical study at the Onderstepoort Veterinary Academic Hospital.

Signed at ……, on the ……. Day of ……..201….

Signature of Owner or Authorized person: ……

Telephone (home): …… Telephone (work): ……

MANUFACTURERS' ADDRESSES

[a]Developed by Cytomedix, Gaithersburg, Maryland, USA, now licensed to Arthex Inc., Florida, USA.

[b]Arthrex Inc., Naples, Florida, USA.

[c]Pall Corporation, Amsterdam, Netherlands.

[d]Biomet Biologics Inc., Warsaw, Indiana, USA.

[e]Siemens Healthcare Diagnostics, Munich, Germany.

[f]Roche Diagnostic Laboratory, Basel, Switzerland.

[g]Eiken Chemical Company, Tokyo, Japan.

[h]Abbot Diagnostics, Illinois, USA.

[i]R&D Systems, Stikland, SA.

[j]StataCorp, College Station, Texas, USA.

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