Cytokine and Growth Factor Profiles in Canine Autologous Conditioned Serum

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ABSTRACT (ACADEMIC)

The object of this study was to compare growth factor and cytokine profiles in canine autologous conditioned serum (ACS) to canine plasma. Blood collected from 16 medium to large breed dogs was used to produce ACS (Orthokine® vet irap 10 syringes) and citrated plasma (control). Canine-specific ELISA assays were run per manufacturers' instructions for interleukin (IL)-10, IL-4, tumor necrosis factor (TNF)-α, insulin-like growth factor (IGF)-1, fibroblast growth factor (FGF)-2, transforming growth factor (TGF)-β1, IL-1β, and interleukin-1 receptor antagonist (IL-1ra). Serum, in addition to plasma and ACS, was collected from an additional 6 dogs for TNF-α, IL-1β, and IL-1ra analysis (total of 22 dogs). Data were analyzed for differences in cytokine concentrations between ACS, plasma, and serum using the Wilcoxon signed-rank test with significance set at $P<.05$.There was a large variability in growth factor and cytokine concentrations between individual dogs in both plasma and ACS. There were no significant differences in IL-10, TNF-α, IGF-1, FGF-2, and TGF-β1 concentrations between ACS, plasma, or serum. ACS concentrations of IL-1β (median, range; 46.3 pg/mL, 0-828.8) and IL-4 (0.0 pg/mL, 0-244.1) were significantly increased compared to plasma (36.6 pg/mL, 0-657.1 and 0.0 pg/mL, 0-0, respectively). IL-1ra concentrations in ACS (median, range; 3458.9 pg/mL, 1,243.1-12,089.0) were significantly higher than plasma (692.3 pg/mL, 422.5-1,475.6), as was the IL-1ra:IL-1β ratio (39.9 and 7.2, respectively).

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ABSTRACT (PUBLIC)

Osteoarthritis (OA) is a common cause of pain and suffering in dogs and is challenging to treat effectively Current treatment for OA merely mask the signs of pain and do not promote regeneration of the damaged cartilage or alter the course of the disease. Many treatments also cause harmful side effects. Autologous conditioned serum (ACS) is a biological therapy that controls inflammation within the joint by specifically blocking the inflammatory cytokine interleukin (IL)-1β. In humans and horses ACS relieves the pain from OA and promotes cartilage regeneration. The purpose of this study was to compare levels of pro- and anti-inflammatory cytokines in canine ACS to that of normal canine plasma with the hypothesis that levels of anti-inflammatory cytokines would be higher in ACS compared to controls and that levels of pro-inflammatory cytokines would remain unchanged. Paired blood samples were collected from 22 large breed healthy dogs and processed in either irap®10 syringes (ACS) or in anti-coagulant as a control. For the last 6 dogs an additional serum sample was collected. Growth factor and cytokine levels were determined using canine-specific ELISAs. One pro-inflammatory cytokine (IL-1β) and two anti-inflammatory cytokines (IL-1ra and IL-4) were significantly higher in the ACS compared to plasma and IL-1ra levels were also higher in serum compared with plasma samples. Other levels were the same between groups. Levels of IL-1ra in ACS were comparable to those measured in humans and horses. Further research is needed to assess the effect of ACS in patients.

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LIST OF ABBREVIATIONS

ACS	Autologous conditioned serum
ARA	Arachidonic acid
COX	Cyclooxygenase
DHA	Docosahexaenoic acid
GAGs	Glycosaminoglycans
EPA	Eicosapentaenoic acid
ICE	Interleukin-1 converting enzyme
IL	Interleukin
IL-1AcP	Interleukin-1 receptor accessory protein
IL-1ra	Interleukin 1 receptor antagonist
IL-1RI	Interleukin-1 receptor one
IL-1RII	Interleukin-1 receptor two
LOX	Lipooxygenase
LTB_4	Leukotriene B_4
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cells
NLRs	NOD-like receptors
NOD	Nucleotide binding and oligomerization domain
OA	Osteoarthritis
PRP	Platelet rich plasma
TNF-a	Tumour necrosis factor alpha
TNFR1	Tumour necrosis factor receptor one
TNFR2	Tumour necrosis factor receptor two
TXA_2	Thromboxane A_2

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Chapter 1: Background and Literature Review

Osteoarthritis

a. Etiology and Pathophysiology

Osteoarthritis is a chronic degenerative disease of joints, whereby deterioration of the articular structures leads to inflammation, pain and loss of function.

i. Normal Joint Structure

A diarthodial, synovial joint consists of hyaline cartilage, subchondral bone, a joint capsule and a joint space containing synovial fluid.[1]

Hyaline cartilage is made from chondrocytes embedded in an extracellular matrix comprising of water, collagen and proteoglycans. The typical chondrocyte content of normal cartilage is 1-12%. The water content varies with the age of an animal and is between 70-80% of the weight of the cartilage. The remaining weight is comprised of approximately 50% collagen, 35% PGs, 10% glycoproteins and 5% minerals, lipids and miscellaneous substances.[2]

The depth of the cartilage is dependent on the area of the joint and is divided into four histological zones (from superficial to deep): tangential, transitional, radiate and calcified zones. The size, number, morphology and orientation of chondrocytes and collagen depend on the zone: The tangential zone contains a high density of flattened chondrocytes that lie parallel to the joint surface. The collagen fibers in this zone also lie parallel to the joint surface and are more densely packed. The transitional zone contains larger, more ovoid to round chondrocytes and the collagen is arranged in a haphazard fashion. The radiate zone contains the largest chondrocytes. In this area, the long axis of the cells and the collagen fibers are arranged perpendicular to the joint surface. Finally,

the calcified zone contains mineralized cells. The junction of the radiate and calcified zones is donated by a "tidemark" which can be seen histologically with Safranin O stain.

The primary collagen in cartilage is type I (90-95%); which is produced by chondrocytes. Type I cartilage is made of three identical collagen molecules that are linearly arranged to be 25% off set from each other. The remaining collagen is comprised of type VI, IX and XI, which help with the organization and stability of the type II cartilage.[3,4] Type XI collagen helps to arrange type II collagen in to fibrils by providing a core for type II collagen to bind, while type IX collagen helps with inter-fibril binding.[3,4]

Proteoglycans are made up of a core protein, to which glycosaminoglycans (GAGs) bind. The main proteoglycan is aggrecan, which accounts for around 85% of the proteoglycans found in cartilage. An aggrecan monomer is comprised of three different GAGs covalently bonded to a core protein through trisaccharide linker: chondroitin-4-sulfate, chondroitin-6-sulfate and keratan sulfate. Each monomer then non-covalently binds to a hyaluronan molecule via a linker protein to form an aggregate; aggregates can comprise of over 100 aggrecan monomers.[5] The GAGs provide cartilage swelling pressure due to being hydrophilic and possessing a high negative change such that they repel each other.[6] This is important in their function of maintaining cartilage volume and buoyancy. The remaining proteoglycans consist of smaller, non-aggregating proteoglycans which, although their exact role is not yet fully defined, are thought to be involved with cartilage repair.[1] Turn over of proteoglycans is estimated to be around 300 days in the dog, this rate is up-regulated in osteoarthritis. Up-regulation of both aggrecan and collagen synthesis may be an early sign of osteoarthritis.[7]

The joint capsule is made of a thick, fibrous outer layer and a thinner inner layer (synovium) that are continuations of the periosteum. The fibrous outer layer is comprised of type I collagen with elastin fibers. The fibers are arranged in parallel fascicles with occasional interspersed fibrocytes and blood vessels.[8,9] Thickening of the joint capsule provides many of the ligaments associated with the joint, for example collateral ligaments.[1] The synovium is made up of two layers: the intimal and the subintimal layers.[10] The inner most layer, the intermal layer, is comprised of 1-4 layers of synoviocytes and is lacking a basement membrane. The main functions of this layer are phagocytocysis (type A synviocytes), protein secretion (type B synoviocytes) and regulation of synovial fluid. Some texts also describe a "type C" synoviocyte, but it is undetermined if this is distinct cell type or a representation of a synoviocyte transitioning between type A and B. The type B synocviocytes are responsible for secreting a wide range of proteins that are important in the composition of the synovial fluid and that contribute to the synovial membrane. Some of these proteins include hyaluronan, collagen, pro-matrix metalloproteinases, lubricin, interleukins and eicosanoids.[2,11] The second layer is the subintimal layer, which is comprised of loosely arranged fibrous and fatty tissues. The subintimal layer has a rich nerve, lymphatic and blood supply.[12,13]

Synovial fluid is an ultra filtrate formed by the passage of plasma through the synovium with the addition of proteins from type B synoviocytes. Generally, the endothelium of the subintimal layer will only allow the passage of molecules less than 10kDa in size, thus excluding larger proteins, oxygen and carbon dioxide. Hyaluronan and lubrican are then added to the ultra filtrate by the type B synoviocytes to help provide lubrication and homeostasis of the joint via steric exclusion of larger molecules.[2,11,14] As

well as lubricating the joint, the synovial fluid functions to provide nutrients to and remove metabolites from the avascular cartilage.[1]

The subchondral bone is located directly below the articular cartilage in the epiphyseal region of the bone. In comparison to cortical bone, the haversian systems run parallel to the joint surface. This configuration allows for greater deformation and better transmission of forces from the cartilage to the underlying cortical bone.[8,10,15]

The blood supply to joints arises from the surrounding arteries and vein, which form a network supplying the joint capsule and the epiphysis. Synovial blood vessels around the margins of the joint form anatomizing loops; known as *circulus articularis vasculosis*. The nerve supply of joints comprises of proprioceptive fibers, nociceptor fibers, and sympathetic visceral efferent/afferent fibers that arise from cutaneous or muscular branches near the joint. Four types of receptors are present in dog, and other mammalian, joints: Pacinian-like and Ruffini-like receptors in the joint capsule, Golgi tendon organs found in ligaments and free nerve endings.[16] Additionally, intraarticular structures that are specific to the joint, such as the meniscus, also contain nerve fibers. The innervation of the joint and associated structures provides proprioception and the recognition of movement.[1]

ii. Propagation of Osteoarthritis

The normal integrity of the joint is crucial to maintaining normal joint function: near frictionless motion, joint congruency and force transmission.[17] Damage to the joint may happen in a multitude of ways including trauma such as articular fractures, development abnormalities such as hip and elbow dysplasia, or degenerative processes

such as cranial cruciate ligament rupture.[17] Regardless of a causative factor, the damage that occurs to the articular cartilage causes a cascade of changes within the joint.[18,19]

Direct damage to chondrocytes causes a release of proteolytic enzymes, favouring a catabolic environment. This leads to a loss of quality and quantity of key components of the matrix, such as aggrecan and collagen. Early signs of OA include an increase in water content of the cartilage and fibrillation, this alters the mechanical properties of the cartilage making it less able to withstand normal forces and thereby propagating the damage. As OA progresses there is measurable cartilage thinning and loss; this causes joint space narrowing on weight bearing radiographs. The narrowing may be asymmetric in cases of focal cartilage loss. Cartilage has a poor capacity for repair; it is replaced by a biomechanically inferior fibrocartilage. Compared with hyaline cartilage, fibrocartilage is composed mainly of type I and III collagen, with a low proteoglycan content. While over time the concentration of type II collagen and aggrecan may increase, at best a hyaline-like tissue is formed.

As the mechanical property of the cartilage is damaged, forces transmitted to the underlying subchondral bone increase.[18] This causes microfractures in the underlying subchondral bone. In order to withstand the increase in forces, the subchondral bone becomes rigid and sclerotic, this occurs under the influence of IL-6.[15,18,20,21] A less common change is subchondral lysis, which may occur due to pressure necrosis due to increased load, joint fluid and the influence of tumor necrosis factor-α (TNF-α).

Although the synovium does not have any direct biomechanical effects on the joint, synoviocytes are responsible for the production of many catabolic products including: proteoglycans, cytokines and matrix metalloproteinases (MMPs). Additionally,

neutrophils and macrophages may also be found in higher concentrations with inflamed synovium, adding to both the production of enzymatic mediators and contributing to the role of phagocytosis.

Histological changes in inflamed synovium include increased vascularity and edema; which has been associated with an increase in joint fluid volume and increased protein concentration. It is the increase in joint fluid volume that is thought to produce increased pressure on the lymphatic draining system causing a decrease in drainage. This occurs simultaneously with a decrease in the quality and quantity of hyluronan; resulting in reduced viscosity of the joint fluid.[22] The increased volume in the joint contributes to the loss of function; while the decrease in viscosity causes increased friction during motion resulting in accelerated cartilage wear.

Pain associated with OA is a result of C-fiber stimulation in the joint capsule, synovium, bone, periosteum and surrounding tendons and ligaments. C-fibers are stimulated in response to both mechanical (stretching) and chemical stimuli. In chronic arthritis, there has been shown to be an up regulation of pain receptors and involvement of the central nervous system.[23]

The net effect of the mechanical and biochemical changes is that the joint is less able to withstand normal forces, thereby perpetuating the cycle.

b. The Role of Cytokines in Osteoarthritis

Cytokines are a diverse group of small signalling molecules that carry both paracrine and autocrine roles. Each cytokine is produced by one or more cell types, and the cytokine goes on to interact with receptors on one or more different cell types to

cause a local or distant response. Due to the function of cytokines involved in OA, they can be broadly divided as pro-inflammatory and anti-inflammatory. It is important to note that cytokine function is also affected by timing, concentration and association with other cytokines.[24]

i. Pro-Inflammatory Cytokines

Although once thought of as a non-inflammatory process, it is now known OA involves a degree of inflammation even in the early stages of the disease.[25-30] In the diseased joint, damaged chondrocytes, synoviocytes and monocytes release pro-inflammatory cytokines, thus causing the joint to enter an inflammatory or catabolic state. In general, pro-inflammatory cytokines stimulate chondrocytes to release MMPs, in particular MMP-1 (interstitial collagenase), MMP-3 (stromelysin 1) and MMP-13 (collagenase 3).[31-33] These three proteinases regulate collagen catabolism. Additionally, pro-inflammatory cytokines induce other inflammatory mediators including inducible nitric oxide synthase, soluble phosholipase A2, cyclooxygenase 2, microsomal prostaglandin E synthase 1, nitric oxide and prostaglandin E_2 (PGE_2). Interleukin (IL)-1β and TNF-α are the main inflammatory factors involved in OA, with IL-1β driving tissue destruction and TNF-α associated with inflammation.[21,34,35]

In addition, other cytokines including IL-6, IL-15, IL-17, IL-18, IL-21, leukemia inhibitory factor and IL-8 (a chemokine) have been implicated.[21]

Interleukin-1β

IL-1β is part of the IL-1 family that consists of three ligands: IL-1α, IL-1β and interleukin receptor antagonist (IL-1ra). IL-1α and IL-1β share approximately 22%

amino acid homology; IL-1ra shares approximately 18% amino acid homology with IL-1α and 26% amino acid homology with IL-1β.[36-38] IL-1β is synthesized in the cytoplasm of several cell types including activated macrophages, neutrophils and synoviocytes as an inactive 31kDa proform (pro-IL-1β).[39] Prior to pro-IL-1β being released from the cell, it is cleaved by interleukin-1 converting enzyme (ICE), otherwise known as caspase-1, to form the mature 17kDa IL-1β.[39,40] IL-1β together with IL-1 receptor accessory protein (IL-1AcP) form a complex with the biologically active (IL-1RI) or inactive (IL-1RII) IL-1 receptors.[39]

IL-1β one of the most potent inflammatory mediators in the body. It has been implicated in several disease processes in people including rheumatoid arthritis, OA, Crohn's disease, gout, pulmonary fibrosis, diabetes mellitus, cardiovascular and central nervous system disease.[39] Once bound to IL-1RI, IL-1β mediates an increase in the production of pro-inflammatory cytokines, prostaglandins and NO. On a systemic level this causes hypotension, decreased white blood cell counts, hemorrhage and pulmonary edema within the host.[41,42] The role of IL-1β in osteoarthritis was first reported by Fell et al, whereby they discovered cultured synovial tissue released a factor (later found to be IL-1β) that activated chondrocytes leading to PGs degradation in adjacent tissues.[43] Following this, multiple reports have implicated IL-1β as a major mediator of inflammation and tissue destruction in OA, with the effect of IL-1β within joints including stimulation of leukocyte infiltration, enhanced proteoglycan degradation, inhibition of proteoglycan synthesis and enhanced collagen degradation.[44-47] Due to the role of IL-1β in OA, IL-1β antagonism is a potential therapeutic avenue in the treatment of OA.

The regulation of IL-1β is very complex and involves several layers of control including production, processing, receptor availability and accessory proteins. Production of pro-IL-1β is dependent on the concentration of cells available to synthesize it; in OA, inflamed synoviocytes and activated macrophages increase the production of pro-IL-1β. Processing of pro-IL-1β to IL-1β is reliant on caspase-1. In OA, products of tissue degradation activate the nucleotide binding and oligomerization domain-like receptors, which in turn leads to the activation of caspase-1 via recruitment to a multiprotein complex termed the inflammasome.[39,40,48,49] The availability of receptor binding can be controlled in two ways: first, IL-1RII is an inactive form of IL-1RI, in that binding of IL-1β to IL-1RII produces no response. Therefore, IL-1RII can negatively regulate cell activation by competing with IL-1RI as a binding site for IL-1β. In addition, IL-1RII can be released from the cell membrane and bind to IL-1β in the cell microenvironment, thus preventing the interaction of IL-1β with the membrane-bound IL-1RI. Second, IL-1ra (see below), is a competitive inhibitor of IL-1β. IL-1ra has a high affinity for IL-1RI but produces no cellular response. Finally, IL-AcP is required for the co-activation of the IL-RI receptor. Following the binding of IL-1 to IL-1RI, IL-AcP is added to the complex. It is the approximation of the cytoplasmic domains of IL-1RI and IL-AcP that goes on to stimulate the intracellular signal; therefore, without IL-AcP there will be no cellular response.[50,51]

Tumour Necrosis Factor-α

Tumour necrosis factor-α is a 17kDa inflammatory cytokine produced by activated macrophages, lymphocytes and neutrophils.[52] TNF-α is initially produced in a transmembrane form, which is then converted to a soluble form by TNF-α converting

enzyme. It is the soluble form of TNF-α that is implicated in OA.[53] TNF-α remains conservative among species with the human cytokine sharing 92% homology with the canine cytokine.[54] TNF-α binds to one of two surface receptors: TNFR1 (55kDa) or TNFR2 (75kDa). TNFR1 is expressed constitutively on most mammalian tissues; however, it is upregulated in OA on chondrocytes and synovial fibroblasts.[55] TNFR2 is inducible and expressed mainly on immune, endothelial and neuronal cells [56,57] TNFR1 is activated by both forms of TNF-α, while TNFR2 is only activated by the transmembrane form.[58] TNF-α perpetuates OA in several ways: first, TNF-α inhibits proteoglycan synthesis, leading to a loss in integrity of the cartilage matrix.[59] Second, TNF-α upregulates the production of MMP3, which in turn causes enzymatic degradation of the cartilage matrix.[60-62] Finally, TNF-α has direct pro-inflammatory effects as well.[63,64] Additionally, as there is an increase in the presence of TNF-α receptors in mildly osteoarthritic joints, TNF-α is thought to have a role perpetuating early OA.[65] TNFR1 has also been implicated in cartilage loss, with the areas of the joint expressing a high number of TNFR1 receptors also experiencing focal cartilage loss.[66]

Homeostasis of TNF-α is maintained by binding of TNF-α to shed soluble portions of TNFR1 and TNFR2 in the circulation, thereby lowering the amount of TNF-α available to bind with membrane receptors.[67,68] In the case of arthritis, there has been research into inhibiting the inflammatory effects of TNF-α while maintaining immunity and immune-modulation. In a study using knockout mice, it was found that TNFR1 signaling has a pathologic effect whereas TNFR2 signaling had an anti-inflammatory effect.[69] Therefore, two main approaches have emerged that specifically target the inflammatory effects of TNF-α: first is to inhibit the soluble form while sparing the

transmembrane form and second is to target TNFR1 and spare TNFR2. Human studies into the use of anti-TNF-α antibodies on autoimmune mediated arthritis have shown positive results.[70-73] Although TNF-α plays a clear role in OA, there is very little research into the clinical effect of TNF-α inhibition. One human pilot study and another case study on the effect of anti-TNF-α treatment found favourable results.[74,75] However, a pilot clinical trial using adalimumab, a human monoclonal anti-TNF antibody drug, found the drug was well tolerated but did not improve symptoms of OA.[76] There are currently no clinical veterinary studies looking at the effects of anti-TNF-α therapy.

Other Pro-Inflammatory Cytokines

Although IL-1β and TNF-α are considered the major pro-inflammatory cytokines in OA, additional pro-inflammatory cytokines also play a role in OA either through direct inflammatory effect or through perpetuating IL-1β and TNF-α.[21]

IL-6 has been found to be elevated in synovial fluid and sera from patients with OA; however, its exact role in OA is controversial.[21,77] The known effects of IL-6 include up-regulation of IL-1β, oncostatin, MMP-1 and MMP-13 expression as well as reducing the expression of type II collagen.[78-80] However, injection of IL-6 into the joints of IL-6 knockout mice with experimentally induced OA reduced cartilage destruction.[81] IL-15 has been found in the synovial fluid of patients during early OA and is suspected to be associated with MMP-1 and MMP-3.[82] IL-17 has been found to induce IL-1β production as well as TNF-α and IL-6. IL-17 also upregulates nitric oxide and MMPs while downregulating proteoglycan levels.[83-86] IL-18 is structurally similar to the IL-1 family, and therefore IL-18 can act through both IL-1 dependent and independent pathways.[87]

ii. Anti-inflammatory cytokines

Interleukin-1 receptor antagonist protein:

Interleukin-1 receptor antagonist protein is a structural variant of IL-1 that acts as a natural antagonist.[50,51,88-93] Initially, IL-1ra was described as a 17kDa protein with variably glycosylated species of 22-24kDa.[94] This form of IL-1ra is secreted by macrophages, monocytes, neutrophils and other cells.[94] Since then, several other intracellular forms of IL-1ra have been described and found to have variable affinity to the IL-1RI receptor.[94] During normal homeostasis, IL-1ra will regulate the effect of IL-1β by competitively binding to IL-1RI receptors. Although IL-1ra will bind to the receptor, it will not allow the binding of IL-AcP and thus prevents initiation of the signaling transduction pathway. Due to the spare receptor effect, IL-1ra concentrations need to be 100 fold that of IL-1β.[95] In the case of OA, there is either a deficiency in IL-1ra or an over production of IL-1β, which does not allow this requirement to be met.

Many papers utilizing both human and animal models support the anti-inflammatory role of IL-1ra.[95-101] It has been found that injection of IL-1ra into animal joints with experimental arthritis suppressed inflammation and tissue destruction.[97-99,101-103] Furthermore, IL-1ra knockout mice not only developed more severe arthritis than the wild type counterparts, but they have also been found to develop spontaneous "rheumatoid-like" arthritis.[100,104] Clinically in people, injections of IL-1ra are an efficacious treatment for arthritis and are well tolerated.[105]

Other Anti-Inflammatory Cytokines

Along with IL-1ra, other anti-inflammatory cytokines including IL-4, IL-10 and IL-13 are elevated in OA tissues. These three cytokines function together to inhibit IL-

1β, TNF-α and proteases while upregulating IL-1ra and tissue inhibitor of metalloproteinase production.[106]

c. Current Treatment Options for Canine Osteoarthritis

A large range of treatment options exists for OA: this in part is due to the low efficacy of long-term treatment and the lack of a "cure". Current mainstays of OA management include weight loss, exercise modification, addition of nutriceuticals to the diet, and the use of systemic and intra-articular pharmacological. Other less common options include cold laser therapy and acupuncture. However, all of these options are aimed more at treating the symptoms rather than the underlying cause.

i. Lifestyle

Weight Management

Achieving and maintaining a healthy weight is an important aspect of OA management as it is estimated that 34% of adult dogs seen by veterinarians are overweight or obese.[107] Weight loss has been well demonstrated in human and canine patients to help with the pain and morbidity associated with OA.[108-112] Weight loss of 6.10% or more in obese dogs can significantly decrease subjectively assessed lameness associated with OA.[110] Objective improvement was also seen with kinematic gait analysis with weight loss of 8.85% or more.[110] It has been demonstrated in humans that being overweight or obese not only increases the load on the joints, it may alter joint alignment as well as induce an inflammatory state with increased concentrations of circulating pro-inflammatory cytokines and leptin.[113-116]

Restrictive diets may have an effect on the development of OA, and they have been advocated as a preventative option in at-risk breeds. Trials were performed on a

cohort of Labrador Retrievers fed ad libitum (control) or on a 25% restricted diet from 8 weeks of age. At the end of the study, 83% of the control dogs had developed OA while only 50% of the diet-restricted dogs developed OA. Additionally, at 8 years of age the prevalence of OA in two or more joints was 77% in the control group and 10% in the diet-restricted group.[117-119] Perhaps the most compelling part of this study is that the control dogs were only mildly overweight.[119]

Exercise

The long-term benefits of exercise in dogs with OA have been ill-defined in the literature. Most of our current recommendations come from human studies where regular exercise has proved to be beneficial in reducing the pain associated with hip and knee OA.[109,120] Exercise therapy may also decrease cytokine and cytokine-related gene levels in the synovial fluid, as well as inhibit inflammatory factor-mediated cartilage degradation.[109,120]

Following the diagnosis of OA, it is generally recommended to start dogs on a regular low impact exercise plan, such as leash walking, treadmill walking, stair climbing, walking up hills or ramps and swimming. Low impact exercise promotes muscle tone and weight loss as well as decreases stiffness and reduces muscle atrophy.[121,122] It is thought that exercise may increase cartilage metabolism and synthesis of proteoglycans through the periodic loading of the articular cartilage. [121,123] Additionally, active-resistive exercises, such as sit to stand, wheelbarrowing or dancing, can help with the animal's strength, stamina and co-ordination.[122,123]

Other aspects of physical therapy include passive range of motion exercises and proprioceptive exercises. Passive range of motion can help restore normal joint motion,

prevent contracture, and improve blood flow and lymphatics by moving the joint though a comfortable range of motion. Passive range of motion has been shown to significantly increase range of motion following 21 days of passive stretching.[124] Passive range of motion has also been shown to reduce the catabolic effects of inactivity on articular cartilage.[125,126] Proprioception exercises can help promote weight bearing on the affected limb by holding up the contralateral limb. Rocker boards may also be used to improve proprioception and balance.[127]

ii. Pharmaceuticals and Nutraceuticals

Nutraceuticals

Nutraceuticals are defined as a food that give medicinal or health benefits in addition to its nutritional properties. Several nutraceuticals have been shown to have beneficial effects for canine osteoarthritis. The most common nutraceuticals are omega-3 fatty acids, glucosamine and chondroitin sulfate.

Omega-3 fatty acids, which contain eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), help to modulate the inflammatory response. The primary omega-6 fatty acid in cell membranes is arachidonic acid (ARA), a precursor for the production of inflammatory eicosanoids found in OA: PGE_2, thromboxane A_2 (TXA_2) and leukotriene B_4 (LTB_4). By including a high level of long chain polyunsaturated omega-3 fatty acids into the diet, ARA is replaced by EPA in the cell membrane, thus inducing the production of the less inflammatory form of these eicosanoids: PGE_3, TXA_3, and LTB_5.[128-130] Additionally, omega-3 fatty acids have been shown to suppress IL-1β and TNFα expression in cartilage.[128,131] Trials in both humans and dogs have shown positive results with omega-3 fatty acid supplementation, with documented changes including

reduced plasma PGE_2 and synovial fluid MMPs, reduced lameness and enhanced weight bearing.[128,131-139] A recent study has also shown dogs with OA supplemented with omega-3 fatty acids required a lower dose of carprofen compared to prior to starting supplementation and to a control group.[138]

Glucosamine and chondroitin sulfate have most commonly been studied together in canine medicine. Glucosamine is thought to have a stimulatory effect on chondrocytes, while chondroitin sulfate is an endogenously produced polysaccharide found in the joint cartilage matrix that works synergistically with glucosamine to reduce inflammation and slow cartilage deterioration.[132,140-142] While studies in humans looking at the effects of glucosamine in OA patients have had mixed results, studies in dogs looking at the effects of glucosamine and chondroitin have had more positive results. However, these trials only contained a positive control, not a placebo, and the measurements were objective.[142-144] In summary, more research needs to be conducted on the effects of glucosamine and chondroitin sulfate.

Many other nutraceuticals have been proposed for use in canine OA including New Zealand green lipped mussel, which is credited for its anti-inflammatory properties, velvet deer antler and antioxidants.[145,146] Large, clinical controlled prospective studies to evaluate these products are lacking.

Pharmaceuticals:

Many different pharmaceuticals are available for the treatment of OA, including local or systemic medications available in oral and injectable forms. Most pharmaceutical drugs fall into the categories of non-steroidal anti-inflammatory drugs (NSAIDs) or corticosteroids.

NSAIDs are commonly used to relieve the pain and inflammation associated with OA. The main anti-inflammatory effect of NSAIDs is by inhibiting the cyclooxygenase (COX) enzymes that induce the conversion of arachidonic acid to prostaglandins and thromboxane. There are two main forms of COX: COX-1, a constitutive enzyme that is responsible for the production of homeostasis prostanoids, and COX-2, an inducible enzyme found in inflamed or injured tissue.[147] During OA, COX-2 is induced and synthesized by activated macrophages and inflammatory cells in response to the presence of pro-inflammatory cytokines, such as IL-1β.[147] Thus, blocking COX-2 decreases the amount of inflammatory mediators in the joint. [148]

In addition to decreasing inflammation within the joint, blocking COX-2 has also been found to decrease joint disease and may actually have a protective effect on subchondral bone when used in early OA.[149-151] One of the proposed mechanisms for this is through the prevention of NO induced cell death. [152]

As well as local effects on the joint, it is also thought that over time COX enzymes play a role in central sensitization. [153,154] It has been shown that not only does the use of NSAIDs reduce the central sensitization, but that in the case of chronic use of NSAIDs the reduction in central sensitization will in turn decrease the progression of the disease process within the joint.[155-158]

In the clinical setting, NSAIDs have been shown to improve joint pain and function. Several common NSAIDs used in small animal medicine include carprofen, meloxicam, robenacoxib and firocoxib. Several studies looking at the efficacy of carprofen have found clinical improvement in over 70% of dogs treated.[159-161] However, complete resolution of lameness is not as common, and the results may be inversely

correlated with the duration of lameness.[161] Likewise, clinical studies looking at the efficacy of other NSAIDs such as robenacoxib, firocoxib and meloxicam in dogs have found similar if not better results as the studies looking at carprofen.[162-165]

Side effects for NSAIDs are common and reported to occur in up to 52% of some study populations; however, most side effects consist of mild gastrointestinal upset and do not preclude administration. [165,166] Other more serious reported side effects are less common and include stomach ulceration, kidney damage and idiosyncratic liver toxicoses with carprofen use.[162,167-169]

Corticosteroids are 21-carbon molecules that contain three 6-carbon rings and a 5-carbon ring.[170] The pharmacological activity of a corticosteroid is determined by the presence of a hydroxyl group at the C-11 location. Corticosteroids are potent anti-inflammatory drugs that can be used as a local intra-articular injection, or less commonly systemically, to reduce inflammation during osteoarthritis; however, the use of corticosteroids is controversial.[171] The most common steroids for intra-articular use are 11β-hydroxyl compounds such as triamcinolone hexacetonide and methylprednisolone acetate, either alone or in combination with hyaluronic acid. Corticosteroids work by stimulating lipocortin which in turn inhibits plasma-bound phospholipase A_2. Phospholipase A is responsible for the conversation of phospholipase to achridonic acid, which would then go on to stimulate the COX and lipoxygenase (LOX) pathways.[148] Therefore, the inhibition of phospholipase A, indirectly inhibits the de novo production of prostaglandins, thromboxane and eicosanoids. Corticosteroids also decrease MMP activity; decreasing catabolic activity in the joint.[171] This may either be through a direct effect of the corticosteroid on MMPs or via inhibition of cytokines that would have gone

on to up-regulate MMP production.[171] Corticosteroids also have several other effects including inhibition of leukocyte infiltration, suppressing leukocyte superoxide synthesis, inhibiting platelet aggregation, decreasing vascular permeability and inhibiting IL-1β and TNF-α.[170] In experimental canine studies, the use of corticosteroids has been found to delay the onset of secondary changes such as osteophytes, decrease the synthesis of inflammatory mediators and may help to protect canine chondrocytes against the effects of IL-1β.[148,172,173]

There are some concerns with the use of corticosteroids for the treatment of osteoarthritis. Several studies have reported that corticosteroids inhibit mitotic activity, cell growth and collagen type II synthesis, which result in decreased chondrocyte viability and decreased matrix production.[148,174-178] Other research suggests that inflamed joints do not exhibit the same side effects as normal joints do.[179] Other reported side effects include septic arthritis, osseous metaplasia and pain following injection. Due to concern that patients may further injure their joint following injection, most authors recommend an arbitrary rest period that may range for 2-6 weeks. Although systemic absorption of steroids injected in the joint is unlikely to be significant, the use of oral steroids can have several side effects including increased drinking and urination weight gain, decreased immune system and with long term use can induce adrenocortical atrophy.

iii. Regenerative Therapies

Regenerative therapies have the potential to not only mitigate the clinical signs of OA, but also may help restore cartilage health. Examples of such therapies include platelet rich plasma (PRP), stem cell therapy and ACS

Platelet Rich Plasma

Platelet rich plasma is a concentrate of plasma containing at least 2-4 times the circulating number of platelets, that has been reported for the use of OA in people, horses and dogs.[181-185] . Depending on the method of production, PRP may also have a leukocyte, red blood cell or fibrin component.[186] It is thought that platelets can enhance the regenerative process within osteoarthritic joints either through direct stimulation of healing via α-granules or recruitment of stem cells from growth factor release.[185,187-191] A small number of leukocytes may be beneficial in the release of anti-inflammatory cytokines; however, they also have the potential for the release of pro-inflammatory cytokine and are therefore mostly minimized.

There are limited reports of PRP use in small animals; however, a recent clinical study found a single intra-articular injection of PRP resulted in significant improvement in joints with mild to moderate OA with respect to improved peak vertical force and client surveys.[182]

Stem Cell Therapy

Adult mesenchymal stem cells are precursor cells found in most adult tissues that are capable of differentiating into different mesenchymal cell lineages: adipocytes, chondrocytes and osteocytes.[192] The main proposed advantage of stem cell therapy is the potential for regeneration of hyaline cartilage, instead of the biomechanically inferior

fibrocartilage. It is also possible to obtain stem cells from fetal tissue. Fetal stem cells are capable of differentiating in to a wider range of tissue types. However, fetal stem cells have a higher rate of complications including reaction and teratoma formation as well as ethical concerns. The richest source of mesenchymal stem cells is from falciform fat.

Currently, there are few clinical canine studies looking at the effects of stem cell therapy in canine OA. Two prospective, randomized, blinded studies have been conducted to investigate the effect of MSC on canine elbow and coxofemoral OA; both studies reported that dogs treated with stem cells had subjectively improved lameness.[193,194] However, it is prudent to note the improvement was only noted by the veterinarian not the owner, and the papers were commissioned by a producer of stem cells.

iv. Surgical

There are no surgical options that directly treat OA. In some cases, such as cranial cruciate ligament rupture, surgery can help slow the progression of OA but will not reverse or completely halt OA. Arthroscopy may be used as a diagnostic and to debride damaged tissue, as in the case of a fragmented medial coronoid process. In severe cases of OA, total joint arthroplasties, arthrodesis or excisional arthroplasties may be indicated.

2. Autologous Conditioned Serum

Autologous conditioned serum (ACS) is produced by incubating blood with medical grade borosilicate spheres.[195] The spheres induce peripheral leukocytes to produce IL-1ra and other anti-inflammatory cytokines by release from intracellular reserves and *de novo* synthesis.[196] So far we do not fully understand the mechanism of

action for ACS. Researchers have looked at the cytokines present in ACS made from human and equine blood, but the list is unlikely to be extensive.[195,197,198] Also, the concentrations of cytokines have a high standard deviation, indicating there is a variance between individuals.[195,198]

a. In Human Medicine

ACS has been used in human medicine for the last 15 years, with a wide range of clinical applications and efficacies reported. Studies looking at the cytokine profile of human ACS have found a 3-140-fold increase in IL-1ra and a moderate increase in a number of other anti-inflammatory cytokines as well as a small but significant increase in IL-1β and TNF-α.[195,197,199]

Several other studies looking at the use of ACS in patients of the age of 18 with knee or hip OA have found ACS improves the clinical signs of OA, such as pain and loss of function. [200-203] Outcome measures in these studies included the use of patient-administered outcome instruments: global western Ontario and McMaster university OA index (WOMAC), knee injury and osteoarthritis outcome score, the knee society clinical rating score and visual analog pain scale. In most studies, dosage of ACS was guided by manufacturer's recommendations: 2ml of ACS, two injections per week for 3 consecutive weeks. In a large clinical trial using ACS for the treatment of OA, there was significant improvement in the global western Ontario and McMaster university OA index (WOMAC) at all time points as compared to hyaluronan and saline placebos. Additionally, ACS was found to have marked clinical and significant therapeutic effects on major symptoms of knee OA over the 6 months, with minimal adverse effects.[204] A similar paper comparing ACS to hyaluronan in 376 patients also found an improvement

in patient-administered outcome instruments.[203] This improvement lasted out to the finial 2-year follow-up.

However, other researchers have found a different result with ACS showing no benefit over placebo in the treatment of OA. [205] A clinical trial of 22 patients with OA looked at the effects of ACS injection on cytokine concentrations in synovial fluid.[197] Patients were treated with either injections of ACS or untreated serum at 0, 3, 7, 10, 14 and 21 days. Prior to each injection a sample of synovial fluid was collected. Additionally, the study looked at the effects of ACS on cultured OA cartilage explants collected from patients undergoing knee replacement surgery. The study found no effect of the ACS on prostaglandin metabolism, or on the concentrations of cytokines in the joint. The authors hypothesized the results may be due to the fast clearance of the cytokines from the joint. A later trail by the same author compared ACS to plasma controls in 74 patients, and found no difference in clinical improvement as based on patient questioners at 12 months.[205] In this study only 20 of the 74 patients opted to be in the ACS group, this caused the study to have a lower power and a selection bias.

Reported adverse effects include mild local reactions (transient knee pain, swelling, tenderness and heat at the injection site), anticipatory pain at injection, and treatment failure due to the laborious nature of the treatment (time and logistics).[203,205]

In addition to application in the treatment of OA, ACS has also shown to be effective in the treatment of sciatic pain[206], tendon healing[207], and to reduce bone tunnel widening following anterior cruciate ligament surgery.[208] ACS has also been shown to enhance muscle healing in both experimentally in mice and in a clinical setting.[209,210]

b. In Equine Medicine

There is a growing interest in ACS in the equine industry; however, few clinical studies looking at the histological and clinical effects of ACS have been published. Similar to human cytokine profiles, horse ACS results in a around a 93 to 119-fold increase in IL-1ra along with a small but significant increase in IL-1β, TNF-α and some other anti-inflammatory cytokines.[198]

A single experimental study exists in equine medicine looking at the effects of ACS on OA. OA was induced in the middle carpal joint of 16 horses, each horse was then treated with 4 injections, 7 days apart of either a placebo or 6ml of ACS.[211] ACS-treated osteoarthritic joints were reported to have significantly less intimal hyperplasia compared to placebo treated and trended towards having lower scores for cartilage erosion and synovial membrane hemorrhage.[211] Clinically, ACS was shown to significantly improve lameness compared to the placebo treated horses.[211]

c. In Small Animal Medicine

To date, there are no clinical trials evaluating the use of ACS to treat OA in small animal medicine. There are reports using dogs as a model for OA, which investigated the effect of intra-articular injections of human IL-1ra genes. These studies found that a local increase in IL-1ra within the joint reduced the progression of experimentally induced OA.[212,213] Similar results were obtained after injection of canine IL-1ra into rabbit stifles.[214] Recently a study looked at the validation of a canine specific IL-1ra ELISA using a commercially available ACS system and found they could produce a 40-fold increase in IL-1ra compared with un-processed serum.[215] Investigation into other cytokines or growth factors in canine ACS has not been performed.

3. Conclusions

Osteoarthritis is a common and extensively studied disease in humans, horses and dogs. However, despite extensive research, current treatment mainstream options are limited to predominately symptomatic treatment. The balance between pro-inflammatory and anti-inflammatory cytokines plays a defined role in the pathogenesis of OA. This role has been exploited and used as a viable treatment target in human and equine medicine. Although further research is required to investigate the efficacy of cytokine therapy in dogs, the outcome appears promising.

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Chapter 2: Cytokine and Growth Factor Profiles in Healthy Canine Autologous Conditioned Serum

5. Introduction

Osteoarthritis (OA) is estimated to affect as many as 20% of dogs over 1 year of age.[1] Conventional treatments such as non-steroidal anti-inflammatory drugs and corticosteroids are non-specific, directed only at managing the symptoms of OA, and have the potential for systemic and/or gastrointestinal side effects.[2-5] The treatment of OA is therefore challenging and new therapies are needed.

Autologous conditioned serum (ACS) is an autologous biological intra-articular therapy used in human and equine sports medicine for the treatment of OA that specifically blocks the major inflammatory mediator, interleukin (IL)-1β.[6,7] Incubation of whole blood in the presence of medical grade glass beads stimulates white blood cells to produce anti-inflammatory and regenerative cytokines, including interleukin-1 receptor antagonist (IL-1ra), the natural antagonist of IL-1β.[8] Supraphysiologic concentrations of IL-1ra block the ability of IL-1β to bind cell surface receptors, reducing inflammation and slowing the progression of cartilage degeneration.[9-14] Higher concentrations of IL-1ra compared to IL-1β are required to effectively block IL-1β receptors and slow cartilage destruction.[7,8,15] In addition to increased concentrations of IL-1ra, increased concentrations of other anti-inflammatory cytokines and anabolic growth factors, without a concurrent increase in pro-inflammatory cytokines, is reported in ACS in other species.[7,8,15] The processing method for ACS takes advantage of harvesting effector molecules from both white blood cells and platelets, resulting in higher cytokine and

growth factor concentrations than those reported for platelet rich plasma.[16] Recently, 10 mL irap syringes have become commercially available, making ACS a practical alternative in canine medicine. To the authors' knowledge, data describing growth factor and cytokine concentrations using the irap 10 syringes in canine ACS are not reported. A recent study reported increased IL-1ra concentrations in canine ACS compared to incubated control serum using another autologous serum processing system (IRAP™ II, Arthrex Vet Systems, Naples, FL).[17]

The purpose of our study was to compare the growth factor and cytokine profile of canine ACS to canine plasma. We hypothesized that anti-inflammatory cytokines (IL- 1ra, IL-4, and IL-10) and growth factors (transforming growth factor (TGF)-β1, insulin-like growth factor (IGF)-1, and fibroblastic growth factor (FGF)-2) in ACS would be significantly higher than plasma controls, but that inflammatory cytokines (IL-1β and tumor necrosis factor (TNF)-α) would not.

6. Materials and Methods

Study Population

Healthy adult dogs (n=22; 11 castrated males, 9 spayed females, and 2 intact females) were recruited from the community for the study. The study was approved by the Institutional Animal Care and Use Committee, the hospital board and written owner consent was obtained. The mean age was 5 ± 3 years and mean weight was 29.8 ± 5.1 kg. Breeds were mixed (6), Golden Retriever (3), Pitbull Terrier (3), Australian Shepherd (3), Labrador Retriever, Catahoula Leopard Dog, Boxer, Coonhound, Husky, and Staffordshire Terrier (1 each). Dogs were deemed healthy based on normal physical and orthopedic examinations and were excluded if lameness was detectable at a walk or trot, if neurologic disease was present, if a clinically significant illness was detectable from physical examination or history, or if medication was administered in the 10 days prior to blood collection.

Blood Collection

For ease of sampling and to minimize variation in circulating cytokine concentrations, all samples were collected at the same time of day in the early morning and food was withheld overnight. Dogs were allowed free access to water. Dogs were sedated with acepromazine (0.02 mg/kg, IV) and held in left lateral recumbencey. A 5 cm^2 area of hair was clipped from over the right jugular vein and the skin prepared for venipuncture using standard aseptic technique. A total of 40 mL of blood was collected from each dog using a 20 gauge butterfly catheter and distributed as follows: 2 Orthokine® vet irap 10 mL syringes (ORTHOGEN Veterinary GmbH, Düsseldorf, Germany) and 2 syringes

containing 1.3 mL acid-citrate-dextrose (ACD-A) each for control plasma. The irap 10 samples were collected first to avoid potential contamination with ACD-A. Care was taken to draw blood slowly to avoid hemolysis and syringes were mixed gently by inversion 10 times immediately following collection. All irap 10 syringes were filled to the same mark on the syringe to ensure equal volumes in each syringe. In the final 6 dogs, an additional 10 mL serum control was collected into a sterile red top blood tube to be used for comparison against plasma. Samples were processed (or incubated in the case of ACS) within 30 minutes of collection.

Sample Processing

All irap 10 syringes were processed according to manufacturer's instructions using aseptic technique. Briefly, the plunger was snapped off and the irap 10 syringe was placed upright in a standard tube rack with the sampling port at the top and incubated at 37°C for 7 hours in a dedicated incubator (Boekel Scientific, Feasterville, PA) as per manufacturer's instructions. Following incubation, tubes were immediately centrifuged at 2900 x g and 22°C for 10 minutes using a standard tabletop centrifuge (5804R, Eppendorf North America, Hauppauge, NY). Tubes were carefully removed, placed in a rack, and disinfected using 70% isopropyl alcohol. The resulting serum was collected using an 18 gauge 3.5 inch spinal needle connected to a 12 mL syringe, the 2 irap samples pooled into a single syringe, and filter sterilized (0.2µm sterile syringe filter, Acrodisc®, Pall Corporation, Port Washington, NY). As samples were filtered, they were aliquoted into polypropylene microcentrifuge tubes. The tubes were labelled and stored immediately at -20°C for ELISA assays.

Immediately following collection, plasma control samples in ACD-A were transferred to sterile red top blood tubes. Control serum samples collected without anticoagulant were allowed to clot at room temperature. Control plasma and serum samples were centrifuged at 2000 x g and 22°C for 10 minutes and the plasma or serum harvested as described above. Identical plasma and serum samples were pooled and filtered as for ACS. Plasma and serum were aliquoted and stored immediately at -20°C as described above.

Cytokine and Growth Factor Quantification

In preparation for quantification by ELISA, samples were thawed on ice to control proteolysis and went through only 1 freeze thaw cycle. Samples were stored for no longer than 6 months before processing. The following ELISA kits were used: IL-1ra, IL-1β, and TNF-α (Kingfisher Biotech Inc, St. Paul, MN), and IL-4, IL-10, TGF-β1, FGF-2, and IGF-1 (NeoBioLab, Cambridge, MN). For the subset of 6 serum samples, ELISA for TNF-α, IL-1β, and IL-1ra were performed (n=22). Remaining ELISA assays (IL-4, IL-10, TGF-β1, FGF-2, and IGF-1) were run only on the first 16 plasma and ACS samples. Cytokines were quantified in duplicate according to the manufacturers' instructions using canine-specific ELISA and standard curves generated, from the average of the duplicates, for each cytokine/growth factor kit.[18] Because of known challenges in accurately measuring IL-1ra in blood products, samples for IL-1ra quantification were diluted 1:3 for serum and plasma and 1:8 for ACS samples using sample buffer (Dulbecco's Phosphate-Buffered Saline with 20% fetal bovine serum) and based on recommendations

from the manufacturer and a previous study.[17] None of the samples were diluted for other ELISA. Plates were read by spectrophotometer (Spectramax 5, Molecular Devices, Sunnyvale, CA).

Data Analysis

All data were skewed to the left and were therefore analyzed using the Wilcoxon signed-rank test (SAS version 9.4, SAS Institute Inc., Cary, NC) with significance set at $P<.05$. For the data sets including serum (IL-1ra, IL-1β, and TNF-α), multiple pairwise comparisons were performed using the Wilcoxon signed-rank test. To estimate the IL-1ra to IL-1β ratio, the lowest detectable concentration of IL-1β (94 pg/mL) was used for values below the detectable limit of the ELISA to avoid a denominator of zero (5 serum, 15 plasma, and 13 ACS samples). The lowest detectable limit was selected as a conservative estimate so as to underestimate the ratio rather than overestimate.

7. Results

There was a large variability in all growth factor and cytokine concentrations between individual dogs. No significant differences between ACS and plasma were identified for IL-10, TNF-a, IGF-1, FGF-2, and TGF-β1 (Table 1). Concentrations of IL-4, IL-1β, and IL-1ra in ACS were significantly higher than plasma (Table 1). There was no significant difference for IL-1ra, IL-1β, and TNF-α between ACS and serum, or for TNF-α and IL-1β between serum and plasma (Table 1). The concentration of IL-1ra was significantly higher in serum compared with plasma. The IL-1ra:IL-1b ratio for ACS (median 39.9, range 4.6-3,300.0) was significantly higher than for serum (9.1, 7.4-32.9; *P*=.04) or plasma (7.2, 1.2-151.2; *P*<.001). The IL-1ra:IL-1b ratio for serum was significantly higher than for plasma (*P*=.04).

8. Discussion

The results of our study demonstrate significantly increased concentrations of IL-1β, IL-1ra, and IL-4, as well as a significantly higher IL-1ra:IL-1β ratio in ACS compared to plasma controls. No significant differences between plasma and ACS for TNF-α, IL-10, IGF-1, TBF-β1, or FGF-2 were identified. Concentrations of IL-1ra and IL-1β in canine ACS were similar to those reported in other species.[7,15,17,19]

Overall, we found a 5-fold increase in IL-1ra between plasma and ACS, which is similar to the 3.2-140-fold increase reported in the human literature,[7,8] but less than the 93-119-fold increase reported in the equine literature.[15] A recent canine study using the IRAP™ II autologous processing system and a 24 hour incubation time reported a 40-fold increase in IL-1ra in ACS compared to serum.[17] This difference compared to our results may be due to use of a different processing system, canine population, or longer incubation time. The two systems have not been compared in the same population of dogs. Production of IL-1ra in ACS is reported to increase with longer incubation times, the number of glass beads, and the quality of glass beads.[8] We chose a 7 hour incubation from the range of 6-9 hours recommended by the manufacturer to enable collection and processing within a standard work day.

In previous studies, serum was used as a control for comparison with ACS.[7,8,15,17,19] Plasma was selected over serum as the control in our study to avoid elevations in growth factors and cytokines resulting from platelet degranulation during clot formation.[20] Because our study design was modified to collect serum in addition to plasma from only

the last 6 dogs, this portion of the sample size lends itself to Type II error when comparing serum to either plasma or ACS.

The fold increase of IL-1β in canine ACS compared to plasma in our study is of similar magnitude to that reported in people and horses.[15,19] The concentration of IL-1β in ACS is an important consideration, as IL-1β and TNF-α are thought to be the main inflammatory factors implicated in OA.[21-23] IL-1β drives cartilage degeneration and TNF-α is associated with pain and inflammation.[21-23] IL-1β at 1,000 pg/mL is commonly used to induce detectable cartilage damage in vitro[24-25]; however, proteoglycan synthesis is inhibited by concentrations as low as 10 pg/mL in vitro.[26] An alternative indicator of therapeutic efficacy is the ratio of IL-1ra to IL-1β.[15] The estimated IL-1ra;IL-1β ratio of 39.9 derived from our data is consistent with human studies suggesting that IL-1ra;IL-1β ratios of 10-1,000 are needed to effectively block the negative effects of IL-1β on cartilage.[27,28]

There was a large variability in growth factor and cytokine concentrations between individual dogs. In people, the importance of timing sample collection is well recognized when quantifying growth factors and cytokines.[29-33] As growth factors and cytokines are released under neuroendocrine control, fluctuations in circulating concentrations follow a diurnal rhythm.[29-31] In order to minimize variability between dogs, all samples were collected at a consistent time in the morning and with the dogs fasted overnight. Other

factors such as recent exercise and disease state can also cause fluctuations in cytokine concentrations.[34,35]

The irap 10 system is easy to use in the clinical setting and is a financially viable therapy for a broad range of small animal veterinarians. No special equipment is required. In addition to the syringes themselves, only a committed space to process samples aseptically and a standard bench top centrifuge and incubator are required. Attention to strict sterile technique must be maintained throughout collection, processing, and injection.

The challenge of our study was in accurately detecting cytokines. Traditionally cytokines are difficult to quantify, particularly from high protein samples such as plasma and serum.[36,37] Factors including pH of the sample, interaction of the cytokine with the protein matrix, the stability of the cytokine, and binding of cytokines to soluble receptors can make cytokine detection inaccurate and difficult to interpret.[38-41] Variations caused by protein in samples can be overcome by sample dilution,[40,42] which may, at least in part, reverse soluble receptor binding.[41,43] Ideally all samples for comparison would be run at the same dilution; however, because of the large difference in concentration between serum and plasma samples compared to ACS, this was not possible. The dilutions used for IL-1ra in our study were based on available data,[17] recommendations from the manufacturer, and dilution trials. Additionally, although the ELISA assays used in our study were canine-specific, with the exception of IL-1ra,[17] none have been validated for

use with canine ACS. Therefore, it is the authors' assumption that the measured values accurately represent those present in the sample.

In conclusion, growth factor and cytokine concentrations in canine ACS are comparable to those reported in other species, with some minor species variations. High concentrations of IL-1ra, proportion of IL-1ra to IL-1β, and low concentrations of inflammatory cytokines in canine ACS create a potentially favorable cytokine profile for the treatment of joint disease in dogs. Based on our results and the literature supporting the efficacy of ACS in other species, future investigations into the use of ACS for the treatment of OA in dogs are warranted.

9. References

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