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PHYSIOLOGIC RESPONSES TO INFLAMMATION IN ISOLATED EQUINE JOINTS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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* * * * *

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ABSTRACT

Articular inflammation is a common finding in equine joints disorders. As in other organs, several aspects of the joint system, including blood flow, transsynovial permeability and fluid exchanges, cellular infiltration and mediator release may be involved in the inflammatory process and contribute to joint morbidity. With inflammation, tissues within the joint contribute to mediator release and stimulate degradation of the cartilage matrix. Perfused oxygenated extracorporeal isolated models have been thoroughly described for organs such as heart, lung and intestine, but have never been created for the joint organ. The studies reported here describe an isolated pump-perfused auto-oxygenated extracorporeal isolated joint model developed in the equine metacarpophalangeal joint. Using this model, the local Starling’s forces in the isolated joint at isogravimetric state are characterized and alterations in Starling’s forces in response to hemodynamic manipulations are described. In a separate series of experiments, articular inflammation was induced in the isolated innervated or denervated joint organ and local hemodynamic, fluid exchanges, metabolic responses, alterations in permeability to small and large molecules, inflammatory mediator release, cellular events and articular cartilage metabolic alterations are described.

Two preliminary studies were performed in the development of the isolated joint model. The first studies was essential to gain a better understanding of the local biomechanical behavior of the fluid filled closed joint cavity. In this study, the investigation of pressure volume curves in the joint revealed a sigmoid relationship, with low compliance at normal subatmospheric pressures, and gradually increasing compliance at supraatmospheric pressures of up to 30 mmHg. Thereafter, compliance of the joint again
decreased exponentially. This study also revealed an important function of synovial fluid to increase joint compliance and hysteresis therefore preventing collapse of the joint cavity and allow fluid exchanges at subatmospheric pressures. Furthermore this study emphasized the importance of joint angle on pressure-volume relationships. In the second preliminary study, a colored microsphere technique was validated for determination of blood flow to the synovial membrane and joint capsule. Furthermore, that study documented a significant decrease in synovial membrane blood flow down to 16% of baseline at intraarticular pressures of 30 mmHg or greater.

The development and physiologic responses of the isolated model were subsequently described. The extracorporeal pump perfused isolated joint unit developed in this model was successfully be maintained for 6 hours. Using this system, isogravimetric state was defined at a circuit arterial pressure of 133 mmHg and venous pressure of 10.5 mm Hg. Starling’s forces were defined for this system. Arterial and venous pressure manipulations revealed that increases in arterial pressure significantly raised transsynovial flow and synovial fluid production, when IAP was maintained at atmospheric pressure. A similar but much less important response was observed with increased venous pressure.

Acute inflammation was successfully induced in the isolated innervated or denervated joint preparation, using interleukin-1β. Acute inflammation induced a significant increase in oxygen consumption and extraction ratio in response to increased metabolic demand. Acute inflammation increased synovial membrane permeability to albumin, but denervation lowered permeability to large molecules (MW 144,000). Inflammation significantly increased synovial fluid production and the permeability surface area product of the synovial membrane.

Acute inflammation was associated with a significant cellular response characterized by neutrophilic leukocytosis in synovial fluid and a neutrophilic vasculitis in the synovial membrane. A significant increase in interleukin-6, prostaglandin E₂ and substance P was
detected in synovial fluid collected from inflamed joints. Inflammation induced a significant degradative response in articular cartilage, even after this short term exposure to an inflammatory mediator.

The isolated joint model described in these studies provided new insights in our understanding of articular physiology, an allowed to study of all pathophysiologic processes involved in articular inflammation. This model should be useful to further our knowledge of the response of joint to various disease processes, and should be very suitable to study the pharmacokinetics and pharmacodynamics of systemic or intraarticular therapies.
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**Book chapters**


**Proceedings and abstracts**

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FIELDS OF STUDY

Major field: Veterinary Clinical Sciences

Studies in Articular Physiology and Pathophysiology
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CHAPTER 1

INTRODUCTION

Nature of the problem

Lameness is the leading cause for decreased performance, days lost to training and wastage in horses. Lameness attributable to joint disease represents greater than 50% of lameness diagnosis. Ten years ago, it was estimated that the cost to the racehorse industry alone was greater than half a billion dollars.

In people, osteoarthritis is the most common form of joint disease. Its prevalence is greater than 75% in people over 70 years old, and radiographic evidence of OA can be found in as many as 80% of people over 55. Joint disease is the leading cause of morbidity in people, particularly the elderly. Joint disease, initiated by trauma, is also the determinant of retirement from athletic endeavors.

Joint disease has been classified by several schemes. A distinction has been made between arthritis, where an inflammatory component predominates, and arthrosis in which degeneration without evidence of inflammation is observed. On an anatomical basis, joint disease has been classified based on the pathologic tissue type, as in synovitis, osteochondral fracture, or cartilage degeneration. Duration of disease can qualify the syndrome as acute, subacute or chronic, and stable or progressive. An etiologic basis for classification of joint disease is most commonly used, because the disease process is
complex, and may involve multiple tissues within the joints, and affect multiple systems (for example lupus erythematosus). Joint disease has been classified as primary or secondary. Primary joint degeneration is a recognized syndrome in people, where joint degeneration is present without a specific etiology. Primary joint degeneration is not a recognized entity in horses, where joint disease can be traced to a specific etiology. Secondary joint disease follow such etiologies as trauma, infection, immune-mediated related problems. Traumatic arthritis has been further specified as traumatic synovitis and capsulitis (type 1 traumatic arthritis), sprains and luxations (type IIA traumatic arthritis), meniscal tears (type IIB traumatic arthritis) or intraarticular fractures (type IIC traumatic arthritis). Immune-mediated joint disease is rarely encountered in the horse, whereas it is a multi-factorial complex commonly recognized in people. All forms of arthritis, given enough time, can lead to chronic degenerative joint disease, also referred to as osteoarthritis or osteoarthrosis.

Models of joint disease have been defined and used in several animal species. Models of osteoarthritis, infectious arthritis, traumatic arthritis, synovitis, and rheumatoid arthritis abound, and even genetically engineered mouse models, predisposed to developing joint degeneration, exist. These models aim to mimic a naturally occurring condition of people, but extrapolation or external validation of these studies and their conclusion should be taken with caution because of such issues as animal species, age, size, weight, gait, posture, and health. For example, rabbits have been shown to heal cartilage defects with hyalin cartilage, whereas in horses and people, fibrocartilaginous tissue predominates in the healing defect. Furthermore, this tissue is unable to withstand the rigors of exercise, and one year follow-up studies have revealed degeneration of the filling tissue. Young animals have significantly greater ability to heal cartilage, because of greater endogenous activity of chondrocytes, than older animals. Active
motion, and to a greater degree, passive motion exercise have been shown to greatly increase the healing of cartilage defects.18,22 Finally, animals with endocrine problems such as diabetes, have decreased wound healing, and more relevantly decreased cartilage healing.23

Because of the importance of joint disease in the horse industry, many equine models of joint disease have been described.17,18,24-28 These models aim to mimic synovitis, look at healing of cartilage defects, mimic naturally occurring osteochondral fractures, infectious arthritis, and most commonly are aimed at investigating therapies. Recently, in vitro models have been explored, in order to provide controlled experimental conditions and maximize use of animals.20,21,29-32 Extrapolation to the in vivo situation is more remote in these latter models, but a greater understanding of disease processes and pathophysiology can be gained at minimal cost and morbidity.

Many models of joint disease address a specific question, and examine a specific tissue or phenomena within the joint. Studies usually focus on the tissue rather than the entire joint as an organ. In reviewing the physiology literature, many aspects of joint physiology such as blood flow, transsynovial fluid exchange, and innervation have not been examined in the horse, and have been only partially described in other species.33-40 In other organ system models, such as heart, lung, liver and intestine it has become evident that many physiologic mechanisms and tissues interact in disease. Such an approach however, had not been examined in the joint. In other organ systems, ex vivo analyses, which allowed manipulation of physiologic mechanisms, were investigated, in order to improve our understanding of organ function, organ homeostasis, and disease process. Classic models, such as the Krogh model,41 the Pappenheimer preparation,42 and the Langendorff heart preparation,43 to name a few, were developed to study organ physiology
and pathophysiology while controlling other systems. Our present study investigated the organ physiology of the joint.

Hypotheses

Our first hypothesis was that we could successfully develop an isolated joint organ preparation that would allow measurement and manipulation of the major Starling's forces regulating capillary exchange, namely capillary, interstitial and articular hydrostatic pressures, and capillary, interstitial and joint oncotic pressure. Our second hypothesis was that manipulation of Starling's forces in the isolated joint would allow the calculation of the forces that regulate capillary fluid exchange to and from the joint cavity. Our third hypothesis was that interleukin-1β could be used as a model of acute inflammation in the isolated joint and that measurable alterations in articular metabolism, cell trafficking, cartilage chondrocyte metabolism and release of inflammatory mediators would result. Our fourth hypothesis was that denervation of the joint would modify the inflammatory response.

Our preliminary studies (Chapters 2 and 3) were designed to overcome and address several technical issues that pertain to an isolated joint organ preparation, such as the relevance of intraarticular pressure to hemodynamics, and the hemodynamic alterations associated with creating a surgical preparation. Appendix A describes the physiologic responses and perfusion of isolated joint preparation. Initially isolation of the equine carpus was attempted, with failure to produce a closed circuit. Extensive soft tissue trauma accompanied total carpal extracorporeal isolation, and partial isolation resulted in significant bone venous outflow and failure to isolate and control venous pressures. The equine metacarpophalangeal (MCP) joint was subsequently used successfully (Appendix A). In chapters 4 and 5 the isolated MCP joint organ was used in an experimentally induced acute arthritis model, to study the physiologic responses to inflammatory insult and the role of innervation in the acute response to this insult.
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CHAPTER 2

REVIEW OF THE LITERATURE

Introduction

Until recently, emphasis has been placed on articular cartilage biochemistry and cellular biology to gain understanding of the pathophysiology of joint disease and as a target for therapeutic efforts. In a recent paper, an extensive review of articular biochemistry in health and disease clearly summarized the importance of biochemistry and molecular biology to describe the pathophysiology of joint disease.  

The joint is an organ composed of several heterogenous tissues, with local blood supply, innervation, and fluid exchanges which function to maintain health and may contribute to the different aspects of disease. This paper will describe the normal articular physiology including blood flow, innervation, fluid exchanges between compartments and situate these physiologic processes when altered by disease.

General Anatomy and physiology

Tissues of the joint cavity have a specialized composition and three dimensional anatomy which ultimately relate them to their specific biomechanical function. As such the articular cartilage, whose ultimate function is to absorb and transfer loads, is a composite of collagen which grants it its tensile strength and highly charged proteoglycan subunits which provide compressive stiffness. In order to maintain cartilage composition and function, a slow but steady turnover of its components occurs, the rate of which is dependent on specific factors including age, mechanical load, and joint environment.  

The building
materials necessary for this turnover are provided through blood flow and exchanges from the synovial membrane and joint capsule, as well as through ultrafiltration and formation of synovial fluid, which provides the ultimate media for these exchanges to occur.6

The supply of nutrients to the avascular but metabolically active articular cartilage is provided by exchanges through the synovial microcirculation and transport in synovial fluid. The synovial membrane is a specialized unit designed to provide a pathway for fluid exchanges, as well as a blood supply, and add to the composition of synovial fluid through hyaluronate synthesis. The synovial membrane is composed of an intima (also referred to as synovium), consisting of luminal layer 1 to 5 cells deep, a capillary plexus which lies 6-11 μm beneath the intimal surface, and a deeper network of lymph vessels, and a subintima (or subsynovium) composed of adipose, areolar or fibrous tissue.6 The intima is designed to favor exchanges between capillaries and the joint cavity: it lacks a basement membrane, intercellular gaps are present (up to 19% in the rabbit knee), and intimal capillaries are fenestrated, with fenestrae are oriented towards the joint cavity.6

Furthermore, the synovial intima adopts a three dimensional architecture which is dependent on its biomechanical environment. In areas of high biomechanical stress, the synovium is flat and rests on a fibrous mechanically strong subintima. This is particularly true of synovium underlying tendons that cross over a joint, such as the extensor tendon overlying the metacarpo-phalangeal joint. In synovial recesses, the intima is thrown into a three dimensional villous architecture. This network is more richly vascular and has been shown to be a preferential site of solute and macromolecule exchanges.7 Hyaluronan synthesis is also greater in synovium from synovial villi.8 Intimal cells include synovial fibroblasts (or type B cells, approximately two thirds of cells) and bone marrow derived mononuclear phagocytes (or type A cells, approximately one third of cells)9. Light
microscopy techniques are not a useful guide for assessing lineage, as subintimal macrophages are often elongated, and intimal fibroblasts may take a rounded appearance. These cells can be separated on the bases of cytochemical staining for uridine diphosphoglucose dehydrogenase, a marker for hyaluronan synthesis, and non-specific esterase, a macrophage marker. These cells are also unique as they are found in close association with specialized components of the intimal matrix. As an example, the adhesion molecule ICAM-1, expressed by synovial fibroblasts, binds to hyaluronan. Although technical difficulty has made the identification and relative numbers of intimal cells of different lineage, there is currently no evidence to suggest that intimal fibroblast can transform to a macrophage phenotype. Although the synovial intima is interrupted and does not possess a basement membrane, interaction of synovial fibroblasts with the specialized matrix of the synovial intima forms a barrier adapted to the regulation of synovial fluid composition. Because of its high hyaluronan content, the synovial intima forms a continuum with synovial fluid for exchanges of fluids and solutes. In addition, other glycoproteins such as fibronectin, laminin, type IV collagen, type V collagen, entactin, and sulfated glycoaminoglycans such as chondroitin sulfate, secreted by the deeper type B synoviocytes, may serve to anchor the intimal cell layer to the underlying connective tissue. The ability of the intima to maintain itself as a layer appears closely related to cell-matrix-cell interactions, through expression of adhesion molecules such as ICAM-1, VCAM-1, fibronectin, laminin and others and their integrin ligands are likely candidates. With inflammation, the relative predominance of these cells types is altered, with subintimal macrophages forming 50-70% of cells in the intima. Integrin expression is also increased, and the morphology of the cells is changed from small cells arranged parallel to the joint surface, to a more superficial location arranged perpendicular to the joint cavity. These cells are also the dominant source of intimal vascular cell adhesion molecule
Expression of cell adhesion molecules by synovial intimal cells serve to direct leukocyte trafficking in disease. Most multinucleate cells within inflamed synovial intima carry macrophage lineage markers. There is also evidence that synovial intimal cells could direct leucocyte trafficking in health and disease, through adhesion molecule expression. Currently, there is no evidence that subintimal macrophages can divide. Intimal fibroblasts do divide in disease, albeit at a slow rate, consistent with synovial proliferation observed with chronic inflammation.

**Articular circulation**

The synovial membrane functions to maintain articular homeostasis by providing a pathway for the exchange of nutrients and metabolic by-products between blood and synovial tissues, including articular cartilage. Optimal oxygen delivery to articular tissues serves to maintain normal synovial fluid composition, chondrocyte metabolism, and ensure normal matrix composition and turnover. The efficiency of exchange between the synovial membrane capillaries and joint cavity is dependent on capillary density, capillary depth and blood flow [Levick, 1990 #41].

Several tissues in the joint are provided with a rich vascular supply which can be affected by physiologic or pathologic states. These include the joint capsule, synovial membrane, intra and periarticular ligaments, and subchondral bone. Articular cartilage is avascular, but the outer third of the menisci is supplied by small vessels arising from the joint capsule. The articular blood supply of most diarthrodial joints is formed by small branches of the epiphyseal arteries running at the junction of the periosteum and the synovial membrane, forming an arterial circle (circulus arteriosus). Larger branches penetrate the bone whereas smaller branches remain at the periphery of the articular cartilage, forming the perichondral circulation. Subchondral bone blood supply is provided
by the epiphyseal arteries, which travel in the epiphysis parallel to the articular cartilage, giving off at interval small perpendicular branches which end in capillary loops at the deep surface of the calcified cartilage. Before physeal closure, this epiphyseal circulation is distinct from the metaphyseal circulation. This segmentation of blood supply may explain the subchondral and epiphyseal location of infection in the equine neonate. \(^{20,21}\)

Conversely, segregation of blood supply to the metaphysis in the older foal provides an explanation for localization of infection to the more mature side of the growth plate. \(^{20,21}\)

The synovial membrane vascular supply is composed of capillaries which are very sparse in areas of high mechanical stress. The angle of reflection of the synovial membrane is composed of a rich vascular plexus and synovial villi are incompletely penetrated by a central arteriole. The richest capillary density in the synovial membrane is within 25 μm of the joint surface. Capillary density is greatest in areolar or adipose synovium and lowest in fibrous synovium. Similarly, blood flow to synovium is greater than in the fibrous joint capsule. \(^{22}\)

Synovial capillaries are fenestrated, with the fenestration oriented towards the joint cavity. \(^{6}\) The rich and superficial blood supply to the synovial membrane explains why hemarthrosis after needle puncture is frequently encountered, and provides an explanation for the hematogenous spread of infection to that area. In addition, the close proximity of highly vascular (synovium) to avascular (cartilage) tissue, referred to as a “borderline area”, makes this anatomical site a preferential location for systemically injected particles. \(^{23}\)

Many joint diseases exhibit greater inflammation at the angle of reflection of the synovium on the articular cartilage, and this site may be more appropriate when synovial membrane biopsies are obtained for culture in septic arthritis. \(^{24}\)

In addition, villous synovial membrane is more vascular than fibrous, and preferential exchanges of small molecules such as albumin, as well as hyaluronan production, occurs in synovial villi. \(^{7,25}\)

There is evidence that the synovial membrane is capable of handling a certain amount of
bacteria without developing infection. The superficial capillaries and small venules have thin fenestrated walls, akin to that observed in the kidney, intestine and choroid plexus, that are well suited for fluid transit. Deeper vessels have thick endothelium and are the site of action of histamine, and transvascular migration of leukocytes. Even in normal joints, deep venules have been shown to have high permeability to particles of up to 250 A, and the preferential localization of intravenously injected bacteria to the joint may be explained in part by this high permeability.

Factors that can acutely affect synovial blood flow include intra-articular pressure (IAP), local temperature, joint motion, vasomotor tone and reflexes, and local release of vasoactive mediators. Although large arteries supplying the joint have pressure approaching systemic arterial pressure, arterioles and capillaries of the synovial membrane have much lower pressure such that a relatively mild increase in IAP can cause significant tamponade. In one study, a significant decrease in blood flow was measured when IAP reached 20 mm Hg. In the horse, a significant decrease in blood flow to the synovial membrane was measured after an increase IAP of 30 mm Hg. In one study, there was an inverse relationship between intraarticular PO₂ and synovial fluid volumes with intraarticular acidosis developing at IAP<45 mmHg.

Chronic pathologic processes can significantly decrease blood flow to the synovial membrane by two mechanisms: the increased fibrosis of the joint capsule can result in a significant decrease in capillary density; and this fibrosis can decrease compliance of the joint capsule, making it more susceptible to be affected by effusion.

The consequences of decreased articular blood flow include chronic ischemia, generation of lactate, synovial fluid acidosis, and generation of free radicals. Chronically hypoxic joints are more affected by exercise than normal joints. Finally, a decreased blood
supply can decrease drug delivery to the joint, and decrease clearance of metabolites from the joint.

Morphometric and morphologic analysis of synovial vessels provide an insight to the contribution of synovial blood flow in disease. Examination of normal synovial vessel morphology demonstrate capillary flattening with intraarticular pressure increases greater than 25 mm Hg, an indication that decreased blood flow, decreased clearance and decreased lymph flow can result from increased intraarticular pressure, and contribute to ischemia, edema, and joint effusion. In chronic arthritis decreased capillary density is observed, and increased intercapillary distance suggests altered transsynovial fluid exchanges, as well as relative ischemia. In immune-mediated conditions such as rheumatoid arthritis, vascular endothelial morphology directly correlated with disease status, with joints in active inflammatory stages showing changes characteristic of high endothelial venules, thus correlating well with the increased lymphocytic infiltration associated with these inflammatory stages.30

**Contribution of ischemia-reperfusion and generation of free radicals in arthritis.**

Generation of free radicals and subsequent membrane and tissue damage has been implicated in the pathophysiology of several disease processes including intestinal strangulation, spinal cord injury, myocardial injury, neoplasia and acute and chronic inflammation. In an attempt to explain the ongoing tissue damage observed in several forms of arthritis, a role of ischemia-reperfusion injury was proposed 31 and subsequently substantiated 32,33. Hypoxic-reperfusion injury is mediated predominantly by oxidative damage precipitated by reactive oxygen species, particularly the superoxide radical and its dismutation product hydrogen peroxide and hydroxyl radical. Generation of free radicals
originates in the generation of xanthine oxidase during the ischemic cycle, with free radical production during reperfusion. Xanthine oxidase has been immunolocalized in synovial endothelium, and production of free radical species by rheumatoid synovium has been documented. Free radicals can degrade articular cartilage matrix, hyaluronan, and cause chondrocyte death. Generation of free radicals is also a feature of the respiratory burst of polymorphonuclear leukocytes, which produce reactive oxygen species responsible for killing microbial pathogens. Thus cellular infiltration during inflammation can be detrimental through production of free radicals. Hypoxia within the diarthrodial joint may result from decreased arterial O₂, decreased synovial membrane permeability, decreased synovial blood flow, and/or increased O₂ utilization through increased metabolic demand. In one study, oxygen consumption was significantly increased in joints with experimentally induced articular inflammation, and a concurrent increase in O₂ extraction ratio was measured until delivery matched demand. Cycles of ischemia-reperfusion can also be generated by joint effusion and increased intraarticular pressure, with subsequent decreased blood flow. The alteration in blood flow caused by effusion is worsened with exercise. Evidence for such mechanisms exist. Oxygen partial pressure in inflamed joint is higher than non-inflamed joints. In one study, oxygen extraction ratio and oxygen consumption was increased in equine joints inflamed with interleukin-1 (Il-1) suggesting inability of delivery to meet demand. These studies support rest and resolution of effusion as part of the treatment of joint disease.

**Role of nitric oxide**

Nitric oxide (NO) is a small molecule that is synthesized from the guanido group of the amino acid L-arginine by a variety of cells. A family of enzymes called the nitric oxide synthases (NOS) are involved in this reaction and two isoforms of the enzymes have been
identified: a constitutive Ca-dependent enzyme and an inducible Ca-independent enzyme. Initially identified as endothelium-derived relaxing factor, NO was shown to have marked effect on vascular smooth muscle, causing vasodilation, and on platelets, neutrophils and macrophages to influence platelet aggregation, bacterial killing, and neutrophil trafficking. Macrophages, peripheral blood monocytes, neutrophils and lymphocytes produce NO by means of the inducible form of NOS during host defense, immunological reactions and septic shock. This inducible form can be activated by endotoxin, or by inflammatory mediators such as interleukin-1, tumor necrosis factor and interferon (IFN), and can be inhibited by dexamethasone. Evidence of a role of NO in arthritis is increasing. Nitrite, the stable measurable end product of NO, has been measured in patients with rheumatoid arthritis and osteoarthritis. NOS inhibitors reduced the severity of several experimentally induced models of arthritis. In vitro studies have recently demonstrated that articular chondrocytes can release NO after stimulation with endotoxin and inflammatory mediators, and that this release could be inhibited by L-NAME, high concentrations of methotrexate and high concentrations of dexamethasone. Low levels of constitutive NOS activity were measured in synovial tissue, probably of endothelial origin, but further release could not be induced from that tissue under stimulation. Nitric oxide is highly toxic, but even more so when combined with superoxide free radical to produce the peroxynitrite anion, causing further tissue injury. In joints, NO decreases proteoglycan synthesis, and increases metalloprotease mediated collagen and proteoglycan degradation. NO also increases PGE₂ synthesis through direct activation of cyclo-oxygenase. The therapeutic benefit of NOS inhibition in arthritis stems from the fact that this pathway is upstream from the point of NSAID anti-inflammatory effects of non steroidal drugs. Implication of NO in arthritis has been suggested, through involvement in the immune response, tissue injury and inflammation. In addition, NO is thought to be responsible for the vasodilator effects of substance P, and thus indirectly could play a role in plasma extravasation and joint...
effusion. Manipulation of NO production may have some therapeutic benefits. Glucocorticoids inhibit the induction of NO, and NSAID may inhibit production of NO by synovial fibroblasts. The ubiquitous nature of NO makes therapeutic targeting difficult and non-specific at this time. Identification and purification of isoforms that are specific to the diarthrodial joint may improve our ability to target NO in joint disease. 37

**Intraarticular pressure**

Intraarticular pressure (IAP) is below atmospheric pressure in most joints at the angle of ease and pressures between -2 to -12 cm H2O have been reported. Recordings of IAP in equine joints yielded similar subatmospheric values of for the midcarpal and metacarpophalangeal joints.38,39 Maintenance of this negative pressure is thought to occur by joint motion, which enhances lymph flow from the interstitium, and by joint flexion which promotes fluid absorption by raising IAP. The normal fluid balance is therefore maintained through 2 pumps in series, one that enhances fluid exchange to the interstitium, and one that enhances lymph flow. Examination of pressure-volume curves in normal joints indicates that this relationship is sigmoid, with low articular compliance at normal subatmospheric pressures, an increased compliance at supraatmospheric pressures up to 30 mmHg, and another increase in compliance at high IAP.39 This relationship can be explained as a resistance to joint distention at IAP in the normal range, followed by accommodation of effusion at IAP up to 30 mmHg. This may prevent collapse of synovium capillaries and preserve joint blood flow. At IAP > 30 mmHg, the decreased compliance may counteract further effusion and articular fluid accumulation. Rupture of the midcarpal joint capsule was documented at IAP of > 80 mmHg in horses, and this rupture was located in the palmar lateral pouch of the midcarpal joint, as has been observed clinically.
The determinants of IAP include joint capsule compliance, joint angle, previous distention history, compliance of the joint capsule, muscle tension, and joint load. In addition, determination of pressure-volume relationships are also dependent on the type of infusate used for measurement. Chronically inflamed joints have thickening and fibrosis of the joint capsule, resulting in decreased compliance. Joint flexion increases IAP. Slow distention-compression cycles result in progressive stress-relaxation of the joint capsule and a gradual increase in articular compliance. However, if rapid successive infusion-withdrawal cycles are performed, a progressive decrease in articular compliance can be measured. If the joint is distended with synovial fluid, an increased compliance is observed compared to infusion with saline, probably because of the lack of a fluid interface which increases surface tension and promotes joint collapse.

Intraarticular pressure is an important determinant of synovial membrane blood flow and transsynovial fluid movement. Synovial membrane capillaries, which have a high density at a depth of approximately 25 μm, are affected by increased IAP. Studies have demonstrated a significant decrease in synovium blood flow at IAP > 20 m (rabbit) or 30 mm Hg (horse). Decreased IA blood flow can lead to synovial fluid hypoxia, acidosis, and lactate accumulation. Chondrocyte metabolism is significantly altered by hypoxia. Furthermore, increased IA pressure and exercise may lead to cycles of ischemia and reperfusion of the synovial membrane, with evidence of reperfusion injury. Superoxide generation, and other oxygen-derived metabolite have been demonstrated in synovial fluid after reperfusion in the synovial membrane. In turn superoxide can also affect chondrocyte metabolism.

Transsynovial fluid absorption is significantly affected by IAP. In the rabbit, transsynovial fluid movement increases 6-fold above a critical pressure of 9.5 cm H2O also termed the breaking pressure. These findings contradict Starling’s hypothesis, which predicts linear
relationship between fluid exchange and extravascular pressure. The clinical relevance of these findings are several fold. First, since flexion increase IAP, an increased IAP is expected with joint effusion and flexion, thus explaining the pain noted clinically with flexion tests performed on effused joints. Secondly, since cycles of reperfusion can be provoked with exercise in the presence of joint effusion, and that reperfusion and generation of free radicals can cause tissue injury, rest is indicated in joints with effusion. The effect of history-dependance on IAP and compliance explains the lack of correlation between the volume of effusion and pressure generated from the effusion. Long term slowly accumulating joint effusion will have relatively low IAP compared to fast-developing effusions. In addition, pain due to effusion is caused by periarticular tension receptors, stress relaxation may explain why rapidly forming effusions are more painful than slower forming ones.

Compartmentation of the joint is the functional separation of joint compartment at physiologic pressure, and has been demonstrated in the rabbit stifle and the equine metacarpophalangeal joint. Because compartmentation was not observed in the non-weight bearing equine midcarpal joint, this would suggest that this phenomena is operative either in small joints or in large weight bearing joints.

The breaking point phenomena is not observed in edematous synovium either in animals with congestive heart failure or in acutely inflamed synovium, further supporting the observation that this phenomena is due to enlargement of the synovium interstitial channels. This phenomena is also present with exercise, such that increased synovium conductance is observed for many hours following exercise. Therefore physiological injections studies of joints should be aware of this phenomena, and therapeutic joint injections should not be made after exercise.

Chronicity of joint effusion affects the magnitude of intraarticular pressure measured at rest and during exercise. Patients with acute traumatic joint effusions had a lower mean resting
intraarticular pressure and generated a significantly lower intraarticular pressure at exercise than patients with chronic effusions. These findings may be explained by the lower compliance of chronically inflamed joints. In chronically inflamed joints, pressures generated at rest but more remarkably at exercise are higher than capillary pressure and may reach values that are higher than systolic pressures. That severe synovial damage does not occur suggests the presence of protective mechanisms to prevent total ischemia to the synovium. Normal exercise does not produce significant fluxes in intraarticular pressures, emphasizing the critical role of effusions on the generation of intraarticular pressure. Interestingly, one of the most important mechanisms for lack of generation of high intraarticular pressures in acute traumatic effusions is quadriceps inhibition. The presence of this reflex has not been documented in horses.

**Synovial fluid dynamics**

Understanding the pathophysiologic mechanisms behind synovial fluid turnover becomes important when one considers the common clinical finding of joint effusion in joint disease, the use of synovial fluid markers as indicators of disease state and progression, and the use of systemic or intraarticular drugs in the treatment of joint disease. The histology, ultrastructure and embryology of the synovium support the theory that the joint cavity is a modified interstitial space. The interstitium is composed of small proteoglycans, hyaluronan and fluid which is an ultrafiltrate of plasma. Similarly, joint fluid is an ultrafiltrate of plasma to which is added hyaluronan and is considered a form of interstitial fluid where 1) the concentration of small solutes is equal to plasma, after allowing for equilibrium for local metabolism and the Gibbs-Donnan effect; 2) the concentration of large solutes (colloids) is lower than that of plasma; 3) the rate of formation and absorption is based on Starlings hypothesis of fluid exchanges.
Current research supports a Starling based model for synovial fluid dynamics, which consists of ultrafiltration of plasma, passage of the ultrafiltrate to and from the synovial cavity via the synovial intima, and absorption into blood vessels and lymphatics. The Starlings forces regulating fluid exchanges can be described by: 

\[ J = K \left[ (P_c - P_i) - \sigma (\pi_c - \pi_i) \right] \]  

where \( J \) = volume flow across the capillary, \( K \) is the filtration coefficient across the capillary wall (and is a function of capillary permeability (P) and surface area available for exchanges), \( P_c \) is the capillary hydrostatic pressure, \( P_i \) is the interstitial hydrostatic pressure, \( \sigma \) is the reflection coefficient, \( \pi_c \) is the capillary oncotic pressure, and \( \pi_i \) is the interstitium oncotic pressure. This equation represents equilibrium at steady state. The dynamics governing fluid exchanges in the joint is best explained by a three-compartment model consisting of the vascular space, the interstitium and the joint space, which each membrane interface regulated by the sum of individual Starlings forces. Several studies have calculated the magnitude of individual forces across the joint and have identified several features of fluid dynamics that are unique to the joint. Increases in arterial and to a lesser degree, venous vascular pressures produces an increase in transsynovial fluid flow rather than interstitial flow. This can be explained by two mechanisms. The low reflection coefficient of the synovial membrane, which favors translocation of colloids into the joint, thus increasing \( \pi_i \), favors fluid flow into the joint. In addition, calculation of the transitional microvascular pressure which is the hydrostatic capillary pressure necessary to initiate synovial fluid or lymph flow yields a lower value for synovial fluid, thus again indicating preferential flow into the joint. The joint cavity therefore represents an overflow system in series with the vascular and interstitial space which can be opened at relatively low pressures. An example of this interaction serves to explain the relationship between the vascular interstitial and articular compartment. In immobilized joints, lymph flow is absent, such that fluid absorption from the joint through is preferentially through
capillaries. It can be demonstrated that in immobilized joint, there is steady increase in joint fluid volume, resulting in a mild increase in joint pressure, illustrating a preferential pathway of filtration to the joint space.

Synovial fluid colloids are an important driving force in joint fluid dynamics. Articular albumin and hyaluronan concentrations are molecules that play a role in oncotic pressures. The coiled three dimensional hyaluronan network creates channels or excluded volumes that are though to be the mainstay of fluid exchanges within the joint. Because of the low synovial membrane coefficient, increases in intraarticular protein concentration are commonly observed with articular inflammation that leads to increased capillary permeability. In turn, this increases the oncotic intraarticular pressure participates in creating joint effusion. Hyaluronan concentration, but not molecular weight, has little influence on clearance of albumin from the joint, suggesting that other mechanisms, such as increased capillary permeability, and increased intraarticular pressure play a more significant role in joint clearance. The half life of the HA molecule in joints is relatively short (12-20 hours) suggesting that alteration in composition and molecular structure can quickly impact joint rheology. The dynamic state of synovial fluid composition raises questions as to the usefulness and pertinence of a one time measurement of markers of disease.

Increased synovial fluid protein concentrations results in two phenomena: increased oncotic pressure, and increased synovial fluid viscosity. Increased oncotic pressure decreases fluid absorption as could be predicted from Starling’s equation, and increased viscosity reduces flow through transintimal and leak channels. Hyaluronate also raises normal synovial fluid oncotic pressure in a ratio that is not proportional to its concentration. For example, a 1% hyaluronate solution doubles osmotic contribution from protein. In joint effusions, oncotic pressure is raised, because of the increased total protein, but because
hyaluronate concentrations are low, total oncotic pressure is closer to the theoretical pressure due to protein.

*Absorption of fluid from the joint.*

The principal determinant of fluid absorption form the joint is intraarticular hydrostatic pressure and the sensitivity of flow (measured by absorption of fluid from the joint) to pressure increases almost six-fold above an intraarticular pressure of approximately 9.5 cm H2O, a limit which has been termed the breaking pressure or breaking point phenomena.\(^5\)

The experimental observation of a breaking point phenomena further yielded the theory that synovial conductivity was a constant below this limit, and a linear function of intraarticular pressure above breaking point. The clinical significance of this observation is that above a critical IAP, synovial membrane conductivity increases linearly with IAP to prevent the formation of effusion. Furthermore, interruption of lymphatic drainage does not affect this relationship.\(^5\)

Lymphatic flow in the immobile joint is not a significant pathway for synovial fluid absorption. Turnover of synovial fluid is an important issue as synovial fluid concentration of a variety of macromolecules has been used to assess joint health in a variety of clinical conditions. In turn, concentration of these markers is determined by their rate of release from the tissue involved but also on factors that affect clearance of the molecule. These factors include volume of synovial fluid, which relates to intraarticular pressure and therefore hydraulic conductance, synovial blood flow, lymph flow, inflammation, joint motion and composition of synovial fluid.\(^5,5\) Fluid absorption from the joint occurs by two parallel pathways, the capillary bed and the interstitial spaces.

Bidirectional flow of fluid across the synovial membrane has been demonstrated, showing that fluid exchanges do occur at static fluid volumes, without the need for cyclical hydrostatic force changes dependent on muscle contraction.\(^9\)
Articular permeability and turnover of synovial fluid

The ease of collection of synovial fluid in the horse has enabled the measurement of many molecules, including markers of osteoarthritis, and therapeutic agents. In humans with OA, several molecules have been measured as markers of disease process, progression and response to treatment. These include keratan sulfate, chondroitin sulfate, proteoglycan core protein, type II collagen polypeptide, link protein, COMP, and others. Serum concentration of some of these molecules, as an estimate of synovial fluid clearance and therefore concentration have also been used as markers. However, the concentration of a molecule in SF is related to its rate of release from cartilage, the volume of synovial fluid, and lymphatic or venous clearance from the joint. For example, one study detected a higher concentration of the 7-D-4 CS epitope and a significantly lower 5-D-4 KS epitope in knee joints with acute traumatic injuries. However, it is possible that the smaller KS epitope was cleared faster from the joint thus resulting in lower concentrations, rather than a reflection of altered cartilage synthesis or degradation. Even though hyaluronan molecules can form three-dimensional networks that can decrease the volume of distribution and clearance of small molecules such as albumin, synovial fluid hyaluronan concentration or molecular weight play little role in clearance of protein from synovial fluid. Low grade synovitis as seen in osteoarthritis can result in significantly increased clearance of small proteins such as albumin from the joint. Therefore the presence of synovitis is a significant variable which must be considered if response to therapy is measured by synovial fluid concentration of a marker protein. Recently, the preferential pathway of albumin exchange in equine fetlock joints has been localized to villous synovium, and a significant increase in albumin permeability was measured in experimentally induced articular inflammation. The same
study also identified a significant effect of denervation, with denervated joints showing a significant decrease in macromolecule (MW: 144,000) permeability. Lymphatic flow is thought to play an important role in protein clearance from the joint. Lymphatic flow, protein permeance and plasma protein clearance was greater in rheumatoid than osteoarthritic joints. Protein permeability was also inversely proportional to molecular radius, suggesting that proteins are cleared from the joint by diffusion. Intraarticular local anesthetics, but not corticosteroids, increased the half life of Xe in rheumatoid knee joints, suggesting a local vasoconstrictive effect of the local anesthetic on synovial vascular tissue.

**Functions of hyaluronan**

Hyaluronan is a non-sulfated glycosaminoglycans consisting of alternating units of D-glucuronic acid and N-acetyl-D-glucosamine. It exhibits polydispersity, but the average molecular weight is in the order of several millions. In dilute solutions, each molecule behaves as a large coil, but as the concentration increases, entanglement of the coils occurs, eventually forming a uniform meshwork. Viscosity is non-linear and increases exponentially as the hyaluronan concentration increases, probably as a result of this three-dimensional network. The effect of hyaluronate on synovial fluid viscosity is proportional to chain length, protein concentration, pH, ionic composition and temperature. Viscosity decreases rapidly with increased shear rate. Viscosity is variable between joints, being high in small joints. In a given joint viscosity varies inversely with volume. The viscoelastic nature and shear dependance of hyaluronan solutions have been show, and a role in boundary lubrication assigned to the hyaluronan molecule. It is apparent however, that the nature and configuration of the molecule, and the existence of receptors to hyaluronan, provide some other insights as to the role of hyaluronan in vivo. Hyaluronan solutions
provide a barrier against water flow, and may act as a barrier against rapid tissue weight changes. The meshwork may also act as a sieve to regulate transport of macromolecules, and exclude macromolecules from space in the system. One theory proposed that the meshwork configuration and the rapid clearance of HA from the joint (20 hours in normal joints) may provide a scavenging function to this molecule. Finally the discovery of hyaluronan binding proteins, also referred to as hyaladherins, provided new insight as to the function of hyaluronan. The first and most known hyaladherin is aggrecans and link protein, which in combination with hyaluronan form the large aggregates of articular cartilage. Other surface receptor proteins, such as CD44 and receptor for hyaluronan mediating motility (RHAMM) may play a role in cell biology. Hyaluronan surface cell binding has been proposed as a mechanism to prevent cell fusion, and these receptors are the first to be recognized in the embryo, before joint cavitation, suggesting a role of hyaluronan to initiate joint formation. RHAMM is thought to activate an intracellular tyrosine kinase and initiate cell locomotion. Finally, cell endocytosis is also thought to be regulated by another hyaladherin, the IEC receptor. The half life of hyaluronan in joints in very low, and is similar for high or low molecular weight preparations. This half life decreases markedly with inflammation, but to date hyaluronan degradation has not been identified in the joint cavity. The liver appears to be a major site of hyaluronan degradation, with some occurring in periarticular tissues. Recently, synovial fluid hyaluronan has also been shown to play an important role in the mechanical properties of the joint cavity, such that the joint capsule is more compliant with synovial fluid than with saline. A role of hyaluronan to decrease joint pain has been demonstrated in a bradykinin induced model of articular pain. In that model, hyaluronan with a molecular weight of greater than 40 kd was shown to have an analgesic effect, and hyaluronan of 860 kd and 2300 kd
produced high and long acting analgesia (72 hours after injection). This effect was not related to binding to hyaluronan receptors or bradykinin receptors.58

There is good experimental and theoretical evidence to support a role for hyaluronan to inhibit rapid efflux of fluid from synovial cavities at increased intra-articular pressures, by its impaction in the synovial intimal matrix.9

Finally, hyaluronan binding protein CD44 and the receptor for hyaluronan mediating motility (RHAMM) suggests an important role of hyaluronan in intercellular reactions and in the immune response and cell traffic.9

Role of articular innervation

The joint is an organ that is richly supplied with large myelinated afferent and efferent nerve endings and small unmyelinated C-fibers. Sensory and motor innervation maintain joint stability through reflex afferent, such that in the absence of these protective reflexes, severe arthropathy may develop if the joint is made unstable.59 Loss of sensory afferent did not lead to degeneration in experimental animals, if an underlying instability did not coexist.60

The role of sympathetic postganglionic fibers in joint circulation is dual: precapillary fibers, in direct contact with blood vessels, have a vasoconstrictive function; postcapillary fibers located in proximity but not in contact with venules indirectly increase plasma extravasation by activation of the cyclooxygenase pathway and synthesis of prostaglandin, particularly PGE2. Activation of the peripheral nervous system can initiate the major features of acute inflammation, which include vasodilation (calor and rubor), plasma extravasation (tumor), and pain sensation and a lower threshold for pain (dolor).61 Plasma extravasation and joint effusion, a cardinal feature of acute articular inflammation, results from the contribution of neurotransmitter release from primary afferent unmyelinated or C-fibers, sympathetic efferents, and mast cells. There is current evidence to show that the effect of
neurotransmitter release is dependent on the downstream presence of sympathetic terminals and mast cells, to cause plasma extravasation. In particular, B2 adrenergic stimulation resulted in severe arthritis, as measured by plasma extravasation or chronic tissue injury. In a similar manner, adrenalectomy significantly reduce the severity of experimentally induced arthritis, illustrating a role of the neuroendocrine system in disease.

The pain of arthritis is relayed both by type IV/C PAN fibers and type III/Aδ fibers. C-fibers are polymodal as they can respond to mechanical, thermal or chemical stimuli. Type III/Aδ fibers are mostly sensitive to mechanical or thermal stimuli. PAN can be directly stimulated by bradykinin, serotonin, and histamine or by potassium or protons, which can act to depolarize PAN. Substance P increases excitation caused by these compounds. Stimulation of small C-fibers causes release of the co-localized neuropeptides CGRP and substance P which act synergistically to exert pro-inflammatory paracrine effects on synovium. In contrast to hormones, which are released in low concentration in circulating blood and depend on specific high affinity receptors for site-specificity, neuropeptides are delivered to the site of action by axonal transport where release is mediated by action-potential induced depolarization and calcium dependent exocytosis of vesicles. Because of these nerve ending release, the presence and density of nerve fibers is directly related to neuropeptide at that site. Furthermore, the activity of neuropeptides in a particular site is limited by the presence of peptidases, the presence of which has been documented in several articular tissues.

Of the many putative neurotransmitters released by unmyelinated primary afferents, substance P has received the most attention. Substance P is an undecapeptide that is synthesized by neurons located in the dorsal root ganglia and stored in small diameter C-fibers nerve endings until release. These C-fibers are localized in the intima and subintima of joints, and in subchondral bone. Small C-fibers containing neuropeptides including calcitonin gene related peptide and substance P have been identified in the
synovium of rat, horses, rabbits. Neurokinin A and calcitonin-gene related peptides are colocalized with substance P in these nerve endings, and also have potent vasodilatory and permeability effects. Upon stimulation, release of substance P from nerve endings serve to transmit pain sensation through spinal pathways. Local antidromic release into the interstitium and joint cavity also serves to contribute to the inflammatory reaction. Substance P has been termed a pro-inflammatory peptide, and it increases vascular permeability, causes vasodilation (through release of nitric oxide), increases mast cells release of inflammatory mediators such as serotonin and histamine, increases leucocyte adherence, and activates lymphocytes. Substance P has a local action and its role is limited by the action of a peptidase, which is localized in the vicinity of its release. The NK1 class of neuropeptide receptor has the greatest affinity for substance P. Variations in receptor density and affinity helps to explain the variable response to inflammation. Specific NK1 receptor antagonists have helped our understanding of the role of substance P and may have a role in controlling the inflammatory response. Although acute inflammation is a necessary and appropriate response to initiate repair following tissue injury, inadequate regulation of this response may lead to excessive tissue damage or chronic inflammation. Physiological release of substance P may be protective in tissue injury to increase blood flow, synovium perfusion, and promote tissue repair. The observation of increased cellular infiltrates in arthritic joints in areas of depleted substance P-immunoreactive fibers and reduction in endothelial NK1 receptor supports a role of neurovascular regulation to resolve chronic arthritis. Substance P does not appear to have a direct role in articular permeability or blood flow. Increased vascular permeability, measured by extravasation of plasma protein, was induced by intraarticular injection of bradykinin, serotonin and histamine; CGRP potentiated the effect of these vasoactive substances to increase vascular permeability. CGRP infusion also significantly increased blood flow to the joint.
A definite role of substance P in joint pain has been demonstrated and recent treatments by the substance P depleting substance capsaicin have become available. Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) is the pungent ingredient in hot paprika or chili peppers. Capsaicin initially activates PAN fibers, resulting in substance P release and pain, but subsequently desensitizes or degenerates PAN fibers, suggesting a mechanisms for pain alleviation with chronic use in articular inflammation.64 Gold sodium thiomalate also causes a selective decrease in unmyelinated axons, resulting in an increased nociceptive threshold.61 A more specific role of substance P to alleviate the symptoms of arthritis has been demonstrated through immunoneutralization studies.65

NSAID are commonly used drugs in the treatment of articular inflammation. The role of prostaglandins in pain is an indirect role, as they act to sensitize PAN fibers to subsequent stimulation. There is some evidence that PGE2 and PGI can also act directly on nociceptors, but this effect is less potent than their sensitizing effect. In order to understand the effect of an inflammatory mediator or of a neuropeptide however, it is essential to remember that the result will be dependent of the density of receptors to the substance. For example bradykinin 2 receptors acutely stimulate pain in inflammation, but chronic inflammation upregulates B1 receptors, thus reversing the hyperalgesia.66 NSAID inhibit PGH2 synthase enzymes (also known as cyclooxygenase) and diminish the formation of PGE1, PGE2, PGF and PGI from arachidonic acid. Corticosteroids inhibit phospholipase A2, thus preventing the formation of arachidonate, a substrate for the cyclooxygenase and lipoxygenase pathways. At high doses, corticosteroids also inhibit IL-1 and TNF, which can also sensitize pain nociceptors. These mechanisms may explain the analgesic effects of NSAID and steroids. In addition to neuropeptide release and receptor activation, degradation must be considered in our understanding of the roles of neuropeptides in pain. Neuropeptide degradation is mediated by several amino and endo-peptidases but can also
be degraded by various reactive oxygen species. Several peptidases have been identified in close proximity to neuropeptide release in the joint, and these enzymes are shown to be increased in synovial tissue and fluid of rheumatoid patients. However, the distance traveled to target cells is much greater than to post-synaptic junction complicating the net effect of neuropeptides in arthritis.\(^{67,68}\) Although neuropeptide Y fibers have been identified in synovial tissues including equine tissue, the role of NPY is less defined, but may be related to diminution of reflex sympathetic discharge. Finally the stress responses and hypothalamo-pituitary-adrenal axis have also implicated in inflammatory arthritis.\(^ {66}\)

The role of neuropeptides in joint disease is currently under investigation, and there appears to be differences in the contribution of neuropeptides to disease process in acute vs chronic inflammatory arthritis. In acute arthritis, loss of sensory nerves may contribute to inflammation, as demonstrated by increased edema formation in denervated limbs.\(^ {63}\) Similar results have been reported in an IL-1 induced model of acute inflammation in the horse, where increased edema and decreased permeability to macromolecule were observed in denervated limbs.\(^ {7}\) The role of innervation in chronic arthritis is complex. Staining for CGRP and substance P was increased in the sciatic nerve, dorsal root ganglia and periarticular tissues, but synovium staining was decreased. It appears that the role of neuropeptides in acute or chronic inflammation may vary as the distribution of sensory nerves is altered with the inflammatory response.

The therapeutic implications of the participation of neuroendocrine mechanisms in arthritis are many. Intramuscular gold or topically applied capsaicin are agents that selectively destroy type IV/C fibers, thus lowering SP levels, and have been found clinically useful. Capsaicin initially causes release of substance P from nerve ending, explaining the burning sensation felt upon initial application. Both type III/A and type IV/C fibers are stimulated by bradykinin, serotonin, histamine and prostaglandin. NSAID
(PGH2 or cyclooxygenase inhibitors) decrease prostanoid production, and intraarticular corticosteroids which inhibit the arachidonic acid cascade are ineffective in the treatment of inflammation and pain in arthritis. In addition, stimulation of primary afferent nociceptive fibers causes release of glutamate and substance P from central spinal pathways. This nociceptive input can be inhibited by stimulation of proprioceptive and tactile type I and II fibers. Stimulation of these fibers can be accomplished by high frequency low intensity transcutaneous neural stimulation, frequently used in physiotherapy.
Mechanisms of joint injury

- Synoviocytes
  - Chondrocytes
- Neutrophils
  - Platelets

Acute joint injury

- Neuropeptides
  - (CGRP, substance P)
- Pain
- Immobilization

Cytokines
- (II-1, II-6, TNF)

Inflammatory mediators
- (PGE2, PAF, TXBA2)

Generation of free radicals

- Increased capillary permeability
- Increased intraarticular volume
- Decreased blood flow
- Increased intraarticular pressure

Cartilage degradation

Hyaluronan degradation

- Hyaluronan degradation
- Decreased blood flow
- Increased intraarticular pressure
References


CHAPTER 3

PRESSURE-VOLUME RELATIONSHIPS IN EQUINE MIDCARPAL JOINTS

Introduction

Normal intra-articular pressure (IAP) is subatmospheric in most joints placed at an angle of ease. Maintenance of normal IAP is important for optimal synovial blood flow and transsynovial fluid exchanges. Joint effusion, a common clinical finding in joint disease, can lead to increased IAP and decreased synovial membrane blood flow. Decreased synovial membrane blood flow may result in altered synovial fluid filtration and composition, articular metabolite accumulation, hypoxemia, and acidosis, all of which can contribute to altered chondrocyte metabolism and promote further articular injury.

Parameters that have been used to describe the pressure volume (PV) relationship of joints include elastance [change in pressure per unit change in volume (ΔP/ΔV)] and its reciprocal, compliance [change in volume per unit change in pressure (ΔP/ΔV)]. Elastance is a measure of resistance to deformation, and compliance reflects the ability of a structure to deform. Other useful parameters are hysteresis is a measure of the difference in behavior of elastic tissues during progressive increased or decreased stress, caused by a greater rate of disengagement of wall elastic elements during unloading than the rate of recruitment during loading. Stress-relaxation is a measure of the decay of pressure over time, under a constant volume or strain, caused by relaxation of the joint capsule. However, because fluid absorption from the joint may contribute to the observed decay in
pressure, the expression "relaxation of IAP" will be used in the present study, since fluid absorption was not measured.

Numerous studies have reported on the PV relationships of joints. These studies have concluded that intra-articular volume, joint capsule compliance, joint angle, joint measurement, site measurement, history of prior distention, and joint activity are important determinants of IAP. More specifically, increased intra-articular volume, decreased capsular compliance caused by thickening and fibrosis, and decreased joint angle (i.e., flexion) contribute to increased IAP. Different joints have different baseline pressures. Compartmentation within a joint has been demonstrated in the rabbit knee and is known to alter the pressure response to fluid infusion through uneven distribution of the infusate. There is net transsynovial filtration during prolonged immobilization that results in a mild increase in intra-articular volume and pressure. Periarticular muscle contraction during locomotor activity increases IAP particularly in the presence of joint effusion. All of these studies have substituted polyionic isotonic solutions or low-viscosity nonabsorbable oil for synovial fluid to describe these phenomena. Furthermore, in some studies, the skin that covers the joint, a major supporting structure, was removed, which may alter the compliance of the joint.

The characteristics of the PV relationships of an organ can be divided into two separate parts: 1) the elastic forces of the tissue and 2) the elastic force caused by surface tension of the fluid that lines the inside of the organ. Synovial fluid is an ultrafiltrate of plasma to which hyaluronate has been added. Hyaluronate is a high-molecular-weight proteoglycan that imparts a high viscosity to synovial fluid. Hyaluronate is also the principal glycosaminoglycan of the interstitium. The viscoelastic non-Newtonian behavior of synovial fluid may yield different PV relationships than that obtained with saline or other fluids. In addition, the use of synovial fluid abolishes the
effect of surface tension that would be present at the interface of two different fluids, as when saline or oil are used, and allows for more accurate determination of the elastic properties of the joint capsule. The boundary lubricant properties of the hyaluronan molecule, which forms an intimate bond with synoviocytes, may also alter the mechanical properties of the joint walls.

The equine midcarpal joint is the most common joint affected by traumatic injury in performance animals. Traumatic injury to the midcarpal joint is accompanied by effusion and lameness. Clinically evident effusion probably results in increased IAP, which may in effect reduce synovial membrane blood flow. The equine midcarpal joint has been used in many models of articular disease for mechanistic or therapeutic investigations. To improve our understanding of the pathogenesis of acute or chronic joint injury and the effect of joint effusion on blood flow, it is important to investigate the normal relationship between synovial fluid volume and IAP within an intact joint.

The purpose of the present study was to describe the PV relationships of the equine midcarpal joint and to determine the effect of joint angle, nature of infusate, and prior distention on these relationships.

Materials and methods

Horses—Twenty-four healthy adult horses (350-500 kg) and 48 midcarpal joints were used for this study. Criteria for inclusion of horses in the study included carpal joints free of disease on the basis of physical examination and gross examination of the articular cartilage and synovium at postmortem. An approved Institutional Laboratory Animal Care and Use protocol was obtained before this study was begun.

Anesthesia—Horses were sedated with xylazine (0.5 mg/kg iv), anesthetized with 5% guaifenesin (intravenously to effect) and thiamylal (4 mg/kg iv), and maintained with
pentobarbital sodium (5-15 mg \cdot kg^{-1} \cdot h^{-1} \text{ iv}). All horses were intubated with a cuffed endotracheal tube, positioned in dorsal recumbency, and mechanically ventilated with 100% oxygen. A 20-gauge catheter was inserted into a facial artery for direct measurement of arterial blood pressure and collection of samples for determination of arterial pH and blood gases. Systolic, mean, and diastolic arterial blood pressures were continuously displayed, and arterial pH and blood gases were determined hourly. Drug administration and mechanical ventilation were adjusted to maintain the mean arterial pressure at > 70 mmHg, arterial PO$_2$ at > 150 Torr, and arterial PCO$_2$ at < 60 Torr.

**Instrumentation**—The horses were positioned in dorsal recumbency, and both forelimbs were suspended from a rack by a rope tied around the pastern. Tension was not exerted on the limb. The midcarpal joints were set at the predetermined angle (90° or 135°) with a goniometer (Fig. 3.1). A 20-gauge needle connected to a stiff-walled polyethylene extension tubing filled with heparinized saline and closed by a three-way connector was inserted into each of the medial and lateral dorsal midcarpal joint pouches and the medial and lateral palmar midcarpal joint pouches. Each connector was attached to a horizontally positioned pressure transducer placed at the same height as its corresponding needle, and each pressure transducer was then calibrated with a mercury manometer before measurement of IAP. Total compliance of the system was 0.45 $\mu$L/mmHg between 0 and 100 mmHg. Transducer drift was 0.008 mmHg/min. The dorsal midcarpal joint was also cannulated with a 20-gauge needle placed through the extensor carpi radialis tendon for infusion and withdrawal of fluid. A calibrated pump adjusted to deliver a rate of 2.47 ml/min was used for infusion and withdrawal of fluids. This rate of fluid transfer was chosen to minimize artifact caused by relaxation of IAP of the joint capsule during infusion while allowing enough time to measure small pressure changes. All pressure tracings were
continuously displayed and recorded on a physiograph. Synovial fluid was obtained from the tarsocrural joints of the same horse and used on the same day. Synovial fluid was considered to be normal based on color, clarity, and viscosity. The viscosity of synovial fluid obtained from normal equine tarsocrural joints has been reported.18

Protocol

Eight experiments were conducted (n = 6 midcarpal joints per experiment). Only one experiment per joint was performed.

**Experiments 1-4: effect of joint angle and infusate.** The PV relationships in the midcarpal joint were determined at a joint angle of 90° by infusion of synovial fluid (Expt 1) or isotonic saline (0.9% NaCl) (expt 2), or at a joint angle of 135° by infusion of synovial fluid (expt 3) or isotonic saline (expt 4).

**Experiments 5 and 6: history dependence of PV curves.** The effect of sequential distention on articular compliance at IAPs in the low (< 30 mmHg) or high (30-80 mmHg) range was determined by continuous infusion at the usual rate of synovial fluid to an IAP of 30 mmHg followed by withdrawal at the same rate. This cycle was repeated twice more, then IAP was increased from 30 to 80 mmHg followed by withdrawal to return to 30 mmHg; similarly, this cycle was repeated twice more (expt 5). In six additional joints (expt 6), IAP was first increased from 30 to 80 mmHg for three cycles, followed by three cycles at low range (up to 30 mmHg).

**Experiments 7 and 8: relaxation of IAP over time.** Relaxation of IAP was measured at angles of 90° (expt 7) and 135° (expt 8) by continuous infusion of synovial fluid in increments of 20 mmHg, up to 80 mmHg. After each 20-mmHg increase in IAP, the infusion was stopped and the pressure decay was measured for 5 min before the next increment.
Analyses

All pressure tracings were analyzed by scanning each tracing into a software program to obtain data points. These data points were used to generate PV curves for each subject and each experiment. These curves were analyzed with linear and nonlinear regression.

Experiments 1-4. Examination of the PV curves revealed a sigmoid relationship that was best fitted to an exponential equation \((R^2 \geq 0.98)\), as previously described. Each PV curve was fitted to the exponential equation \(\text{IAP} = A \times e^{(B \times \text{volume})} - C\), where \(B\) is the fractional change in pressure per unit change of volume, thus defining the curvature of the relationship, and \(A\) and \(C\) on constants. The term \(B\) was used for statistical analysis in testing for the effect of joint angle, fluid infused, hysteresis, and compartmentation. All parameter values were obtained by an iterative algorithm by using the Levenberg-Marquardt method, with \(R^2 \geq 0.98\). Elastance was calculated for each increment in pressure and volume \((\Delta P/\Delta V)\) and was plotted against mean IAP. This IAP/V vs. IAP relationship was linear below or above atmospheric pressure, and the slope of the linear regression equation was compared at pressure ranges below and above atmospheric pressure. Hysteresis was computed by measuring the areas under the infusion and withdrawal curves and calculated as percent \(\left[\frac{\text{area under infusion} - \text{area under withdrawal}}{\text{area under infusion}}\right]\).

Experiments 5 and 6. PV relationships were linear below and above 30 mmHg \((R^2 \geq 0.99)\). Linear-regression equations of the form \(\text{IAP} = a + b \times \text{volume}\) where \(a\) is the intercept and \(b\) is the slope \(\Delta P/\Delta V\), were fitted to the low (baseline to 30 mmHg) or high (30-80 mmHg) PV curves by using the least squares method. The slope of each curve, representing elastance, was used for statistical analysis.
Experiments 7 and 8. Articular relaxation at a given pressure was best described as a logarithmic decaying function of time. The relationship $P_t = P_i - s \ln t$, where $P_t$ is pressure at a given time $t$, $P_i$ is initial pressure, and $s$ is slope of the curve, was determined for each relaxation of the IAP curve at each 20 mmHg increment. The slopes of each stress relaxation curve were compared among incremental pressures for each joint angle.

Statistical Analysis

Student's t-test was used to compare baseline IAPs between the two joint angles. A four-way fixed-model analysis of variance was used to compare the B coefficients of the PV curves, with joint angle, infusate, joint compartment, and infusion of withdrawal as the independent variables. Body weight of the horses was tested and found not to correlate with the data, so it was not included as a covariate. A four-way repeated-measure analysis of variance was used for comparing the linear slopes for joint angle, compartment, and low or high pressure, with cycles as the repeated measure. A two-way analysis of variance was used to compare the hysteresis ratio between infusates (saline or synovial fluid) and joint angle (90° or 135° angle). A three-way repeated-measure analysis of variance was used to compare the relaxation of IAP function between joint angles and among pressure increments, with slope as the repeated measure. For all analyses, when a main effect or an interaction was detected, a Newman-Keuls post hoc comparison test was used to identify the different means. Significance was set at $P \leq 0.05$, and data are expressed as means ± SE.
Results

Baseline IAPs measured -2.7 ± 0.1 mmHg [95% confidence interval (CI) = -4.84, -0.5] at 135° and 0.3 ± 0.1 mmHg (95% CI = -2.18, 2.44) at 90° midcarpal joint angle. IAP was significantly higher (P = 0.0062) at 90° than at 135° angle.

Description of PV Relationship in Midcarpal Joints

The PV relationship in equine midcarpal joints was sigmoid and was best defined with two exponential equations (Fig 3.2 and 3.3):

for subatmospheric pressures:

\[ IAP = -7.20 \times e^{-3.46 \times \text{volume}} - 3.61 \] (1)

and for supra-atmospheric pressures

\[ IAP = 40.74 \times e^{0.11 \times \text{volume}} - 35.46 \] (2)

Elastance (E) was linearly related to IAP below or above atmospheric pressure (Fig. 3.4). The regression equation below atmospheric pressure was

\[ E = 19.13 - 3.276 \times \text{IAP} \ (R^2 = 0.90, \ P = 0.0008), \] (3)

and that above atmospheric pressure was

\[ E = 4.38 + 0.108 \times \text{IAP} \ (R^2 = 0.99, \ P \leq 0.0001). \] (4)

Examination of the slopes of the regression equations (3.27 vs. 0.0108) demonstrates the much greater resistance to distention of the joint (greater elastance, lesser compliance) at subatmospheric than supra-atmospheric pressures.

In the present study, we were unable to demonstrate significant compartmentation of fluid during infusion or withdrawal, indicating that in dorsal recumbency for joint angles of 90° or 135° angle, fluid was distributed evenly between all four joint pouches.
Hysteresis was detected for all joint angles for synovial fluid or saline infusion (Fig. 3.5, A and B). Hysteresis at 135° angle was 38 ± 2 and 22 ± 2% for synovial fluid and saline infusion, respectively. Hysteresis at 90° was 57 ± 3 and 30 ± 2% for synovial fluid and saline infusion, respectively. There was significantly greater hysteresis when synovial fluid was used as the infusate with a joint angle of 90° (P ≤ 0.005).

In two horses, rupture of the joint capsule with subcutaneous leakage of synovial fluid was observed at a joint angle of 135° and IAP of 80 mmHg. The rupture occurred in the lateral palmar articular joint pouch.

Effect of Joint Angle

Decreased joint angle from 135 to 90° significantly increased subatmospheric (P = 0.028) and supra-atmospheric (P = 0.0016) articular elastance (Figs. 3.2 and 3.3).

Effect of Type of Infusate

Infusion of saline resulted in a significantly higher articular elastance than that measured with infusion of synovial fluid (Fig. 3.6) at 135° (P = 0.045) and a trend toward higher elastance at 90° (P = 0.063).

Effect of Prior Distention on PV Relationships

Sequential distention cycles (history dependence) did not significantly affect PV relationships at IAPs < 30 mmHg. At high IAPs (30-80 mmHg) a significant increase in elastance was observed at the second and third distention cycles, when the high-pressure cycles followed low-pressure cycles (P ≤ 0.0001) (Fig. 3.7). Elastance at IAPs ≤ 30 mmHg was 8.00 ± 0.78 mmHg/ml and was not different between cycles. Elastance at the first high-pressure cycle after three low-pressure cycles was 14.87 ± 1.00 mmHg/ml, and values for subsequent high-pressure cycles were 17.09 ± 1.25 and 17.27 ± 1.24
mmHg/ml. There was a trend toward an effect of joint angle on elastance for low or high IAP (P = 0.087).

**Relaxation of IAP**

Sequential increases in IAP by 20-mmHg increments allowed to better define elastance at supra-atmospheric pressures ranging from 0 to 20, 20 to 40, 40 to 60, and 60 to 80 mmHg. There was a significant difference (P < 0.005) between all incremental pressures, and the lowest pressure range was associated with the lowest elastance (greater compliance) (Fig. 3.8). A significant relaxation of IAP was observed when pressures were increased to 40, 60, or 80 mmHg. At 20 mmHg, relaxation of IAP was minimal for that measurement period. The slope of the stress relaxation curve of the pressure-time relationship was a positive logarithmic function of IAP (Fig. 3.9).

**Discussion**

The PV relationship of the equine midcarpal joint is a characteristic sigmoid curve that is best described by two exponential equations, one for subatmospheric pressures and one for supra-atmospheric pressures. This dual relationship reflects the difference in compliance observed at sub- or supra-atmospheric pressures, indicating that the joint capsule resists distention at normal (subatmospheric) pressures; however, after increases in intra-articular volume, as occurs with effusion, there is relaxation of the joint capsule to accommodate the increased volume. Examination of the relationship between elastance and IAP better illustrates this relationship, as the regression coefficient is much greater at subatmospheric than supra-atmospheric than supra-atmospheric pressures (3.27 vs. 0.108/ml). This sigmoid PV relationship has been described in other species and is comparable to that observed in the interstitial space, supporting the statement that the joint cavity, because of its lack of a basement membrane, is an expansion of the interstitial
space. The low compliance observed at subatmospheric pressure may help prevent edema formation and help maintain a normal negative IAP, which favors fluid exchanges. The more compliant joint observed at supra-atmospheric pressures up to 30 mmHg may help prevent impaired blood flow and preserve synovial membrane metabolism as well as decrease joint pain. The decreased compliance at higher pressure may impede net filtration of fluid and prevent further effusion.

In our study, compartmentation of fluid within the different midcarpal joint pouches was not observed. This finding contrasts with that observed in the rabbit stifle (knee) joint and may reflect the non-weight-bearing position of the leg, the larger joint compartments of the horse, or the difference in the anatomy of the stifle joint vs. the midcarpal joint. This finding validates the future use of the dorsal joint compartments in studies that investigate IAP of the equine midcarpal joint in the non-weight-bearing position. However, we found that the palmar joint pouches were more difficult to cannulate because of their smaller size, particularly in smaller subjects.

We observed that the response to volume infusion was different when saline was used instead of synovial fluid, with saline infusion resulting in a lower joint compliance than when synovial fluid was used. Another study reported increased compliance with a polyionic solution compared with a nonabsorbable oil. The viscosity of synovial fluid is given by its high hyaluronate content. This high-molecular-weight molecule has properties that are shear-rate dependent, i.e., it is more viscous at low shear rates. In addition, synovial lining cells have receptors for hyaluronate, and the cell-hyaluronate unit may yield different properties to the joint capsule than each component taken separately.

In the present study, we measured significant hysteresis, reflecting a difference in the PV relationship between volume infusion or withdrawal. Compared with saline, hysteresis was significantly greater when synovial fluid was used as the infusate. This
observation is similar to that observed in the lung when saline is used instead of surfactant. In the lung, saline inflation markedly decreases hysteresis in the absence of surfactant; with surfactant, normal hysteresis is restored. The high-molecular-weight hyaluronate molecule may play a similar role in the joint, where, by preventing joint collapse, it helps maintain subatmospheric pressure, which favors fluid exchanges. Furthermore, it is doubtful that all hyaluronan molecules are initially removed when saline or oil are used as infusate in PV studies. It is more likely that they are progressively washed out of the joint with each PV loop. Therefore, when fluids other than synovial fluid are used there is a fluid-synovial fluid interface, the composition of which changes with each cycle of infusion or withdrawal. Our findings suggest that when fluids other than synovial fluid are used, the mechanical properties of the hyaluronan-synoviocyte unit are altered; perhaps this illustrates the important boundary properties of hyaluronan.

As previously described, joint flexion (135 to 90° angle) significantly decreased articular compliance, probably by increasing tension on the joint capsule. This finding may explain the clinical observation that with severe effusion most horses will resent joint flexion and prefer to hold their joint at ~ 135°.

In the present study, increased elastance or decreased compliance was measured after the first infusion cycle at high IAP. The total compliance of the joint is the sum of the compliance of each part. The decreased compliance observed with subsequent cycles at high pressure may reflect distribution of fluid within the joint during the first cycle or may be related to the relatively rapid rate of infusion. Normal human and dog lungs undergo a decrease in compliance over time when ventilated spontaneously or mechanically. In a previous study by Knight and Levick, compliance increased with subsequent infusions, in contrast to our findings. Knight and Levick attributed this increased compliance to plastic deformation of the joint capsule. In their study, infusion rate was slower than our rates. In
addition, the skin overlying the joint had been removed, which may have changed the mechanical properties of the periarticular tissues.

Relaxation of IAP was significant, particularly at higher IAPs. In our study, stress-relaxation was a better function of the natural log of time than a linear function, which suggests that with prolonged observation a non-zero asymptote was being approached. Relaxation of IAP is a measure of pressure decay under constant volume or strain. Alternative explanations for the decay in IAP include fluid absorption from the joint space, slow change in joint angle, and slow fluid distribution within the joint space. Calculation of relaxation of IAP at 80 mmHg gives a value of 7.56 mmHg/5 min (see equation in Fig. 9 legend). Compliance (1/elastance) at 80 mmHg was 0.045 ml/mmHg (see Fig. 5), such that absorption of 0.225 ml (225 µl) of synovial fluid in 5 min would have been necessary to decrease IAP by 7.56 mmHg. Hydraulic conductance of synovial fluid in the equine midcarpal joint at normal or increased IAP is unknown. In other species, the rate of turnover of synovial fluid estimated by three different methods (rate of change in pressure, protein influx, and protein efflux) yielded values ranging from 1.3 to 6.7 µl/cm² of synovium per hour for normal joints; however, these values probably are different with increased IAP, considering that hydraulic conductance for Ringer solution was increased almost sixfold with IAP > 9 and ≤ 25 cmH₂O. The effect of increased IAP on synovial membrane blood flow and clearance also needs to be considered. We have shown that blood flow in synovial membrane of equine midcarpal joints (joint angle of 135°) was decreased from 108 ± 36 to 12 ± 7 and 11 ± 3 µl/min ·1 ·g⁻¹ at IAPs of 30 and 60 mmHg, respectively. Such low blood flow could alter fluid clearance from the joint cavity such that extrapolation of hydraulic conductance values obtained at 25 cmH₂O may not be valid. Therefore, we cannot conclude on the contribution of fluid absorption from the joint to the decrease in IAP over time. In our study, horse limbs were secured and fixed in position.
for a minimum of 1 h before the start of the study. Our study did not demonstrate compartmentation in the midcarpal joint. Infusion of fluid was associated with an immediate increase in IAP in all four compartments. Therefore, it is unlikely that fluid distribution would be responsible for the decay in pressure over time. In our study, we used a short measurement period (5 min) to measure the rate of pressure decay. This short measurement period allowed us to use a simple logarithmic transformation to linearize the pressure-time relationship. The characteristics of biomechanical relaxation of tissue is a function of the length of the experiments, and long-duration experiments are necessary to accurately describe this phenomenon. These experiments are mathematically more complex, and therefore were not performed. Our goal was to describe the short-term effect of time on IAP, and we demonstrated that in future studies, particularly at high IAPs, measurements should be made immediately after pressure increases to minimize the effect of relaxation of IAP.

In conclusion, our study described the PV relationships of the equine midcarpal joint and highlighted the importance of joint angle, nature of infusate, and time on these relationships. Our findings will serve as a useful basis for future studies that investigate articular PV relationships.

Footnotes

a ABL 500, Radiometer, Copenhagen, Denmark
b P23 XL Statham transducer, Gould, Cleveland, OH
c Personal communication from Omega Corp., Cleveland, OH
d Model 940 infusion-withdrawal pump; Harvard Apparatus, Dover, MA
e Model 7D Polygraph, Grass Instruments, Quincy, MA
f Flexitrace, Tree Star, Campbell, CA
Figure 3.1. Illustration of forelimb of horse, demonstrating position of needles for measurement of intra-articular pressure in midcarpal joints.
Joint Angle 90° or 135°

To physiograph

To physiograph
Figure 3.2. Pressure-volume relationships in equine midcarpal joints demonstrating sigmoid shape of curve and difference between sub- and supra-atmospheric pressures. Elastance is significantly lower (greater compliance) at 135° than 90° angle (P=0.0016).
Figure 3.3. Pressure-volume relationships in equine midcarpal joints at subatmospheric (normal) pressures. Elastance is significantly lower (greater compliance) at 135° than 90° angle ($P=0.028$).
Figure 3.4. Elastance as function of IAP at joint angle of 135°. Regression slope is steeper below than above atmospheric pressure.
Elastance (mm Hg/ml)

Intraarticular pressure (mm Hg)
Figure 3.5. PV relationships in equine midcarpal joint during infusion or withdrawal of saline or synovial fluid at joint angles of 90° (A) or 135° (B). Hysteresis calculated as (area under infusion curve-area under withdrawal curve)/area under infusion (in %), is significantly greater with synovial fluid than saline (P<0.005) and at 90° than at 135° angle (P<0.005).
Intraarticular pressure (mm Hg)

Volume (ml)

SYNOVIAL FLUID

SALINE

22%

57%

37%
Figure 3.6. PV relationships in equine midcarpal joints at 135° infused with isotonic saline or synovial fluid. Elastance was significantly greater with saline than with synovial fluid (P=0.045).
Figure 3.7. A: PV relationships at 3 low-pressure cycles (<30 mmHg) followed by 3 high-pressure cycles (30-80 mmHg) and at 3 high-pressure cycles followed by 3 low-pressure cycles. B: inset of A; shows that elastance significantly increased at high-pressure cycles after prior distention at low pressures.
Figure 3.8 Relaxation of IAP as a function of natural log of time for 20-mmHg increments of IAPS (joint angle 135°). Slope of IAP-relaxation (IAP-R) curve is significantly increased with increasing IAP (P ≤ 0.005).
Initial IAP = 80 mm Hg
Initial IAP = 60 mm Hg
Initial IAP = 40 mm Hg
Initial IAP = 20 mm Hg
Figure 3.9 IAP-R as a function of IAP. There is a significant increase in slope of IAP-R curve with increased initial IAP. $\text{IAP} = -8.37 + 3.00 \times \ln(\text{IAP})$ ($R^2 = 0.99$, $P \leq 0.0001$).
References


CHAPTER 4

DETERMINATION OF SYNOVIAL MEMBRANE BLOOD FLOW IN EQUINE JOINTS BY COLORED MICROSPHERES AND EVALUATION OF THE EFFECT OF INCREASED INTRAARTICULAR PRESSURE

Introduction

The synovial membrane functions to maintain articular homeostasis by providing a pathway for the exchange of nutrients and metabolic by-products between blood and synovial tissues, including articular cartilage. Optimal oxygen delivery to articular tissues serves to maintain normal synovial fluid composition, chondrocyte metabolism, and ensure normal matrix composition and turnover. The efficiency of exchange between the synovial membrane capillaries and joint cavity is dependent on capillary density, capillary depth and blood flow.

Previous investigations have used indirect methods such as clearance of small solutes or radioactive tracers to measure blood flow to articular tissues. These methods provide a relative measure of alterations in flow and do not account for the heterogeneity in blood flow distribution among different articular tissues. Direct quantitation of blood flow to the synovial membrane of normal joints may provide a better understanding of the local influence of the circulation on joint health. Radioactive microspheres were used in a study that compared blood flow in hard and soft tissues of the radiocarpal and stifle joints of normal dogs at rest and during treadmill exercise. That study illustrated the usefulness of the microsphere technique to quantify blood flow in tissues, but had the inconvenience of
using radioactivity. Colored microspheres provide a quantitative, safe and relatively simple method of blood flow measurement for use in small tissue samples, and have been used to measure blood flow in several different organs, including the heart and gut 11-13.

Indirect methods of blood flow measurements have helped to define the determinants of synovial membrane blood flow to be defined, which include local temperature, vasoactive substances, and intraarticular pressure. In turn, intraarticular pressure is dependent on synovial fluid volume, joint angle and joint capsule elastance. In chronic inflammatory arthritis, current evidence suggests that a state of hypoxic acidosis exists, and that the worst hypoxia and acidosis occurs in joints with advanced fibrosis, marked effusion and poor blood flow 3,4,14,15. Furthermore, flexion or usage of inflamed joints exacerbates increases in intraarticular pressure, impairs blood flow, and aggravates hypoxia 14. Intraarticular pressures of up to 170 mmHg have been measured in rheumatoid knee joints of human patients submitted to isometric quadriceps contractions 16. In these rheumatoid patients, oxygen partial pressures fell significantly during exercise. Significant reactive hyperemia occurred after exercise and evidence of reperfusion injury was demonstrated by an increase in lipid peroxidation products in synovial fluid of exercised knees, and by an increased formation of fluorescent monomeric and polymeric IgG 16. Significant hypoxic acidosis was measured at intraarticular pressures greater than 45 mmHg 14. Hypoxic acidosis can contribute to articular inflammation by impairment of synovial membrane and chondrocyte metabolism 17,18, lysosomal enzyme release, acceleration of inflammatory processes, and synovial membrane proliferation 9. Therefore synovial membrane blood flow plays a critical role in the pathogenesis of chronic arthritis. Little is known of the influence of these determinants in normal joints. Determination of blood flow and blood flow distribution to normal joints would allow comparison to blood flows in acute or chronic articular disorders. Knowledge of the influence of the articular
environment, including joint angle, intraarticular pressure, temperature, innervation and vasoactive agents on blood flow to the joint would provide a better understanding of the dynamic events that occur during joint disease.

In the present study, the synovial membrane was defined as that tissue consisting of an intimal layer or synovium, 1-5 cells in depth, adjacent to the joint cavity, containing a rich capillary plexus and deeper lymph vessels, and a subintimal or subsynovial layer, principally composed of fibrous, areolar or adipose tissue depending on the function and location within the joint as originally proposed by Key. Morphometric studies have demonstrated that areolar synovial membrane has the greatest capillary density, and that the capillary density is greatest within 25 µm of the synovial surface. It is thought that this most superficial capillary plexuses of the synovial membrane contributes to the majority of solute exchanges.

The purpose of the present study was to quantify the normal regional distribution of synovial membrane blood flow in the horse using colored microspheres, and to determine the effect of acute increases in intraarticular pressure on synovial membrane blood flow.

Material and methods

Horses - Six healthy adult horses (350-500 kg) and 8 forelimbs were used for this study. Criteria for inclusion of horses in the study included midcarpal and metacarpophalangeal joints free of disease based on physical examination and gross examination of the articular cartilage and synovial membrane at post-mortem. An Institutional Laboratory Animal Care and Use protocol was approved for this study.

Experimental design and protocol - Blood flow to the synovial membrane of the dorsal antebrachiocarpal; dorsal, lateral palmar and medial palmar midcarpal; and dorsal and
palmar metacarpophalangeal joints was determined from a randomly selected forelimb in all horses (n=6), and from the contralateral limb of 2 horses. The fibrous capsule of the dorsal pouch of the midcarpal joint (n=8) was also harvested for blood flow determination and comparison with synovial membrane blood flow at the same site. One forelimb from each horse (Group 1, n=6) was used for blood flow determination at baseline (trial 1) and following increased midcarpal joint intraarticular pressures of 30 (trial 2) and 60 mmHg (trial 3). In 2 horses, the contralateral limb (Group 2; n=2) was instrumented and blood flow was determined at corresponding times (Trials 1, 2, and 3) but without increased midcarpal joint intraarticular pressure.

Horses were allowed to stabilize under general anesthesia for 1 hour before each experiment. Drug administration and mechanical ventilation were adjusted to maintain mean arterial pressure > 70 mmHg, \( P_{aO_2} > 150 \) mmHg and \( P_{aCO_2} < 60 \) mmHg. After 1 hour, a randomly selected forelimb was suspended from a rack, which placed the midcarpal and metacarpophalangeal joints at approximately 40 cm and 64 cm above the level of the right atrium, respectively. The limb was positioned to produce a 135° angle between the radius and third metacarpus (verified with a goniometer) and instrumented with catheters for microsphere determination and needles and pressure transducers for intraarticular pressure measurement and synovial fluid infusion (Fig 1). Regional blood flow to the selected forelimb (Group 1) were determined at baseline (trial 1) and following increased midcarpal joint pressures of 30 mmHg (trial 2) and 60 mmHg (trial 3) with the use of a different microsphere color for each trial. Intraarticular pressure was increased by intraarticular infusion of synovial fluid collected from the tarsocrural joint of the same horse. To counter for stress-relaxation,\(^{22}\) intraarticular pressure was maintained at the predetermined value by continuous infusion of synovial fluid for the duration of blood flow measurement (fixed pressure, variable rate of infusion). Intraarticular pressure was allowed to return to baseline
for 10 minutes before each increase in intraarticular pressure. The contralateral forelimb
was instrumented in 2 horses, and regional blood flow was determined at three consecutive
times, with a minimum interval of 10 minutes between each determination (Group 2, Trials
1, 2, 3), but without increasing intraarticular pressure. Cardiac output was determined prior
to each experiment and mean arterial blood pressure was recorded before each trial. Horses
were euthanized with an overdose of pentobarbital at the end of each study.

Instrumentation- Each horse was instrumented for drug administration and measurement of
cardiac output by a thermodilution technique before induction of anesthesia 23. Briefly, a 7-
F, double-lumen, 110-cm long thermodilution catheter was inserted through an introducer
into the right jugular vein until the distal port was positioned in the pulmonary artery.
Polyethylene tubing (PE-240) for injection of cold solution was advanced through an
 introducer inserted in the right jugular vein proximal to the thermodilution catheter until the
tip was positioned in the right atrium. Catheter position was verified by observing
characteristic pressure waveforms. Horses were sedated with xylazine (0.5 mg/kg, IV) and
anesthetized with 5% guaifenesin (IV, to effect) and thiamylal (4 mg/kg, IV) and
maintained with sodium pentobarbital (5-15 mg·kg⁻¹·hr⁻¹, IV). Horses were intubated,
positioned in dorsal recumbency, and mechanically ventilated with 100% oxygen. A 20-
gauge catheter was inserted in a facial artery after anesthesia induction, for direct
measurement of arterial blood pressure and collection of arterial blood samples for
determination of arterial pH and blood gas. The zero-pressure reference point for all
pressure measurements was the point of the shoulder 24. Systolic, mean and diastolic
arterial blood pressures were continuously displayed and arterial blood gases were
determined hourly. Mean arterial blood pressure was electronically derived. Cardiac outputs were determined hourly and before each experiment and expressed in ml·kg⁻¹·min⁻¹.

**Measurement of joint pressures** Midcarpal joint pressure was measured by inserting a 20-gauge needle in the dorsal compartment of the midcarpal joint, 1 cm medial to the extensor carpi radialis tendon. We have documented that intraarticular pressures are equivalent in the medial or lateral compartment of the dorsal midcarpal joint pouch in horses. The needle was connected to tubing filled with heparinized 0.9% saline (heparin 10 IU/ml). Care was taken to ensure that the tubing was closed to the horse prior to insertion of the needle, in order to measure negative intraarticular pressure. The tubing was filled with heparinized saline to prevent clotting that could occur as a result of hemorrhage during needle insertion and was connected to a horizontally positioned pressure transducer placed level with the midcarpal joint (approximately 40 cm above heart level). The recorded pressures were continuously displayed on a physiograph precalibrated by a Hg column to allow measurements ranging from -10 to +80 mmHg pressures. Intraarticular pressure was increased by infusion of synovial fluid using a 20-gauge needle placed through the extensor carpi radialis tendon and into the midcarpal joint. Synovial fluid was collected from the tarsocutaneous joints of the same experimental horse immediately before use to avoid potential effect of temperature. Intraarticular pressures was increased and maintained at the predetermined values by continuous monitoring on the physiograph.

**Measurement of regional synovial membrane blood flow** Regional synovial membrane blood flow was determined using 15 μm-diameter polystyrene colored microspheres. Red, blue and yellow were the selected colors. The median artery served as the injection
portal and the medial palmar artery as the arterial blood reference sample withdrawal portal. The accessory cephalic vein was used to obtain venous blood reference samples for detection of arteriovenous shunts (Fig 1). The median artery was cannulated in a proximo-distal direction with a heparinized 22-gauge polytetrafluoroethylene catheter. A second heparinized 22-gauge polytetrafluoroethylene catheter was inserted in a disto-proximal direction in the medial palmar artery. A third heparinized 22-gauge polytetrafluoroethylene catheter was also placed in the accessory cephalic vein for withdrawal of the reference venous sample (Fig 1).

Colored microspheres (red, blue or yellow) were thoroughly dispersed by vortex mixing for 30 seconds, suspended in 5 ml of saline containing 0.02% polyoxyethylene sorbitan monoooleate (Tween 80) and separately injected at a constant rate in 2-3 sec into the inflow median artery catheter. Reference arterial blood samples were withdrawn for 60 seconds with a calibrated withdrawal pumps set at a withdrawal rate of 10.89 ml/min starting 10 seconds before microsphere injection. This rate was selected because it was much smaller than the flow to the limb (previously determined, approximately 100 ml/min) and allowed collection of approximately 10 ml of blood in one minute, which simplified analysis according to the manufacturer’s recommendations. The withdrawal rate of the pump was verified independently at the end of each experiment. To ensure a desired microsphere count of 1000 spheres per tissue sample, 3x10^6 (trial 1) and 6x10^6 (trials 2 and 3) microspheres were used for injection. These values were estimated based on the percent weight of an average synovial tissue specimen of 2 g, compared to the average weight of the limb distal to the arterial injection portal of 6000 g (0.033%), from which the total amount of microspheres to inject was calculated so that each tissue sample would contain approximately 1000 spheres (Number of microspheres to inject = 1000 x 100 / 0.033 = 3 x 10^6).
Collection of tissue - The joint capsule (including fibrous layer and synovial membrane) was collected immediately after euthanasia from the dorsal, lateral and medial palmar midcarpal joint pouches, the dorsal antebrachialcarpal joint, and the dorsal and palmar metacarpopalangeal joint pouches. The synovial membrane (synovium and subsynovium) was dissected from the fibrous joint capsule and separated into 0.5-2.0 g specimens. One or two specimens were obtained from each site, discarding specimens that had evidence of irritation or bruising at the site of needle insertion. All sampled joints were inspected for evidence of cartilage fibrillation, or synovial membrane thickening to determine if inclusion criteria were met (see above).

Tissue processing - Reference blood samples and tissue specimens were digested and filtered for recovery of microspheres. Tissue digestion was performed as follows: after weighing, tissues specimens were placed in glass tubes and 7 ml of 4M KOH with 2% Tween 80 were added to each tube. Blood samples were digested with 4 ml of 2% Tween 80, 4.4 ml of 16M KOH and 2 ml of 20% Tween 80 for each 10 ml of blood. Blood and tissue specimens were allowed to digest overnight in a 37°C water bath. Each digest was then filtered under vacuum using a 8-μm 25-mm diameter polyester filter placed in a stainless steel syringe holder. Digestion tubes were rinsed with 2% Tween 80 and 70% ethanol to ensure collection of all spheres. The filter containing the microspheres was placed in a 1.5 ml Eppendorf tube, to which 100 μl of NN-dimethylformamide was added to release the dye. Each tube was vortex mixed for 30 s and centrifuged for 3 minutes at 2000 g.

Microsphere assay technique and calculations - The concentration of each dye solution (proportional to number of microspheres) was measured by spectrophotometry at
wavelengths of 448 nm (yellow), 530 nm (red) and 672 nm (blue) using
dimethylformamide as the blank. Nine standards were used to develop the standard matrix
before each assay. A matrix inversion technique was used to correct for overlap of
composite spectra. Samples were diluted when necessary to maintain absorbance of each
wavelength under 1.3 absorbance units (AU), to ensure linearity of the
absorption-concentration curve according to the Lambert-Beer law. All tissue samples
containing less than 400 spheres were discarded from analysis.
Blood flow to each tissue specimen was calculated as follows:

Blood flow to tissue sample (μ/min) =
\[
\frac{\text{AU of tissue sample} \times \text{reference blood flow (ml/min)}}{\text{AU of reference sample}}
\]  

Blood flow was expressed in µl-min⁻¹-g⁻¹ of tissue.

Statistical analysis- Distribution of data for all sampled populations were tested for normal
distribution using the Kolmogorov-Smirnov test for continuous data, and for equality of
variances using the F test. The assumption of normal distribution was met in all instances.
However, the variances about the mean of midcarpal blood flow for trials 1, 2 and 3 were
unequal, with trial 1 showing a higher variability. Therefore a nonparametric test (Friedman
test for repeated measures) was used to test for differences among trials. If a significant
effect was detected, a Dunn’s comparison test was used to compare individual means.
Mean dorsal and palmar metacarpophalangeal pouch blood flow and mean fibrous capsule
and synovial membrane dorsal midcarpal joint blood flow were compared using a
two-tailed paired Student’s t test. The coefficient of repeatability of blood flow
determination within each animal and among colors was determined from the control.
animals using the mean square error of the analysis of variance table performed on the log-transformed data as there was an association between the magnitude of measurement and repeatability. A 95% confidence interval for the coefficient of repeatability was also constructed for each pair of color, using log-transformed data. The coefficient of variation of blood flow within each animal was calculated for each site that had two tissue specimens available for blood flow determination. The coefficient of variation among animals was calculated from the pooled mean and standard deviation for each site. Mean arterial blood pressure obtained before each trial was compared using a one-way analysis of variance with repeated measures. Data are reported as mean ± SE. The level of significance for all tests was set at P < 0.05.

Results

Cardiac outputs determined before the start of each experiment measured 51.7 ± 3.7 ml·min⁻¹·kg⁻¹. Mean arterial blood pressures recorded before each trial were 119.2 ± 3.0 (trial 1), 116.8 ± 3.7 (trial 2), and 116.5 ± 3.9 (trial 3). Mean PaCO₂ was 38.00 ± 1.57 mmHg and mean arterial pH was 7.41 ± 0.02 for all horses and all trials. There was no significant difference in mean arterial blood pressures, pH and PaCO₂ among trials.

Regional synovial membrane blood flow - Baseline regional synovial membrane blood flow for each joint was 103 ± 8 (dorsal radiocarpal), 108 ± 36 (dorsal midcarpal), 61 ± 12 and 50 ± 11 (lateral and medial palmar midcarpal joints) and 17 ± 3 and 26 ± 5 (dorsal and palmar metacarpophalangeal joints) μl·min⁻¹·g⁻¹ (Table 1). Blood flow to the dorsal aspect of the metacarpophalangeal joint was significantly less than that of the palmar aspect (p=0.048). Synovial membrane blood flow was significantly higher than fibrous capsule flow in the dorsal pouch of the midcarpal joint (96 ± 32 vs 24 ± 6 μl·min⁻¹·g⁻¹, p=0.034).
Microspheres were not detected in venous blood reference samples obtained from the accessory cephalic vein at baseline or after increased intraarticular pressure.

**Midcarpal joint pressure** - Baseline intraarticular midcarpal joint pressures measured -2.86 ± 1.45 mmHg, with a median value of -2.00 mmHg and 95% confidence intervals of -6.42 and 0.70 mmHg. Increases in intraarticular pressures of 30 and 60 mmHg in the midcarpal joint resulted in a significant decrease (p=0.0224) in synovial membrane blood flow to 16% of baseline values (baseline: 108 ± 36 μl-min⁻¹·g⁻¹, 30 mmHg: 12 ± 7 ml-min⁻¹·g⁻¹, 60 mmHg: 11 ± 3 μl-min⁻¹·g⁻¹) (Fig. 2). There was no difference between synovial membrane blood flow measured at intraarticular pressures of 30 and 60 mmHg. Sequential synovial blood flow of control limbs were not significantly different between trials (trial 1: 109 ± 51 μl-min⁻¹·g⁻¹, trial 2: 94 ± 48 μl-min⁻¹·g⁻¹, trial 3: 128 ± 52 μl-min⁻¹·g⁻¹).

**Repeatability**: The overall coefficient of variability for the method, calculated from log-transformed data from the group 2 horses on 39 samples from all sites was 47%. The 95% confidence interval for blood flow determination between the first and second blood flow determination was 13% and 27% (i.e. the blood flow could be expected to range between 13% below and 27% above the value obtained), between the second and third blood flow determination was 16% and 30% and between the first and 3rd determination was 21% and 22%.

**Limits of agreement for the same site**: For 26 samples where 2 tissue specimens from the same site were available, the limits of agreement were determined from the log-transformed data, with 25 degrees of freedom, giving an interval of 8% below to 16% above the value obtained.
Coefficient of variation- Within each horse, the coefficient of variation of blood flow for all sites averaged 29% (range 25% -34%). The mean coefficient of variation of blood flow between horse at each site was 64% (range 54%-73%).

Discussion

Our study is the first to examine synovial blood flow and tissue specific blood flow in joints with the use of colored microspheres. Regional synovial membrane blood flows in equine joints at baseline ranged from 17± 3 (dorsal fetlock) to 108± 36 (dorsal midcarpal joint pouch) μl·min⁻¹·g⁻¹ (mean ± SE). These results are comparable to stifle and hip joint blood flows obtained in dogs as measured with radioactive microspheres 29-31.

Our synovial membrane tissue samples included synovium and subsynovium. Previous studies have shown that capillary density is highest within 25 μm of the joint boundary, and decreases thereafter 32,33. Our tissue samples were obtained by gross dissection, and thus contained synovium and subsynovium. The inclusion of less vascular tissue may have resulted in lower blood flow values than if only the most superficial, capillary dense, 25 μm of synovial membrane had been obtained.

We were able to demonstrate significant heterogeneity of blood flow from different sites within a joint. Blood flow to the dorsal aspect of the metacarpophalangeal joint was significantly lower than that of the palmar aspect. Morphologically, the synovial membrane of the dorsal aspect of the metacarpophalangeal joint is classified as fibrous, because the synovium rests on a fibrous, relatively avascular subsynovial layer 2. In contrast, synovium of the palmar aspect of the fetlock joint pouch is classified as areolar as it contains numerous synovial villi resting on areolar subsynovial tissue. Synovial villi are very vascular, which correlates well with our findings of a higher blood flow at that site.

Our study further characterized the blood flow to specific tissues within the midcarpal joint,
and demonstrated that blood flow to the fibrous joint capsule is lower than that of the synovial membrane.

An intraarticular pressure of 30 mmHg was sufficient to cause a significant and marked reduction in articular tissue blood flow in horses in dorsal recumbency. The effect of hydrostatic pressure, capillary pressure and thus blood flow occlusion pressure may vary with limb and body position. Venous pressure in the feet of human beings standing absolutely still approximates 90 mmHg, and it can be inferred that a similar or greater value would be obtained in standing still horses. Locomotor activity decreases venous pressure in the feet of adult humans to less than 25 mmHg because of the combined effects of the pumping action of skeletal muscles and venous valves. We are confident that a similar effect would occur in active horses. We have measured the capillary pressure at the level of the metacarpophalangeal joint in laterally recumbent horses by the venous occlusion technique, and obtained values of 13.3 mmHg. In another study, the more rigid tissues of the distal digit (hoof) of laterally recumbent horses yielded capillary pressures of 37 ± 2 mmHg. This capillary pressure is higher than that in the dog hindpaw, and was counterbalanced by a higher tissue pressure of 25.6 ± 2.5 mmHg, thus preventing edema formation. In our study, the use of an upright limb position may have resulted in a progressive increase in capillary hydrostatic pressure because of inactivity, thus altering the intraarticular pressure necessary to occlude blood flow.

The formation of synovial fluid, an ultrafiltrate of plasma, is dependent on synovial capillary pressure as there is no synovial basement membrane. Capillary pressure in our study was likely less than 30 mmHg, such that reduction of blood flow was significant with intraarticular pressures of 30 mmHg or more. We speculate that a reduction in blood flow may lead to decreased capillary hydrostatic pressure, decreased filtration and consequently decreased synovial fluid production. In addition, articular clearance of small
solute which are dependent on venous flow would be expected to be decreased. At intraarticular pressures of 25 cm H$_2$O, morphologic synovial capillary compression has been observed. The deleterious effects of increased intraarticular pressure on synovial fluid metabolite accumulation has been reported. Relative joint ischemia resulted in synovial fluid acidosis, increased CO$_2$ pressure, and lactate accumulation, reflecting poor clearance and anaerobic metabolism. Although it was initially reported that intraarticular pressures above systolic blood pressure were necessary to obtain these effects, subsequent studies have shown that intraarticular pressure greater than 45 mmHg resulted in significant lactate and metabolite accumulation. These previous studies served as the basis for our choice of intraarticular pressures above and below 45 mmHg. Geborek reported a significant decrease in synovial blood flow (measured by doppler flowmetry) when intraarticular pressure was increased to 20 mmHg. Our results corroborate that relatively low intraarticular pressures can produce significant reduction in blood flow. The effect of intraarticular pressure on synovial membrane blood flow is, however, dependent on synovial membrane capillary hydrostatic pressure, which in turn will vary with positioning, locomotor activity, vasomotor tone and venous pressure. Our findings suggest that articular decompression and resolution of joint effusion may be beneficial as part of the treatment of joint disease.

There was marked variability in individual blood flow values between horses in the present study. This finding is similar to that of studies performed with radioactive microspheres, where blood flows from the same sites varied between animals, and was suggested to be function of animal variation rather than variability in the method. Differences in vascular tone or cardiac output may contribute to these differences. Blood flow values were more consistent within each horse and suggest that colored microspheres
are a useful method for measurement of synovial membrane blood flow in the horse when a baseline value is obtained.

Microspheres have been used successfully to measure regional blood flow to several organs, including bone, myocardium, endocardium, lung, ocular and renal tissues [12,41-46]. High agreement has been demonstrated between blood flow measured by radioactive and colored microspheres [12]. Colored microspheres eliminate the need to use radionuclides, use different colors to allow serial measurements, and are biocompatible, such that chronic implantation is possible. Other methods of articular blood flow determination include venous occlusion plethysmography, clearance of rapidly-diffusing radioactive solute ($^{23}$Na, $^{131}$I, $^{132}$Xe, tritiated water) or heat, and laser Doppler flowmetry [8,38]. These methods provide relative estimates of perfusion, assume homogeneity of tissue, do not account for flow to skin or periarticular tissues (articular soft tissues are estimated to form only 15% of an articular segment [47,48]), and in the case of clearance studies, assume that solute clearance is only flow limited. The use of microspheres for synovial blood flow measurement allows an absolute measure of blood flow, which can be determined from small tissue samples, and allows quantification of flow from different anatomical sites or within different tissue types.

Aggregation of microspheres, maldistribution of blood flow including lamination of flow, and arteriovenous shunting are problems that may be encountered when using a microsphere method to determine blood flow. Aggregation of spheres results in nonuniform distribution of the microspheres and may occlude blood flow to the tissue of interest. Microsphere aggregation is prevented by the addition of the surface-active polyoxyethylene sorbitan monooleate (Tween 80). The cardiovascular effects of Tween 80 are virtually non-existent at the concentration used in our study (0.02%), particularly since regional rather than systemic injection was performed [49]. Cardiac output and mean arterial
blood pressure values throughout our study were comparable to those obtained in other studies in horses under general anesthesia.\textsuperscript{50,51} We believe that maldistribution of blood flow, which may be caused by the laminar flow of microspheres within the inflow artery, was insignificant, because the coefficient of variation of blood flow was small within each horse for a given site. We were unable to demonstrate arteriovenous shunting in any horse in our study during altered blood flow conditions such as increased intraarticular pressure.

In conclusion, this study successfully used colored microspheres for the measurement of articular blood flow in horses. Significant heterogeneity of blood flow distribution was demonstrated between the synovial membrane and fibrous joint capsule. Increased intraarticular pressure to 30 mmHg markedly decreased synovial membrane blood flow. Increased intraarticular pressure may contribute to synovial membrane pathology via an ischemic pathway.

Footnotes

- a Swan-Ganz thermodilution catheter, Columbus Instruments, Columbus, OH
- b ABL 500, Radiometer Copenhagen, Copenhagen, Denmark
- c Spectramed P23XL Transducer, Oxnard, CA
- d Grass Model 7D Polygraph, Grass Instruments Co, Quincy, Mass
- e Dye-Trak microspheres, Triton Technology Inc., San Diego, CA
- f Angiocath, Becton-Dickinson, Sandy, UT
- g Model 940 Infusion/Withdrawal pump, Harvard Apparatus Co, Dover, Mass
- h Triton Technology Inc., San Diego, CA
- i Beckman D-70 Spectrophotometer, Scientific Instrument Division, Beckman Instrument, Fullerton, CA
**Figure 4.1:** Medial aspect of the left forelimb of a horse illustrating normal anatomy and instrumentation for microsphere determination. The scale indicates approximate height above the heart of the metacarpophalangeal joint.
Figure 4.2: Synovial membrane blood flow in the dorsal pouch of normal equine midcarpal joints in dorsal recumbency at a joint angle of 135° after increased intraarticular pressure (IAP) (Group 1) or in normal joints (Group 2). Data are mean +/- SE.

* indicates significant difference from baseline
Trial 1
IAP = 30 mm Hg

Trial 2
IAP = 30 mm Hg

IAP = 60 mm Hg

Trial 3

Flow (ml/min/1.g-1)

Control
Increased IAP
Figure 4.3. Scattergram of the coefficient of variability between three simultaneous blood flow determinations obtained with three different microsphere colors, plotted against number of microspheres. This graph illustrates the higher variability associated with low microsphere numbers.
Figure 4.4. Bland and Altman plot of the difference between three serial (one minute interval) blood flow determinations in the same tissue specimen, to illustrate the repeatability of blood flow determinations. Indicated are the mean difference and 95% confidence interval for serial blood flow determinations.
<table>
<thead>
<tr>
<th>Joint</th>
<th>Tissue</th>
<th>n</th>
<th>Blood flow μl/min/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorsal radiocarpal</td>
<td>Syn</td>
<td>16</td>
<td>103 ± 8</td>
</tr>
<tr>
<td>Dorsal midcarpal</td>
<td>Syn</td>
<td>8</td>
<td>108 ± 36</td>
</tr>
<tr>
<td>Dorsal midcarpal</td>
<td>Fibrous</td>
<td>8</td>
<td>24 ± 6*</td>
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<tr>
<td>Lateral palmar midcarpal</td>
<td>Syn</td>
<td>7</td>
<td>61 ± 12</td>
</tr>
<tr>
<td>Medial palmar midcarpal</td>
<td>Syn</td>
<td>9</td>
<td>50 ± 11</td>
</tr>
<tr>
<td>Dorsal metacarpophalangeal</td>
<td>Syn</td>
<td>15</td>
<td>17 ± 3**</td>
</tr>
<tr>
<td>Palmar metacarpophalangeal</td>
<td>Syn</td>
<td>15</td>
<td>26 ± 5</td>
</tr>
</tbody>
</table>

**Table 4.1** Blood flow to articular soft tissues of normal midcarpal and metacarpophalangeal joints in horses positioned in dorsal recumbency, with the midcarpal joint at 135° angle. Data are mean ± SE.

Syn = synovial membrane (synovium and subsynovium). Fibrous = fibrous joint capsule.

* Midcarpal joint fibrous capsule blood flow significantly lower than midcarpal joint synovial membrane ($P = 0.034$)

** Dorsal metacarpophalangeal joint blood flow significantly lower than palmar ($P = 0.048$)
References


CHAPTER 5

LOCAL HEMODYNAMICS, PERMEABILITY AND O₂ METABOLISM OF INNERVATED OR DENERVATED ISOLATED JOINTS IN AN IL-1 MODEL

Introduction

Synovial joints form functional units that facilitate relative motion of masses under load.¹ The blood flow, fluid exchange and innervation of joints have adapted to this biomechanical role and to the preservation of a supply of nutrients to a tissue under compressive load, the articular cartilage. Articular cartilage is an avascular, aneural but metabolically active tissue. Nutrients to cartilage must cross the blood-joint barrier, which consists of a fenestrated capillary endothelium in series with the overlying synovial intima, and in parallel with the synovium interstitium. In turn, the exchange characteristics of the blood-joint barrier can be modified by alterations in plasma and synovial fluid composition, capillary and joint cavity hydrostatic pressure and capillary and synovial membrane permeability. The means of acutely affecting these changes are through blood flow and innervation.

A Starling’s based mechanism has been proposed and substantiated to explain the pathways of fluid exchange across the blood-joint barrier. Starling proposed that the rate of fluid exchange across a physiologic barrier is the result of differences of hydrostatic and oncotic pressures acting across a semi-permeable barrier and modified by the filtration and oncotic reflection coefficients of the tissues. The influence of various osmotic fluids on joint fluid filtration and intraarticular pressure have clearly supported the use of Starling’s forces to explain joint fluid exchanges. The presence of local heat, periarticular edema and
joint effusion in articular disease indicates an imbalance between vascular permeability and tissue clearance that leads to tissue swelling, which warrants further investigation.2 Separate clinical and experimental studies have examined the physiology of blood flow, innervation, and fluid exchanges of joints in health and their alteration with disease.3-6 However, these mechanisms have not been examined in an isolated model or during acute articular inflammation. Extracorporeal isolated organ preparations improved our understanding of co-dependent physiopathologic mechanisms of organs such as heart,7 lung, liver, intestine8 and others.

In this study, we use an isolated joint organ preparation to examine pathophysiologic alterations of the joint, including blood flow, permeability, fluid exchanges, and oxygen metabolism associated with innervation or denervation in a model of acute joint inflammation.

Materials and Methods

Horses - Twenty-four adult horses (body weight 330-550 kg, 8 females, 8 geldings, 8 stallions) of various breeds were used. Horses were determined free of metacarpophalangeal joint disease by thorough clinical examination, synovial fluid analysis, and gross post-mortem examination. All experiments were conducted in accordance with the Animal Care and Use Guidelines of The Ohio State University.

Experimental procedures

Horse instrumentation - Horses were instrumented for cardiac output measurements and intravenous drug administration prior to induction of general anesthesia. Horses were sedated with xylazine hydrochloride (0.25 mg/kg) and the skin over both jugular furrows
was clipped, aseptically prepared, and catheter insertion sites were locally infiltrated with lidocaine hydrochloride. A flow-directed thermodilution catheter was inserted in one jugular vein through a catheter introducer and advanced until the distal tip was located in the pulmonary artery. This catheter was used for thermal dilution estimates of cardiac output. Polyethylene tubing for administration of cold dextrose solution was inserted proximal to the thermodilution catheter and advanced until the distal tip was positioned in the right atrium. Catheter placement was guided and position was confirmed by observation of characteristic pressure waveforms. The opposite jugular vein was cannulated with a 14 gauge teflon catheter for drug administration. Cardiac output was recorded as the mean of 3 measurements, using a cardiac output computer. After induction of general anesthesia, a facial artery was cannulated for direct measurement of arterial blood pressure.

**Anesthesia and monitoring**

Only one horse was studied on a given day. Horses were sedated with xylazine (0.5 mg/kg) and anesthesia was induced with guaifenesin (25 mg/kg) followed by sodium thiopental (2 mg/kg, iv). Anesthesia was maintained with sodium pentobarbital (loading doses 4 mg/kg, maintenance 5-15 mg/kg/hour, iv, to effect) and controlled ventilation with 100% oxygen. A bipolar ECG (base-apex lead) was recorded throughout each study for determination of heart rate and rhythm. Arterial pressure and ECG were continuously displayed and semicontinuously recorded on a multichannel recorder. Systemic arterial blood gases, packed cell volume and total protein were determined hourly. Intravenous polyionic isotonic fluids were administered (5-10 ml/kg/hr) throughout each study. Mean systemic arterial pressure was maintained at ≥ 70 mm Hg, and ventilation was adjusted to maintain PCO₂ ≤ 55 mmHg. Heparin (50,000 IU iv) was administered every 90 minutes.
Joint isolation procedure and instrumentation (Fig. 5.1)

One metacarpophalangeal joint (MCP) was randomly selected and the horse was positioned with the selected limb lowermost. All circuit tubing was thoroughly flushed with heparinized saline. The medial palmar artery and vein were isolated at the level of the proximal third of the metacarpus. The artery was cannulated with polyethylene tubing (PE 320) in a disto-proximal direction proximal to the anticipated site of joint isolation, and in a proximo-distal direction distal to the anticipated site of joint isolation. Blood was diverted from the cannulated artery, circulated through a precalibrated peristaltic pump and infused via the distal cannulated medial artery to the joint preparation. In a similar fashion, the medial palmar vein and the medial and lateral palmar digital veins (PE 240) were cannulated and connected to a Y-connector and venous outflow was diverted to a reservoir and reinfused to the horse via the median vein using a second peristaltic pump. Venous pressure was controlled by adjusting the height of the extracorporeal reservoir. The medial palmar digital artery was cannulated for collection of a reference sample for blood flow determination by the microsphere method (see below). Final isolation of the joint was achieved by transection of all soft tissue, sectioning of the bones at the level of the midmetacarpus and dislocation of the proximal interphalangeal joint. In innervated preparations, the medial and lateral palmar nerves were isolated and preserved. Hemorrhage was controlled by electrocoagulation or ligation. A 20 gauge 2.5 cm needle was inserted in the MCP joint using a palmaro-medial approach by insertion of the needle between the distal aspect of the medial sesamoid bone and the palmar aspect of the first phalanx, immediately distal to the medial metacarpo-sesamoidean ligament. After baseline synovial fluid collection, the needle was connected to a heparinized saline filled tubing connected to a three way stopcock and pressure transducer, for continuous intraarticular measurement (first 240 minutes) and for synovial fluid collection (at 240, 300 and 330 minutes). The isolated joint was suspended from a calibrated FT03 transducer connected to
a physiograph for continuous measurement of weight changes. All circuit pressure transducers were placed at the level of the joint preparation. Initial circuit arterial flow was set at 10 ml/min until completion of the preparation and establishment of isogravimetric state (see below). This joint isolation technique established a pressure-driven auto-oxygenated, pump-perfused, extracorporeal isolated joint model.

**Experimental protocol**

The joint preparation model was randomly assigned to one of four groups: 1-Control; 2) Control-denervated; 3)Inflamed; 4)Inflamed-denervated. Inflammation was induced by intraarticular injection of 0.35 ng/kg BW of recombinant human interleukin-1β (IL-1β). After joint isolation, isogravimetric state (no gain or loss of weight in the isolated preparation) was established and maintained for 20 minutes by adjustment of arterial flow. The preparation was maintained for 6 hours, after which horses were euthanized with an overdose of pentobarbital (100 mg/kg, iv) and specimens collected. Baseline (time 0) was considered as the time after establishment of isogravimetric state and collection of baseline synovial fluid sample.

**Sample collection**

Circuit arterial and venous blood samples were collected at baseline, and hourly thereafter, in heparinized syringes, and transported on ice for blood gas determination. Packed cell volume and total protein (refractometer) were measured in the circuit arterial blood. Synovial fluid samples were collected after joint isolation at baseline (time 0), 240, 300 and 330 minutes into appropriate vials as described below. All available fluid was collected at each collection time. Between the beginning of the experiment (time 0) and 240 minutes, synovial fluid was allowed to accumulate in the joint, and intraarticular pressure
was continuously recorded, using a pressure transducer placed at the level of the isolated joint and connected to a precalibrated physiograph module\(^d\). Local hemodynamic and oxygen metabolism measurements were obtained hourly. Synovial membrane blood flow was obtained at time 0, 60 and 330 minutes. Synovial membrane permeability to albumin and dextran was determined from 330 to 360 minutes. At 360 minutes, the preparation was disassembled and weighed, and each joint was carefully examined for evidence of abnormalities such as cartilage lesions, synovial membrane proliferation, and periarticular osteophyte formation. The presence of such findings excluded the horse from the study. Synovial membrane specimens were obtained for fluorescence studies and determination of wet/dry ratio. Specifics of each sample and specimen collection and analysis are described below.

**Local hemodynamics measurements and calculations**

After baseline measurements were recorded, circuit arterial pressures and flows were recorded every 5 minutes for one hour to examine the influence of II18 on local articular hemodynamics. Circuit arterial and venous pressures were then adjusted as needed to maintain pressures at isogravimetric state, respectively, and circuit arterial flow values were recorded hourly to determine alterations in hemodynamics. Total vascular resistance (TVR) of the isolated joint was calculated as follows:

\[
TVR \text{ (mm Hg/ml/min)} = \frac{(P_{art} - P_{ven})}{Q_{art}}
\]

(1)

where \(P_{art}\) is the arterial pressure to the circuit (mm Hg), \(P_{ven}\) is the venous pressure and \(Q_{art}\) is the arterial flow to the circuit (ml/min).

Synovial fluid collection was re-initiated at 240 minutes, and production of synovial fluid was measured from 240 to 300 minutes, and 300 to 330 minutes by collection into a graduated cylinder.
Synovial fluid total protein were measured using the BCA method. Synovial membrane permeability surface area product (PS) was estimated at 330 minutes using the following calculation:

\[ \frac{dm}{dt} = PS \times (C_p - C_f) \]  

where \( \frac{dm}{dt} \) is the protein flux across the synovial membrane, and \( C_f \) and \( C_p \) are the total protein concentration in synovial fluid and arterial plasma, respectively.

The protein flux \( \frac{dm}{dt} \) was measured by the following equation:

\[ \frac{dm}{dt} = V \times \frac{dC_f}{dt} \]  

where \( V \) is the volume of synovial fluid produced between 300 and 330 minutes, and \( dC_f \) is the difference in synovial fluid protein concentration between 300 and 330 minutes. This time period was chosen because IAP was 0 mm Hg (atmospheric pressure) during that time and synovial fluid flow was not influenced by the increased IAP observed at 240 minutes.

**Oxygen metabolism measurements and calculations**

Circuit arterial and venous blood were analyzed for \( \text{PO}_2 \), \( \text{PCO}_2 \) and pH and corrected to body temperature at the time of collection. Total hemoglobin (Hb) and arterial oxygen saturation (\( \text{SaO}_2 \)) were measured in each sample using a co-oximeter, and arterial oxygen content (\( \text{CaO}_2 \)) in each sample was calculated as follows:

\[ \text{CaO}_2 \text{ (ml/dl)} = \text{Hb x SaO}_2 \times 1.36 + \text{PaO}_2 \times 0.003 \]  

Circuit oxygen delivery (\( \text{DO}_2 \)) was calculated as

\[ \text{DO}_2 \text{ (ml/min/g)} = \text{CaO}_2 \text{ (ml/dl)} \times \text{Q}_\text{art} \text{ (ml/min)} / W \]  

where \( \text{Q}_\text{art} \) is the arterial flow and \( W \) is the weight in grams of the isolated joint. Circuit oxygen consumption (\( \text{VO}_2 \)) was calculated as
\[ \text{VO}_2 (\text{ml/min/g}) = C(a-v) O_2 \times Q \text{art} / W, \]  
\[ \text{(6)} \]

where \( C(a-v) O_2 \) is the arteriovenous \( O_2 \) content difference. Oxygen extraction ratio (\( O_2 \) ER) was calculated as
\[ O_2 \text{ER} = \frac{C(a-v) O_2}{C\text{a}O_2}. \]
\[ \text{(7)} \]

Synovial fluid blood gases were measured at baseline, 240, 300 and 330 minutes.

Synovial fluid \( O_2 \) gradient was calculated as:
\[ O_2 \text{gradient}_{SF} = P_{a}O_2 - P_{S}O_2 \]
\[ \text{(8)} \]

where \( P_{S}O_2 \) is the oxygen tension in synovial fluid at each time period.

**Determination of blood flow by colored microspheres**

Blood flow to synovial tissue was determined at baseline after joint isolation, 240 and 330 minutes, using a colored microsphere technique. Blue, red or yellow microspheres were injected into the circuit arterial port and a reference blood sample was simultaneously collected from the medial digital artery reference sample port. Synovial tissue collected at the end of the experiment (15 mm x 10 mm) was dissected and blood flow to this sample was determined. Briefly, blood and synovial tissue were digested KOH and ethanol, filtered on 8 \( \mu \text{m} \) polystyrene filter. Dye was then released from the microspheres using dimethylmethylformamide, and the concentration of the dye, proportional to the concentration of microspheres, was determined by spectrophotometry, after standardization and correction for overlap of individual spectra. Blood flow was then calculated as follows:
\[ \text{Blood flow to tissue sample} = \frac{\text{AU of tissue sample X reference blood flow (ml/min)}}{\text{AU of tissue sample (\( \mu \text{L/min/g} \))}} \]
\[ \text{(8)} \]

Blood flow was expressed in \( \mu \text{L/min/g} \) of tissue.
Synovial membrane permeability to FITC-dextran (MW 144,000)

Synovial membrane permeability to dextran (MW 144,000) was measured by injection of 2 ml of a 0.5 mg/ml FITC-Dextran solution in normal saline, prepared before each experiment, and kept protected from light in an amber bottle. A circuit venous blood sample was obtained immediately before injection, every 2 minutes for 20 minutes, and every 5 minutes for another 10 minutes after injection. All vials were protected from light, and serum immediately separated, stored in covered vials and frozen until assay.

FITC-dextran concentrations were measured by spectrophotofluorometry with excitation/emission wavelengths of 485 nm and 530 nm respectively. All background values were subtracted for analysis. Normal horse serum was used to prepare standards.

Synovial membrane permeability to albumin

Synovial membrane permeability to albumin was determined by intraarticular injection of 2 ml of Evans blue-albumin (40 mg/ml of bovine serum albumin, MW 68,000), performed at 330 minutes, and allowed to absorb for 30 minutes. Evans blue-albumin was prepared by dissolving 0.6 g of Evans blue and 4 g of bovine serum albumin in 0.9% NaCl solution, a solution which results in negligible amounts of free dye. After euthanasia, sections of synovial membrane and underlying fibrous capsule (20 mm x 10 mm) were harvested from the dorso-medial aspect of the MCP joint, packed in OCT and snap frozen in liquid nitrogen and preserved at -70°C. Frozen sections (7μm) were prepared on poly-L-Lysine coated slides using a sliding microtome.

Amount and depth of Evans blue fluorescence of the synovial capsule (intima, subintima, and fibrous layer) was analyzed on a computer image acquired with a 5 watt argon laser and image analysis system at an excitation wavelength of 488 nm, and
collection of fluorescence with a 605 nm long pass filter. Scanning was performed at a microscope setting of 40x, with step sizes of 4μm, and an image size of 500 x 300 points. On each specimen, background tissue fluorescence was measured, and depth of dye penetration was recorded as the distance from the synovial intima to a point in the subsynovial tissue where fluorescence was equivalent to background. Five measurements, separated by a 20 μm distance, were obtained in the villous and fibrous synovial membrane respectively of each specimen, and fluorescence was reported as the mean of these 5 measurements for each region.

**Wet/dry ratio**

Synovial specimens (20 mm x 10 mm) were harvested from the dorsal aspect of the MCP joint, weighed and dried in an oven at 80° C for 48 hours and the ratio of wet to dry weight was calculated.

**Statistical analysis**

A three factor analysis of variance (model (innervated or denervated), treatment (control or inflamed) and time) with repeated measures (time or site) was used to test for differences among groups and across time in the means of systemic and circuit hemodynamics, systemic and circuit blood gas tensions, synovial fluid total protein, oxygen metabolism parameters, FITC-dextran permeability and depth of fluorescence of Evans blue albumin between sites. Assumptions of normality and homogeneity of variances were not met for synovial fluid production and for intraarticular pressure, so data were normalized for these parameters by log transformation (synovial fluid production) or square root transformation (intraarticular pressure) prior to analysis. A two factor analysis of variance (model, treatment) was used to test for differences in the means of synovial fluid production, intraarticular pressure at 4 hours, synovial membrane PS and wet-dry
ratio. For all parameters, if a significant F test was obtained, a post hoc Student Neuman Keuls multiple comparison test was used to detect individual differences among means. Significance for all tests was set at P≤0.05, and trends are reported when P≤ 0.15. Data are expressed as mean ± SE.

Results

Systemic hemodynamics

Systemic mean arterial pressure and PaCO₂ were maintained within stated limits of mean arterial pressure >70 mmHg and PaCO₂ < 55 mmHg (Table 5.1). There were no difference detected in systemic hemodynamics or blood gas analysis measurements among groups.

Local dynamics and fluid exchanges

Isogravimetric state was established for all joints for 20 minutes before the beginning of each experiment. Isogravimetric state was obtained at a circuit arterial flow of 14.29 ± 4.64 ml/min, circuit arterial pressure of 151.45 ± 2.42 mmHg, and circuit venous pressure of 10.12 ± 0.40 mmHg (Table 5.2). There was no difference among groups in baseline isogravimetric state.

From time 0 to 60 minutes (when circuit flow was not adjusted to control pressure), significant changes in arterial pressure or total vascular resistance were not detected over time. However there was a trend (P=0.12) for a progressive increase in arterial pressure in the inflamed innervated groups (groups 3) (Fig 5.2), and a trend for increased arterial (P=0.11) and total vascular resistance (P=0.10) in the innervated groups (Groups 1 and 3) (Fig 5.2).

During the remainder of the study period, circuit arterial blood flow significantly increased over time in all groups, from 14.29 ± 4.64 to 27.24 ± 8.06 ml/min (Table 5.2).
There was no difference in this parameter among groups. Similarly, total vascular resistance significantly decreased over time in all groups (P=0.009), and there was no difference among groups in this parameter (Fig 5.3). Blood flow to the synovial membrane was significantly increased (P=0.013) over baseline at 60 and 330 minutes in all groups (Fig 5.4). There was no difference in blood flow to the synovial membrane among groups.

Synovial fluid production was significantly increased in the inflamed groups (groups 3 and 4) compared to the control groups (groups 1 and 2) (24.81 ± 7.7 ml vs 5.6 ± .64 ml respectively, P=0.001), and there was no difference detected with denervation in this parameter (Fig 5.5). Intraarticular pressure was significantly greater at 240 minutes in the inflamed groups (groups 3 and 4) compared to control groups (groups 1 and 2) (6.46 ± 1.65 mm Hg vs 1.21 ± 0.41 mmHg, respectively, P=0.005), and there was no difference detected with denervation (Table 5.3).

Synovial fluid total protein significantly increased over time (P<0.0001). There was no difference detected in synovial fluid total protein among groups (Table 5.3). Synovial membrane permeability surface area product was significantly increased with inflammation (Groups 3 and 4) compared to control (Groups 1 and 2). Permeability surface area product was 0.00057 ± 0.0001 ml/min in control joints (Groups 1 and 2) and 0.0037 ± 0.00085 ml/min in inflamed joints (Groups 3 and 4) (P= 0.0002).

Parameters of oxygen metabolism

Oxygen delivery (DO$_2$) significantly increased (P < 0.0001) in all groups over time (Fig 4.5) and was not different among groups. Oxygen consumption (VO$_2$) significantly increased over time in all groups, however, there was a significantly greater increase (P=0.01) in oxygen consumption in inflamed joints (groups 3 and 4) (Fig 5.7). There a trend (P=0.10) for increased oxygen consumption in innervated joints (groups 1 and 3).
Oxygen extraction ratio (O$_2$ ER) was significantly increased (P = 0.04) for the first three hours in inflamed joints (groups 3 and 4) (Fig. 5.7). There was a trend (P=0.09) for increased extraction ratio in denervated joints (groups 2 and 4).

Synovial fluid PaO$_2$ and synovial fluid pH were significantly decreased in all groups at 240 minutes (P<0.005) and was not different among groups (Table 5.3). There was a significant effect of model, treatment and time on synovial fluid O$_2$ gradient. In control groups (Groups 1 and 2) O$_2$ gradient significantly decreased at 240 and 300 minutes with denervation (P= 0.0001). Inflammation significantly decreased synovial fluid O$_2$ gradient (P=0.01). In inflamed groups (Groups 3 and 4) O$_2$ gradient significantly increased with denervation (P=0.02).

**Wet/dry ratio**

Synovial tissue wet/dry ratio was significantly higher in the denervated group (groups 2 and 4) (P=0.009) (Table 5.4). Denervated joints (Groups 2 and 4) had approximately 7% more edema fluid than innervated joints (Groups 1 and 3).

**Permeability of the synovial membrane to albumin**

The preferential pathway of albumin absorption was localized at the synovial villi. Depth of penetration of Evans blue-albumin was significantly greater in inflamed joints (groups 3 and 4) (P=0.023), and was most marked in the villous area (P=0.0003) (Figure 5.8 and Table 5.5).

**FITC-dextran permeability**

There was a significant increase in FITC-Dextran concentration in the venous effluent of innervated joints (groups 1 and 3) (P=0.02). There was a trend (P=0.07) for
increased permeability in inflamed joints (groups 3 and 4) (Fig 5.9). Denervation decreased permeability to FITC-dextran by approximately 26%.

Discussion

The principal findings of this study are a significant effect of denervation to decrease synovial membrane permeability to large molecular weight dextrans, and to induce synovial membrane edema. Inflammation increased parameters of oxygen metabolism, synovial fluid production, and synovial membrane permeability to albumin, and increased synovial membrane permeability surface area product of proteins.

Our findings of increased permeability to a large molecule such as dextran (MW: 144,000) in innervated joints suggests a role of innervation in molecular selectivity of the blood-joint barrier.

The rate of appearance of dextran in venous blood is a function of the rate constant for fluid absorption from the joint and rate of removal from the interstitium of the synovial membrane into capillaries. Intraarticular hydrostatic pressure can significantly increase fluid absorption from the joint, and fluid absorption becomes a linear function at IAP greater than 9 mm Hg, a pressure that has been termed the breaking point or yield pressure. In the present study, IAP was returned to and maintained at atmospheric pressure after 240 minutes. It is possible that the increased IAP obtained at 240 minutes with inflammation caused the opening of interstitial channels that remained open after return to baseline. However, the greater effect of innervation to increase permeability of large molecular weight dextran necessitates an alternative explanation. Synovial capillaries are richly innervated with sympathetic as well as small C-fibers. Experimentally induced plasma extravasation by intra-articular infusion of bradykinin is decreased by approximately 60%
with sympathetic denervation of the joint. Furthermore, bradykinin-induced plasma extravasation is dependent on intact postganglionic sympathetic fibers. In our model, postganglionic fibers are intact in the joint capsule, such that the local vasoactive response mediated by postganglionic sympathetic fibers is preserved. In acute denervation, such as in that performed in this model, neuropeptides stored at nerve endings may still have been released in response to stimulation. Neuropeptides such as substance P and calcitonin gene related peptide (CGRP) are known to induce many of the changes occurring in acute inflammation, namely, vasodilation, increased vascular permeability, infiltration of leucocytes in venules, stimulation of phagocytosis, and mast cell degranulation. CGRP, but not substance P was shown to have a significant effect on articular vascular permeability. CGRP and substance P are collocated on small C-fiber nerve endings and can be released in the face of a stimulus such as interleukin-1. Chronic denervation by neonatal capsacin has no effect on experimentally induced plasma extravasation. Therefore sympathetic denervation provides an explanation of our findings of decreased FITC-dextran in denervated joints in venous outflow from denervated joints.

The influence of denervation to increase the tissue wet dry ratio, a measure of tissue edema, can also be explained by a decreased absorption of fluid from the interstitium. In our preparation, absorption of large molecules was decreased with denervation, which may also decrease convective flow of fluid, leading to tissue edema.

Intraarticular inflammation produced significant increases in oxygen metabolism of the joint unit. Oxygen delivery and extraction ratio provide an index of the efficiency with which tissues can extract oxygen for a given metabolic rate. In normal tissues at normal or high levels of delivery, oxygen consumption is constant and independent of delivery. If delivery decreases or metabolic demand increases, tissue extraction ratio increases. In the present study, when oxygen delivery was below 0.61 ml/min/g significant increase in
extraction ratio was required to meet the increased oxygen demand of inflamed joints. As delivery increased, extraction ratio subsequently returned to baseline and still met O₂ demand (Fig 5.7). Although intraarticular pressure was allowed to increase during the first four hours of the study, this increase did not affect the ability of the tissue to increase extraction ratio in response to demand. Furthermore, the resulting IAP of 6.46 ± 1.65 mm Hg observed at 4 hours in inflamed joints was well below the values of 20 mm Hg³ and 30 mmHg⁴ reported in previous studies to significantly decrease synovial membrane blood flow. However, in clinical situations, where equine metacarpophalangeal joint intraarticular pressure increases of greater than 30 mmHg have been reported,²⁰ decreased synovial membrane blood flow and maldistribution of flow within the joint may result in a defect in extraction efficiency, leading to oxygen debt and anaerobic metabolism.²¹ In our study, the absence of a difference in synovial fluid oxygen tension in inflamed compared to control joints is explained by the low and insufficient increase in IAP to induce such changes.²² Other mechanisms by which extraction efficiency may be affected include a decrease in capillary density due to capsular fibrosis in chronic arthritis, edema formation resulting in increased diffusion distance, perivascular cuffing caused by severe inflammatory changes, microthrombi formation, or in bacterial arthritis the direct effect of LPS to decrease cellular oxygen extraction efficiency. When extraction ratio increases in response to increased demand, a greater dependency on delivery exists. If demand continues to increase, for example if an inflamed joint is exercised, extraction ratio reaches a maximal value, and a critical level of oxygen delivery can be reached below which anaerobic metabolism is initiated, contributing to the pathology of the disease.

Synovial tissue blood flow values in our study in latter recumbency were greater than that previously reported for the equine dorsal MCP joint in dorsal recumbency. Baseline blood flow in control-innervated joints were 43 ± 17 μL/min/g whereas previously
reported values were $17 \pm 3 \, \mu l/min/g$. In the study with horses in dorsal recumbency, the fetlock joint located approximately 64 cm above the heart base. In our study, isolated limbs were placed horizontally, but more importantly flow to the limb was controlled to maintain physiologic arterial pressures.

Synovial fluid is formed by ultrafiltration of plasma in the fenestrated capillaries of the synovial membrane. Increases in arterial pressure and to a lesser degree venous pressure significantly increase synovial fluid production by increasing capillary hydrostatic pressure. In inflammatory processes, synovial fluid production is increased by increasing capillary permeability. In our study, the increased synovial fluid production was associated with an increase in articular pressure, however, as was previously reported, the relationship between increased volume and pressure is non-linear, such that predicting intraarticular volume from pressure is not possible unless that relationship is known.

The permeability surface area product of the blood joint barrier reflects the permeability of the capillary endothelium and overlying synovial intima, and the surface available for fluid exchanges. This parameter was used to calculate protein permeability rather than the osmotic reflection coefficient because the latter must be measured at flow independent states. To obtain flow independent states, marked increases in venous pressures are required, which would have produced confounding effects such as tissue edema. Because synovial fluid production, and thus IAP were increased in inflamed joints, we measured PS from 300 to 330 minutes, when IAP was at atmospheric pressure, as the value of PS is increased with increased IAP. Total protein concentration was measured in circuit arterial blood as an estimate of capillary total protein concentration, because we were interested in blood-to-joint protein flux. In addition, for large slowly exchanging molecules such as protein, arterial or mixed venous protein concentrations provide good estimates of capillary concentration. Our estimates of PS in control joints compare to those
previously calculated for the blood-joint barrier of the normal human knee.\textsuperscript{1} Inflamed joints had protein permeability that were raised approximately 5 times that of control joints. A similar observation has been reported for human rheumatoid knees,\textsuperscript{27} which also reported a greater increase in protein permeability with increased solute dimension.

Synovial membrane permeability to albumin was significantly increased in inflamed joints, and this increase was more significant in the synovial villi region of the joint. Synovial villi are more vascular and receive a more abundant blood flow than the fibrous synovial membrane.\textsuperscript{4} Increased albumin clearance has been previously reported in acute articular inflammation, but the effect of synovial membrane type or of innervation had not been examined. The present study illustrates the importance of synovial villi for synovial fluid turnover. Therapeutic synovectomy occasionally performed for the treatment of joint disease may alter clearance of metabolites and synovial fluid kinetics, and these potentially detrimental effects should be considered.

Experimental models of limb isolation demonstrate no significant difference in vascular reactivity between freshly amputated limbs and limbs stored for 12 hours,\textsuperscript{28} indicating that short term ischemia, such as occurred during surgical preparation of the model, would not contribute to alter vascular responsiveness. In denervated preparations, blood flow would no longer be under control of the sympathetic nervous system, but rather locally by vascular endothelium or metabolic substances. However the lack of effect of denervation to affect the progressive decrease in vascular resistance supports the preservation of vascular reactivity, and the relative importance of local vasomotor function rather than sympathetic innervation to control vascular responsiveness. These findings are in contrast with skeletal vascular responsiveness, where isolated preparations showed a decreased resistance in the immediate post-preparatory period, followed by a progressive increase over time.\textsuperscript{29} However, recordings without treatment were not performed after 75
minutes of equilibration, so it is unknown whether vascular resistance would then decrease again with time. These findings may also reflect a different response of skeletal vascular endothelium in comparison with digit vascular endothelium.

Even though attempts were made to minimize trauma during surgical manipulations, there was significant vascular reactivity which appeared to last approximately 2 hrs. Interestingly, but not unexpected, total vascular resistance of the isolated joint was higher in innervated preparation again illustrating the importance of innervation in vascular responsiveness.

In conclusion, we characterized the hemodynamic, metabolic, and fluid exchange responses of innervated and denervated joints and their response to inflammation. The altered permeability observed with denervation illustrates the importance of regional innervation in transsynovial exchanges, as was further corroborated by our findings of significant tissue edema. The significant increase in oxygen demand with inflammation confirms the importance of appropriate oxygen delivery to articular tissue during inflammation, and supports a role of ischemia in the pathogenesis of joint disease. Beneficial therapeutic intervention that can be gained in arthritis from such conclusions include improvement of oxygen delivery to the joint by promoting blood flow and minimizing edema formation, and decreasing demand by encouraging joint rest. The increased permeability to small molecules observed with inflammation has direct implications for local therapeutic intervention, and raises the question of extrapolation of pharmacokinetic studies performed in normal joints.
Footnotes

a Cardiomax II, Columbus Instruments, Columbus, OH.
b Honeywell VR12, Electronics for Medicine Inc, Pleasantville, NY
c Masterflex variable speed pump, Cole Palmer Int, Chicago, IL
d Model 7D Polygraph, Grass instruments, Quincy, MA
e Cellular Products Inc., Buffalo, NY
f Micro-BCA protein assay, Pierce Chemical Co, Rockford, IL
g ABL 500, Radiometer, Copenhagen, Denmark
h Coherent, Palo Alto, CA
i ACAS 570 Interactive laser cytometer, Meridian Instruments, Inc. Okemos, MI
j Cytofluor 2350 Fluorescence Measurement System; Astrascan Ltd., Isle of Man, British Isles.
Figure 5.1. Illustration of the isolated pump-perfused auto-oxygenated isolated joint model used in the study.
Figure 5.2. Circuit arterial pressure (A) and total vascular resistance (B) among groups and across time in isolated metacarpophalangeal joints from Baseline to 60 minutes. (Group 1 - ■ Control; Group 2 - ● Control-Denervated; Group 3 - ▲ Inflamed; Group 4- ❈ Inflamed-denervated). There was no significant difference among groups.
Figure 5.3 Total vascular resistance (TVR) of among groups and across time in isolated metacarpophalangeal joints. (Group 1 - ■ Control; Group 2-● Control-Denervated; Group 3- ▲ Inflamed; Group 4- ♦ Inflamed-denervated) There was a significant decrease in TVR over time in all groups (P=0.009), and there was no significant difference detected among groups.
Figure 5.4. Synovial membrane blood flow determined by colored microspheres among groups and across time in isolated metacarpophalangeal joints. (Group 1 - ■ Control; Group 2-● Control-Denervated; Group 3- ▲ Inflamed; Group 4- ♦ Inflamed-denervated)

*Synovial blood flow was significantly increased (P=0.013) at 60 and 330 minutes compared to baseline and there was no significant difference detected among groups.
Figure 5.4
Figure 5.5. Synovial fluid production among groups from 240 to 330 minutes in isolated metacarpophalangeal joints.* Indicates a significant difference between inflamed (groups 3 and 4) and control joints (Groups 1 and 2) (P=0.001).
Figure 5.5
Figure 5.6. Oxygen delivery among groups and across time in isolated metacarpophalangeal joints. (Group 1 - ■ Control; Group 2-● Control-Denervated; Group 3- ▲ Inflamed; Group 4- ♦ Inflamed-denervated) DO$_2$ significantly increased across time (P<0.0001). There was no significant difference detected among groups.
Figure 5.6
Figure 5.7. Oxygen consumption (VO$_2$) and extraction ratio (O$_2$ ER) among groups and across time in isolated metacarpophalangeal joints. (Group 1 - ■ Control; Group 2-● Control-Denervated; Group 3- ▲ Inflamed; Group 4- ♦ Inflamed-denervated) * Indicates a significant difference between control (Groups 1 and 2) and inflamed joints (Groups 3 and 4) for VO$_2$ (P=0.01) and O$_2$ ER (P=0.04).
Figure 5.8. Photomicrographs (20 x) of laser argon acquired images of evans-blue albumin fluorescence of synovial membrane among groups and at fibrous or villous sites, demonstrating depth of absorption. Mean depth of absorption in the villous region for inflamed groups (groups 2 and 4) was 964.85 ± 141.55 μm.
CONTROL

INFLAMED

FIBROUS

VILLOUS
Figure 5.9. FITC-dextran concentration in circuit plasma effluent among groups and across time in isolated metacarpophalangeal joints from 330 to 360 minutes. (Group 1 - ■ Control; Group 2-● Control-Denervated; Group 3- ▲ Inflamed; Group 4- ♦ Inflamed-denervated) * Indicates a significant difference between control (Groups 1 and 2) and inflamed joints (Groups 3 and 4) (P=0.02).
Figure 5.9
Table 5.1 Systemic hemodynamics and blood gas analysis (mean ± SE) among groups in horses submitted to isolated joint preparation.

* indicates a significant difference from baseline.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>60 minutes</th>
<th>120 minutes</th>
<th>180 minutes</th>
<th>240 minutes</th>
<th>300 minutes</th>
<th>330 minutes</th>
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<td><strong>Cardiac output (L/min/kg)</strong></td>
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<tr>
<td>Group 1 (Control-Innervated)</td>
<td>0.103 ± 0.008</td>
<td>0.091 ± 0.008</td>
<td>0.086 ± 0.007</td>
<td>0.084 ± 0.009</td>
<td>0.082 ± 0.009</td>
<td>0.079 ± 0.011</td>
<td>0.081 ± 0.011</td>
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<tr>
<td>Group 2 (Control-Denervated)</td>
<td>0.110 ± 0.02</td>
<td>0.1 ± 0.02</td>
<td>0.087 ± 0.01</td>
<td>0.087±0.014</td>
<td>0.083±0.016</td>
<td>0.083±0.013</td>
<td>0.084±0.013</td>
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<tr>
<td>Group 3 (Inflamed-Innervated)</td>
<td>0.100±0.009</td>
<td>0.093±0.005</td>
<td>0.093±0.007</td>
<td>0.086±0.006</td>
<td>0.091±0.008</td>
<td>0.083±0.004</td>
<td>0.078±0.004</td>
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<tr>
<td>Group 4 (Inflamed-Denervated)</td>
<td>0.098±0.016</td>
<td>0.057±0.012</td>
<td>0.057±0.011</td>
<td>0.065±0.015</td>
<td>0.060±0.013</td>
<td>0.057±0.013</td>
<td>0.056±0.014</td>
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<tr>
<td>Overall</td>
<td>0.094 ± 0.007</td>
<td>0.084 ± 0.006</td>
<td>0.080 ± 0.005*</td>
<td>0.080 ± 0.006*</td>
<td>0.078 ± 0.008*</td>
<td>0.074 ± 0.008*</td>
<td>0.074 ± 0.008*</td>
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<td>Group 1 (Control-Innervated)</td>
<td>120.7 ± 10.7</td>
<td>123.8 ± 11.5</td>
<td>127 ± 6.1</td>
<td>128.5 ± 7.7</td>
<td>124.3 ± 7.6</td>
<td>120.3 ± 9.5</td>
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<td>98.7 ± 14.9</td>
<td>117 ± 8.1</td>
<td>116.7 ± 11.2</td>
<td>115.5 ± 12.5</td>
<td>108.7 ± 10.4</td>
<td>111 ± 11.8</td>
<td>113 ± 12.1</td>
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<td>Group 3 (Inflamed-Innervated)</td>
<td>114.0 ± 9.3</td>
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<td>119.3 ± 4.2</td>
<td>117.5 ± 3.7</td>
<td>111.8 ± 5.5</td>
<td>111.2 ± 4.7</td>
<td>110.1 ± 4.9</td>
<td>108.8 ± 5.5</td>
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<td>153 ± 11.2</td>
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<td>137.8 ± 15.8</td>
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<td>144.6 ± 14.5</td>
<td>138.5 ± 15.9</td>
<td>141.3 ± 15.9</td>
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<td>132.6 ± 8.8</td>
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<td>128.9 ± 11.4</td>
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<td>120.8 ± 15.1</td>
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<td>Overall</td>
<td>143.4 ± 8.1</td>
<td>149.9 ± 5.2</td>
<td>144.5 ± 7.3</td>
<td>136.0 ± 8.2</td>
<td>141.0 ± 5.8</td>
<td>138.5 ± 5.9</td>
<td>133.7 ± 7.2</td>
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<td><strong>Systemic PaO2 (mmHg)</strong></td>
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<tr>
<td>Group 1 (Control-Innervated)</td>
<td>311.1 ± 31.1</td>
<td>291.4 ± 37.5</td>
<td>284.9 ± 40.7</td>
<td>276.2 ± 42.3</td>
<td>216.8 ± 38.2</td>
<td>294.2 ± 44.4</td>
<td>305.7 ± 38.8</td>
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<td>190.4 ± 25.6</td>
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<td>149.5 ± 32.2</td>
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<td>Group 3 (Inflamed-Innervated)</td>
<td>169.6 ± 38.3</td>
<td>146.9 ± 35.5</td>
<td>148.9 ± 35.7</td>
<td>150.6 ± 33.3</td>
<td>198.9 ± 42.3</td>
<td>180.1 ± 52.5</td>
<td>181.7 ± 35.6</td>
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<td>Group 4 (Inflamed-Denervated)</td>
<td>228.9 ± 41.0</td>
<td>191.7 ± 45.1</td>
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<td>211.1 ± 41.4</td>
<td>196.6 ± 48.9</td>
<td>250.8 ± 27.9</td>
<td>263.8 ± 38.5</td>
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<td>212.9 ± 21.2</td>
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<td>44.5 ± 1.4</td>
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<td>Group 3 (Inflamed-Innervated)</td>
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<td>Group 4 (Inflamed-Denervated)</td>
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<td>48.4 ± 4.8</td>
<td>42.5 ± 2.2</td>
<td>41.4 ± 2.2</td>
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<td><strong>Circuit arterial flow (ml/min)</strong></td>
<td><strong>Circuit arterial pressure (mmHg)</strong></td>
<td><strong>Circuit venous pressure (mmHg)</strong></td>
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<td><strong>Baseline</strong></td>
<td><strong>60 minutes</strong></td>
<td><strong>120 minutes</strong></td>
<td><strong>180 minutes</strong></td>
<td><strong>240 minutes</strong></td>
<td><strong>300 minutes</strong></td>
<td><strong>330 minutes</strong></td>
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<tr>
<td><strong>Overall</strong></td>
<td>14.29 ± 4.64</td>
<td>15.88 ± 5.38</td>
<td>17.17 ± 5.05</td>
<td>18.42 ± 5.96</td>
<td>23.77 ± 6.89</td>
<td>23.15 ± 6.65</td>
<td>27.24 ± 8.06</td>
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<td><strong>Group 1</strong></td>
<td>13.88 ± 7.86</td>
<td>13.05 ± 8.62</td>
<td>15.67 ± 8.42</td>
<td>16.92 ± 8.86</td>
<td>22.05 ± 8.39</td>
<td>16.50 ± 8.23</td>
<td>21.25 ± 8.73</td>
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<td><strong>Group 2</strong></td>
<td>12.42 ± 2.98</td>
<td>13.92 ± 3.87</td>
<td>15.17 ± 4.52</td>
<td>15.17 ± 6.99</td>
<td>18.67 ± 4.49</td>
<td>22.67 ± 5.96</td>
<td>25.67 ± 6.07</td>
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<td><strong>Group 3</strong></td>
<td>13.00 ± 2.36</td>
<td>15.42 ± 2.83</td>
<td>14.92 ± 4.52</td>
<td>16.67 ± 5.99</td>
<td>27.24 ± 6.65</td>
<td>17.67 ± 4.49</td>
<td>23.15 ± 7.73</td>
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<td><strong>Group 4</strong></td>
<td>17.26 ± 2.94</td>
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<td>13.88 ± 4.62</td>
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<td><strong>120 minutes</strong></td>
<td><strong>180 minutes</strong></td>
<td><strong>240 minutes</strong></td>
<td><strong>300 minutes</strong></td>
<td><strong>330 minutes</strong></td>
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<tr>
<td><strong>Overall</strong></td>
<td>151.45 ± 2.42</td>
<td>148.23 ± 7.03</td>
<td>150.74 ± 4.35</td>
<td>150.20 ± 3.48</td>
<td>152.90 ± 4.88</td>
<td>150.95 ± 3.79</td>
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<td>149.00 ± 2.73</td>
<td>148.67 ± 2.92</td>
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Table 5.2 Local hemodynamics (mean ± SE) among groups and across time in isolated joints.

* indicates a significant difference from baseline.
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<th>Time</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
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<td>Mean SE</td>
<td>Mean SE</td>
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<td>3.37 0.37</td>
<td>3.14 0.47</td>
<td>3.09* 0.35</td>
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<td>3.03 0.42</td>
<td>3.20 0.28</td>
<td>3.27 0.52</td>
<td>3.49 0.47</td>
<td>3.25* 0.42</td>
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<td>330 minutes</td>
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<td>3.37 0.38</td>
<td>3.28 0.47</td>
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**Synovial fluid pH**

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<td>7.32 0.04</td>
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**Synovial fluid PaO2 (mmHg)**

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<th>Group 3</th>
<th>Group 4</th>
<th>Overall</th>
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<td>99.50 10.00</td>
<td>130.60 15.00</td>
<td>114.45 3.35</td>
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<td>240 minutes</td>
<td>46.27 5.53</td>
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<td>51.90 19.60</td>
<td>95.47 15.50</td>
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<td>300 minutes</td>
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<td>129.20 41.90</td>
<td>82.45 5.85</td>
<td>89.24 20.55</td>
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<td>151.20 0.00</td>
<td>73.80 0.00</td>
<td>23.30 0.00</td>
<td>156.97 88.47</td>
<td>103.82 22.12</td>
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<tr>
<td>Mean</td>
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<td>76.18 11.41</td>
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**Synovial fluid PaCO2 (mmHg)**

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<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Overall</th>
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<tbody>
<tr>
<td></td>
<td>Mean SE</td>
<td>Mean SE</td>
<td>Mean SE</td>
<td>Mean SE</td>
<td>Mean SE</td>
</tr>
<tr>
<td>Baseline</td>
<td>48.85 0.63</td>
<td>48.71 2.88</td>
<td>48.60 0.36</td>
<td>47.02 1.22</td>
<td>48.30 1.27</td>
</tr>
<tr>
<td>240 minutes</td>
<td>66.17 1.17</td>
<td>71.34 7.08</td>
<td>66.77 3.70</td>
<td>59.31 2.82</td>
<td>65.90* 3.69</td>
</tr>
<tr>
<td>300 minutes</td>
<td>52.78 0.80</td>
<td>53.55 3.77</td>
<td>51.97 1.19</td>
<td>54.94 1.42</td>
<td>53.31 1.74</td>
</tr>
<tr>
<td>330 minutes</td>
<td>45.45 1.25</td>
<td>40.36 6.12</td>
<td>51.17 4.47</td>
<td>49.39 5.29</td>
<td>46.59 4.78</td>
</tr>
<tr>
<td>Mean</td>
<td>56.30 1.17</td>
<td>59.00 4.37</td>
<td>54.00 1.97</td>
<td>54.30 1.42</td>
<td>54.00 1.74</td>
</tr>
</tbody>
</table>

**Synovial fluid O2 gradient (mmHg)**

<table>
<thead>
<tr>
<th>Time</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean SE</td>
<td>Mean SE</td>
<td>Mean SE</td>
<td>Mean SE</td>
<td>Mean SE</td>
</tr>
<tr>
<td>Baseline</td>
<td>195.95 32.35</td>
<td>151.10 14.29</td>
<td>73.87* 33.42</td>
<td>129.14*¥ 35.41</td>
<td>173.53 28.87</td>
</tr>
<tr>
<td>240 minutes</td>
<td>226.67 42.72</td>
<td>127.28 24.02</td>
<td>82.25* 41.34</td>
<td>121.08*¥ 28.23</td>
<td>176.28 36.58</td>
</tr>
<tr>
<td>300 minutes</td>
<td>205.27 43.02</td>
<td>59.93* 23.14</td>
<td>79.77* 48.32</td>
<td>164.68*¥ 22.49</td>
<td>205.27 34.24</td>
</tr>
<tr>
<td>330 minutes</td>
<td>177.00 40.39</td>
<td>52.55* 23.63</td>
<td>71.32* 29.80</td>
<td>124.37*¥ 35.48</td>
<td>177.00 32.32</td>
</tr>
<tr>
<td>Mean</td>
<td>190.50 31.95</td>
<td>112.20 13.92</td>
<td>83.87* 33.42</td>
<td>129.14*¥ 35.41</td>
<td>173.53 28.87</td>
</tr>
</tbody>
</table>

**Permeability surface area product (ml/mln)**

<table>
<thead>
<tr>
<th>Time</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean SE</td>
<td>Mean SE</td>
<td>Mean SE</td>
<td>Mean SE</td>
<td>Mean SE</td>
</tr>
<tr>
<td>330-330 minutes</td>
<td>0.00057 0.00014</td>
<td>0.00036 0.00006</td>
<td>0.0039 0.0015</td>
<td>0.0037 0.0003</td>
<td>0.0021 0.0005</td>
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</table>

**Intraarticular pressure (mmHg)**

<table>
<thead>
<tr>
<th>Time</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean SE</td>
<td>Mean SE</td>
<td>Mean SE</td>
<td>Mean SE</td>
<td>Mean SE</td>
</tr>
<tr>
<td>240 minutes</td>
<td>1.08 a 0.27</td>
<td>1.33 a 0.80</td>
<td>7.00 b 3.17</td>
<td>6.00 b 1.70</td>
<td>3.85 1.49</td>
</tr>
</tbody>
</table>

Table 5.3 Synovial fluid total protein, pH, PaO2, PaCO2, O2 gradient, permeability surface area product and intraarticular pressure among groups and across time in isolated joints

* indicates a significant difference from baseline
¥ indicates a significant difference between group 3 and group 4 for that time period

different letters are significantly different
<table>
<thead>
<tr>
<th>Wet/dry ratio</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Control-Innervated)</td>
<td>1.02 ± 0.03</td>
</tr>
<tr>
<td>Group 2 (Control-Denervated)</td>
<td>1.11 ± 0.0†</td>
</tr>
<tr>
<td>Group 3 (Inflamed-Innervated)</td>
<td>1.06 ± 0.02</td>
</tr>
<tr>
<td>Group 4 (Inflamed-Denervated)</td>
<td>1.12 ± 0.03†</td>
</tr>
</tbody>
</table>

Table 5.4 Synovial membrane wet/dry ratio among groups in isolated joints. † indicates a significant difference between innervated (Groups 1 and 3) and denervated (Groups 2 and 4) joints. (P=0.009)
Table 5.5 Depth of Evans blue-albumin fluorescence (µm) among groups in isolated equine joints
*indicates a significant difference between villous and fibrous synovium (P=0.000)
¥ indicates a significant difference between inflamed (groups 3 and 4) and control (groups 1 and 2) joints (P=0.023).

<table>
<thead>
<tr>
<th>Depth of fluorescence</th>
<th>Fibrous synovium</th>
<th>Villous synovium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Control-Innervated)</td>
<td>493.3 ± 83.5</td>
<td>685.7 ± 96.7*</td>
</tr>
<tr>
<td>Group 2 (Control-Denervated)</td>
<td>615.6 ± 229.5</td>
<td>774.0 ± 143.1*</td>
</tr>
<tr>
<td>Group 3 (Inflamed-Innervated)</td>
<td>489.0 ± 113.2</td>
<td>1075.0 ± 112.7*¥</td>
</tr>
<tr>
<td>Group 4 (Inflamed-Denervated)</td>
<td>433.7 ± 1010</td>
<td>854.7 ± 170.4*¥</td>
</tr>
</tbody>
</table>
References


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CHAPTER 6

CELL TRAFFICKING, MEDIATOR RELEASE AND ARTICULAR Cartilage Metabolism in Acute Arthritis of Innervated or Denervated Isolated Joints

Introduction

Acute articular inflammation is clinically recognized by joint effusion, periarticular edema, local heat and pain, lameness of the affected limb, and decreased range of motion of the joint.¹ Routine synovial fluid analysis in acute articular inflammation reveals variable degrees of neutrophilic leukocytosis, increased protein concentration, and decreased mucin precipitate quality.¹ Synovitis accompanies most forms of joint disease, and predominates in acute articular inflammation.² Inflamed synovial cells can release mediators such as interleukin-1 and prostaglandin E₂ (PGE₂)³ which in turn activate adjacent synoviocytes,⁴ and alter the balance of chondrocyte matrix synthesis and degradation towards a degradative pathway.⁵

The investigation of acute articular injury is important because single articular trauma can lead to progressive degenerative joint disease if not identified or treated appropriately. Therapeutic efforts may be more successful to restore joint health in acute injury, if gross changes have not occurred. Several models of acute articular inflammation have been developed in equids, including intra-articular injection of endotoxin,⁶ carrageenan,⁷ amphotericin B,⁸ and bacteria.⁹ These models provide important clinical and clinicopathologic information that allowed the investigation and comparison of therapeutic
modalities, however, the acute dynamic events including cellular migration and mediator release occurring in acute inflammation were not documented.

Recent advances in joint disease emphasized an important role of innervation in modulating articular inflammation.\textsuperscript{10-12} Clinical and experimental data suggest that a decreased inflammatory response occurs in denervated joints. Antidromic release of neuropeptides, particularly substance P, from small C fibers, has been shown to occur in articular inflammation,\textsuperscript{13} and neuropeptide depletion has been associated with improvement in clinical signs of disease.\textsuperscript{14} Increased substance P concentrations have been measured in synovial fluid of horses with joint disorders,\textsuperscript{15} and substance P staining nerve fibers have been identified in equine synovial membrane.\textsuperscript{15-17} In vitro studies have indicated a synergistic effect of substance P and interleukin-1 to degrade articular cartilage.\textsuperscript{18} Despite this information, no experimental or clinical information is available on the effect of denervation to modulate articular inflammation in the horse. Investigations of the early events in articular inflammation, and the possible role of neural modulation needed to be performed in an appropriate model.

An isolated joint preparation model for the study of joint physiology,\textsuperscript{19} has been used to characterize the permeability, fluid exchange and oxygen metabolism in acutely inflamed, innervated or denervated isolated joints.\textsuperscript{20} We documented increased permeability, oxygen metabolism and oxygen debt with acute inflammation, and an effect of denervation to decrease articular permeability to large molecular weight molecules and increase edema formation.

The purpose of the present study is to describe the cellular events (synovial and chondrocytic), cytokine, eicosanoid and neuropeptide release in acute inflammation of innervated or denervated isolated joints, and associate these events with the physiologic alterations of innervated or denervated joints.
Materials and methods

Horses
Twenty-four adult horses (body weight 330-550 kg, 8 females, 8 geldings, 8 stallions) of mixed breeds were used. Horses were determined free of metacarpophalangeal joint disease by thorough clinical examination, synovial fluid analysis, and gross post-mortem examination. All experiments were conducted in accordance with the Animal Care and Use Guidelines of The Ohio State University.

Experimental procedures

Instrumentation

Horses were instrumented for hemodynamic measurements and intravenous drug administration prior to induction of general anesthesia as previously described (see Chapter 5).

Anesthesia and monitoring

Only one horse was studied on a given day. Horses were sedated and anesthesia was induced as previously described (see chapter 5). Anesthesia was maintained with sodium pentobarbital (loading dose 4 mg/kg, maintenance 5-15 mg/kg/hour, iv, to effect) and controlled ventilation with 100% oxygen. A bipolar ECG (base-apex lead) was recorded throughout each study for determination of heart rate and rhythm. Arterial pressure and ECG were continuously displayed and semicontinuously recorded on a multichannel recorder. Systemic arterial blood gases, packed cell volume and total protein were determined hourly. Intravenous polyionic isotonic fluids were administered (5-10 ml/kg/hr) throughout each study. Mean systemic arterial pressure was maintained at ≥ 70
mm Hg, and ventilation was adjusted to maintain PCO$_2$ ≤ 55 mmHg. Heparin (50,000 IU iv) was administered every 90 minutes.

**Joint isolation procedure and instrumentation (Fig. 6.1)**

One metacarpophalangeal joint was randomly selected and the horse was positioned with the selected limb lowermost. All circuit tubing for circulation through precalibrated pumps was thoroughly flushed with heparinized saline. The MCP was isolated as previously described (see Chapter 5). In innervated preparations, the medial and lateral palmar nerves were isolated and preserved. The isolated joint was suspended from a calibrated FT03 transducer connected to a physiograph for continuous measurement of weight changes. All circuit pressure transducers were placed at the level of the joint preparation. Initial circuit arterial flow was set at 10 ml/min until completion of the preparation and establishment of isogravimetric state (see below). This joint isolation technique established a pressure-driven auto-oxygenated, pump-perfused, extracorporeal isolated joint model.

**Experimental protocol**

The joint preparation model was randomly assigned to one of four groups: 1-Control; 2) Control-denervated; 3)Inflamed; 4)Inflamed-denervated. In groups 3 and 4, inflammation was induced by intraarticular injection of 0.35 ng/kg BW of recombinant human interleukin-1β (IL-1β). After complete joint isolation and instrumentation, isogravimetric state (no gain or loss of weight in the isolated preparation) was established and maintained for 20 minutes by adjustment of arterial flow. In all groups, the preparation was maintained for 360 minutes, after which horses were euthanized with an overdose of pentobarbital (100 mg/kg, iv), and specimens collected. Baseline (time 0) was considered
as the time after establishment of isogravimetric state, collection of the baseline synovial fluid sample, and before injection of IL-1β in Groups 3 and 4.

**Blood sample collection**

Circuit arterial and venous blood were collected at baseline and hourly thereafter, in heparinized syringes, and transported on ice for blood gas determination, and measurement of packed cell volume and total protein (refractometer).

**Synovial fluid collection and analysis**

Synovial fluid was collected at time 0, and at 240, 300 and 330 minutes. Synovial fluid volume was recorded from 240 to 300 minutes and from 300 to 330 minutes by collection into a graduated cylinder. Fluid for clinical pathological analysis was collected into 2 ml evacuated tubes containing 1.5 mg of K₃EDTA/ml. The total number of nucleated cells was determined by use of an automated hematology analyzers. Differential leukocyte counts were made by counting 100 cells on direct smears that were air dried and stained with a Wright-Giemsa stain. Synovial fluid samples for the analysis of the cytokines IL-1β, and interleukin 6 (IL-6) were collected in Eppendorf tubes and centrifuged, and the cell-free supernatant was stored at -20°C for 24 hrs then at -80°C until assay. Cytokine assays were performed using commercially available ELISA kits. These assays employ the quantitative ‘sandwich’ enzyme immunoassay technique, using monoclonal antibodies raised against human cytokines, as well as human recombinant cytokines as standards. Sensitivity of the assays were 1.3 pg/ml and 3.2 pg/ml for IL-1β, and IL-6, respectively. Synovial fluid for the PGE₂ assay was collected in chilled Eppendorf tubes containing 25 µl of 100 mM Na₂EDTA and 25 µl of 10 mM meclofenamic acid. Tubes were centrifuged for 10 minutes, and the cell-free supernatant was collected and stored at
-20°C for 24 hours, then at -80°C until assayed. PGE₂ concentration was determined by use of commercially available PGE₂ EIA kits. Absorbance was read at 405 nm by use of a microplate reader. Absorbance was correlated with concentration by use of a standard curve ranging from 10 to 5000 pg/ml. Samples with >5000 pg/ml PGE₂ concentrations were reanalyzed after dilution. Synovial fluid for substance P determination was collected in chilled Eppendorf tubes and centrifuged at -4°C. The cell-free supernatant was collected, placed in Eppendorf tubes, immediately frozen in liquid nitrogen and stored at -80°C until assay. Substance P concentrations were quantified in duplicate, using standard radioimmunoassay techniques. The primary antibody was used as a dilution of 1:10,000. Standard curves were constructed using 1²⁵I-radiolabelled ligand/100µl (5,000 counts per min) and increasing concentrations of unlabeled standard ligands for the SP assays.

Concentration ratios for II18 and II6 were calculated using group 1 as the reference group to assess relative differences. PGE₂ results for each time within each group are expressed as a ratio to baseline values because marked variations in baseline values existed, as previously reported. Interleukin-1β, interleukin-6 and substance P results are expressed as concentrations (pg/ml), content (pg) and ratio. Content was calculated by multiplying the average of concentrations at 240 and 300 minutes and 300 and 330 minutes by volume of synovial fluid collected between 240 and 300 minutes, and 300 and 330 minutes. Total content for these 90 minutes was obtained by adding these values.

Synovial membrane and cartilage collection

The dorsal aspect of the isolated MCP joint and the contralateral MCP joint were aseptically prepared. FITC-dextran and Evans blue albumin were injected into the isolated and contralateral joint 30 minutes before euthanasia. After euthanasia, the preparation was disassembled and weighed, to expose articular cartilage to the same treatments as that in the
isolated joint (see chapter 5). Using aseptic technique, the dorsal skin and joint capsule were incised and the joint cavity was exposed. The joint was carefully inspected for evidence of cartilage erosions, synovial membrane proliferation or presence of osteophyte. The presence of gross abnormalities excluded the preparation from the study.

**Synovial membrane collection:** Three synovial membrane and underlying joint capsule specimens (20 mm x 10 mm each) were collected from both joints and placed on a wooden support to maintain shape. One specimen was placed in fixative (4ml 25% glutaraldehyde, 25 ml 0.2M sodium phosphate buffer, 21 ml water, 1.7 g sucrose) for 24 hours, followed by storage in 70% alcohol, for histopathology and substance P immunostaining. These specimens were processed and stained within 2 weeks of collection. One specimen was packed in OCT, frozen in liquid nitrogen and stored at -70°C. This specimen was processed for fluorescence studies (see chapter 5). One specimen was used for determination of wet/dry ratio and microsphere determination (see chapter 5).

**Cartilage collection** Full-thickness cartilage specimens were obtained from the medial aspect of the sagittal ridge of each distal metacarpi using a 2 mm diameter bone biopsy trocar. Care was taken to collect cartilage from the non-weight bearing aspect of the joint surface, and to limit collection to within 0.5 cm of the cartilage edges. Cartilage specimens from each joint were placed in sterile DMEM medium and transported on ice to the laboratory. Under a laminar flow hood, approximately 100 mg of pooled cartilage from each joint were aliquoted to each of 4 wells of a six well plate: Well 1-18 hour $^{35}$S incorporation; control joint; Well 2- 18 hour $^{35}$S incorporation; isolated joint; Well 3- Cartilage degradation study; control joint; Well 4- Cartilage degradation study; isolated joint.
Cartilage proteoglycan synthesis and degradation

Cartilage synthesis

One well from each joint was used for synthesis studies. In each well, 2.5 ml of standard medium (DMEM with 10% fetal calf serum, 100 μg/ml penicillin and 100 μg/ml gentamicin) and 40 μCi of $^{35}$S were added. Cartilage specimens were incubated for 18 hours at 37 °C in 100% humidity and 5% CO$_2$. After 18 hours, cartilage specimens were placed in a separate vial and extracted for 48 hours at 4°C with 4 ml guanidine-HCl in 0.1 M Na-acetate (pH 5.8) containing protease inhibitors (100 mM 6-aminohexanoic acid, 10 mM Na$_2$EDTA, 5 mM benzamidine). Extracted samples were centrifuged for 5 minutes at 4°C to remove unextractable material and dialyzed against deionized water at 4°C for 24 hours (membrane cut-off: 6,000-8,000) to remove unincorporated radiosulfate and to allow for reaggregation of the proteoglycan with endogenous hyaluronan. An aliquot (100 μl) of each extract was counted on a scintillation counter, and samples were stored at -70°C until assay for proteoglycan and glycosaminoglycan concentrations. Total protein in each sample was measured by the bicinchoninic acid assay kit, with bovine serum albumin as standard. Proteoglycan synthesis was expressed as CPM/μg protein. Endogenous proteoglycan concentration was measured by determination of uronic acid concentration determined on cartilage extracts by the carbazole-borosulfuric acid method using glucuronolactone as standard. Proteoglycan concentration is expressed in μg/μg protein. Total glycosaminoglycan concentration was measured by dimethylmethylene blue assay followed by sequential digestion with chondroitinase ABC and keratanase, to measure chondroitin sulfate and keratan sulfate content, respectively. Chondroitin sulfate was used to construct standard curves. Glycosaminoglycan concentrations are expressed in μg/μg protein.
Cartilage degradation

One well from each joint was used for degradation studies. In each well, 2.5 ml of standard medium (DMEM with 10% fetal calf serum, 100 µg/ml penicillin and 100 µg/ml gentamicin) and 40 µCi of $^{35}$S were added. Cartilage specimens were incubated for 72 hours at 37 °C in 100% humidity and 5% CO2. After 72 hours, half of each cartilage specimen was collected, placed in a separate vial, and extracted as described above. The remainder of the cartilage specimens were rinsed three times in PBS and further incubated in standard medium without radioactive material. Medium was collected and exchanged every 24 hours for 72 hours. After 72 hours (144 hours total incubation), cartilage was placed in a separate vial and extracted as described above. All cartilage and culture medium were dialyzed against water to remove unbound sulfur. All dialysis volumes were recorded. Scintigraphic counts were obtained on aliquots of cartilage extracts obtained at 72 and 144 hours, and on daily collected culture medium. Proteoglycan concentrations in daily collected media were determined using the carbazole-borosulfuric acid method (see above). Newly synthesized proteoglycan release in media are expressed as CPM for each time, and as a ratio of experimental to control joints. Endogenous proteoglycan released in media are expressed as µg, and as a ratio of experimental to control joints. The percentage of total proteoglycan (µg) and newly labelled proteoglycan (CPM) released from the cartilage explants into culture media was expressed as follows:

$$\% \text{ release at time } X = \frac{\mu\text{g or CPM of PG in medium at time } X}{\text{sum (µg or CPM) of PG in all collected media} + \mu\text{g or CPM of PG in cartilage extract at 144 hrs}} \times 100$$

Each value was expressed as a percentage and as a ratio to the contralateral limb.

Substance P and Nerve Specific Enolase (NSE) immunostaining
Synovial membrane specimens were fixed, paraffin embedded sectioned and processed for immunostaining as follows. After deparaffinization in xylene, specimens were rehydrated in decreasing concentrations of ethanol, followed by 3% peroxide to block endogenous peroxidase. Specimens were then coated with normal goat serum to block non-specific binding, followed by coating with the primary antibody (rabbit anti-substance Pm or rabbit anti-NSE) and incubated for 1 hour in a humidified chamber to prevent specimen drying. After rinsing with PBS, the secondary antibody (anti-rabbit biotinylated) was added and incubated for 20 minutes. After rinsing with PBS the tertiary antibody (streptavidin-peroxidase complex) was added to bind to the secondary antibody and incubated for 20 minutes. The specimens were then coated with freshly prepared diamonobenzidine-peroxide substrate for reaction with the tertiary antibody to produce the brown color. Specimens were dehydrated in increasing concentrations of ethanol, and coverslip mounted.

For each specimen, a blank was prepared where the rabbit anti-neuropeptide was omitted. Slides were examined under oil immersion and 10 adjacent high powered fields were scrutinized in the fibrous and villous synovium specimens. The number of positive staining nerve filament per field was recorded. The location (perivascular or interstitium) was also recorded.

**Histopathology scores**

Synovial membrane specimens collected from the dorsal aspect of the experimental and contralateral control metacarpophalangeal joints were fixed, paraffin embedded, sectioned and stained with hematoxylin and eosin for histopathologic examination. Specimens were evaluated at 20X and 100X (oil immersion) by three investigators blinded as to the specimen examined. Semi-quantitative morphology scores were assigned on villous and fibrous sections of synovial membrane for extent of edema, hemorrhage,
neutrophilic vasculitis, distribution of infiltrate and morphology of the intima in both the villous and the fibrous regions of the synovial membrane. Scores were assigned for each category (see table 6.1). A interobserver mean score was obtained for each category and each site.

Statistical analysis
A three factor analysis of variance (model (innervated or denervated), treatment (control or inflamed) and time) with repeated measures (time) was used to test for differences among groups and across time of synovial fluid white blood cell count, white blood cell differential, IL-1β, IL-6 and substance P concentrations, content and ratios, cartilage extracts and culture medium scintillation counts, hexuronic acid concentration, cartilage extract glycosaminoglycan values and cartilage degradation ratios. Cartilage extract values were obtained for incubations at 18, 72 and 144 hours. Degradation values were obtained at 24, 48 and 72 hours. For all cartilage and culture medium analysis, age of the animal was entered in the analysis as a covariate. Histopathology scores were compared with a three way analysis of variance comparing the factors model, treatment and joint (experimental or contralateral control). Synovial fluid PGE2 concentrations were expressed as a ratio of baseline samples. Natural logarithmic and arcsine transformations were used to stabilize variances of data for white blood cell total count and differential, respectively, before analysis. For all analysis, if a significant F test was obtained, a post-hoc Neuman-Keuls multiple comparison test was used to detect difference in means among groups. A significance level of P≤0.05 was assigned for all tests. A coefficient of repeatability among observers was calculated for histopathology scores by doubling the standard deviation of the differences between scores and mean.
Results

Systemic hemodynamics

Systemic mean arterial pressure and \( \text{PaCO}_2 \) were maintained within stated limits of mean arterial pressure > 70 mmHg and \( \text{PaCO}_2 < 55 \) mmHg (See chapter 5). There were no difference detected in systemic hemodynamics or blood gas analysis measurements among groups.

Synovial fluid analysis

All baseline synovial fluid WBC and nucleated cell differential counts were within accepted normal range\(^2\) (total nucleated count < 1.0 \( \times \) 10\(^3\) cells/\( \mu l \), neutrophils < 10%). Synovial fluid WBC in groups 1 and 2 significantly increased across time over baseline, with a peak mean WBC of 2.3 \( \times \) 10\(^3\) cells/\( \mu l \) at 240 minutes. IL\( \beta \)-treated isolated joints (groups 3 and 4) had significantly greater synovial fluid WBC than control isolated joints (groups 1 and 2) (Fig 6.2) (\( P=0.001 \)). Mean synovial fluid WBC in joints of groups 3 and 4 peaked at 330 minutes and was 9.5 \( \times \) 10\(^3\) ± 2.2 cells/\( \mu l \). Maximal WBC in inflamed joints was 26.9 \( \times \) 10\(^3\) cells/\( \mu l \), and was observed at 330 minutes. Synovial fluid WBC differential was within normal reported ranges\(^2\) at baseline in all isolated joints (Fig 6.3). In all groups, % neutrophils significantly increased (\( P<0.001 \)) and % large and % small mononuclear cell significantly decreased over time. There was no significant effect of treatment or denervation on synovial fluid nucleated cell differential.

Synovial fluid interleukin-1, interleukin-6, PGE\(_2\) and substance P concentrations

Interleukin 1\( \beta \) concentration was significantly greater (\( P < 0.0001 \)) in groups 3 and 4 compared to groups 1 and 2 up to 300 minutes after intraarticular injection of
interleukin-1β (Table 6.2). Similarly, II1β content was greater in groups 3 and 4 than groups 1 and 2 (P<0.05) (Table 6.2). When inflamed groups only were considered, II1β concentrations, content and ratio were significantly greater (P=0.04) in group 3 than group 4 at 240, 300 and 330 minutes (Fig 6.4 and table 6.2). At 240 minutes, II1β concentrations in inflamed-innervated joints (Group 3) were 19 times greater than control, vs 8.6 times in inflamed-denervated joints (Group 4). In inflamed-denervated joints II1β concentration, content and ratios had returned to levels to significantly different from control by 300 minutes.

Interleukin-6 concentration was significantly greater (P=0.01) in groups 3 and 4 compared to groups 1 and 2 at 240, 300, and 330 minutes (Table 6.3). In contrast to II1β, II6 concentrations showed an increase across time. Similarly, II6 content was greater in groups 3 and 4 than groups 1 and 2 (P<0.05) (Table 6.3). The concentration ratios of II6 were significantly increased (P=0.01) in inflamed (Groups 3 and 4) compared to control (Groups 1 and 2) with a peak ratio of 21 times that of the reference group observed at 330 minutes in inflamed-denervated joints (group 4). There was no effect of denervation detected on II6 concentration, content, or ratio.

Synovial fluid PGE2 ratio significantly increased over time in all groups (P=0.029). Inflammation (groups 3 and 4) significantly increased PGE2 ratio over control joints (groups 1 and 2) (P=0.038) (Fig 6.6). Synovial fluid from Groups 1 and 2 had a maximal elevation in PGE2 of 2.5 ± 0.6 times baseline values whereas inflamed denervated joints (groups 4) had a mean elevation of 16.9 ± 11.9 times baseline values. These peak ratios were observed at 330 minutes.

Total substance P content in synovial fluid was significantly greater (P=0.025) in groups 3 and 4 than groups 1 and 2 at 300 and 330 minutes (Fig 6.7). There was no
significant difference in denervated joints in total substance P amount released in synovial fluid.

**Chondrocyte proteoglycan synthesis and content**

Proteoglycan synthesis (CPM/µg protein) and endogenous proteoglycan content (µg/µg protein) and their respective ratios of experimental to control leg were not significantly different among groups or across time (18 hours, 72 hours or 144 hours) (Table 6.4). Total glycosaminoglycan, chondroitin and keratan sulfate concentrations were not significantly different among groups (Table 6.5).

**Proteoglycan degradation**

The ratio of newly synthesized proteoglycan released in culture medium was significantly decreased at 24 and 48 hours in Inflamed -denervated group (Group 4) (P=0.03) (Table 6.6). There was no difference in total uronic acid concentration (endogenous proteoglycan) released in culture medium among groups or across time (Table 6.7).

**Histopathology scores**

There was no significant effect of joint isolation, inflammation or denervation on edema scores. Isolated joints (Groups 1-4) had significantly greater (P=0.02) hemorrhage scores than their contralateral control joints. However there was no effect of inflammation or denervation on this score. There was a significant effect of site within the joint, joint isolation and inflammation on neutrophilic vasculitis scores. Villous synovial membrane had greater neutrophilic vasculitis scores than fibrous synovial membrane. Isolated joints had significantly greater neutrophilic vasculitis than their contralateral control joints. Inflamed joints (groups 3 and 4) had significantly greater (P=0.01) neutrophilic vasculitis
scores than control isolated joints (groups 1 and 2) (Figure 6.8 and table 6.8). There was no effect of denervation on this score. The coefficient of variability among observers was 0.68, indicating that 95% of observations were within a score value of 0.68.

**Substance P and NSE immunostaining**

Occasional substance P positive peroxidase stained nerve fibers were observed in the interstitium and perivascular space of synovial membrane specimens (Plate 6.2). Similarly, NSE-positive nerve fibers were observed at those sites. Subjectively, there was a greater density of nerve fibers in the villous area of the synovial membrane compared to the fibrous zone. The number of positive staining fibers were too few and variable to justify quantitative measurements.

**Discussion**

Interleukin-1β injected in equine joints induced a rapid cellular inflammatory response identified by a synovial fluid leucocytosis at 240 minutes after induction, a significant neutrophilic vasculitis particularly of the villous synovium at 360 minutes after induction, and a significant release of the mediators II6 and PGE2. Acute denervation during the induction of acute intraarticular inflammation by II16 resulted in lower increases in II1β, and a more rapid return to baseline values than that observed with innervation. In denervated joints, the ratio of PGE2 was greater with inflammation than with innervation. Denervated joints also had less cartilage degradative response to inflammation than denervated joints.

Synovial fluid leukocytosis was observed in II1β injected joints. The magnitude of increase in synovial fluid WBC and the neutrophilic response were comparable to that observed in clinical cases of synovitis. This model of II1β (0.35 ng/kg) produced a
milder synovitis than that reported after carrageenan or amphotericin B injection where WBC values are in the $100$-$150 \times 10^3$ cells/µl range. Synovial fluid WBC values after intraarticular endotoxin were dose-dependent.6

Interleukin 1β concentrations were significantly higher at 240 minutes ($P<0.0005$) in inflamed joints, but had returned to baseline values at the end of the study. Mean II-1β concentrations at 240 minutes in inflamed joints was $19.9 \pm 6.5$ pg/ml, with a peak value of $85.25$ pg/ml. These values agree with the reported range of II1β concentrations obtained in synovial fluid collected from horse with various joint disorders. Total II1β content (Group 3: $164.6 \pm 46.7$ pg/ml, Group 4: $107.19 \pm 28.18$ pg/ml) were much smaller than the amount originally infused, suggesting that the majority of II1β is cleared by transsynovial pathways or degraded. Control joints had II1β concentrations that were less than 3.0 pg/ml. This single II1β injection presumably did not result in increased or sustained endogenous II1β synthesis, as concentrations continuously decreased following the initial sampling time. The significantly greater II1β in innervated joints suggests a role of sympathetic innervation to contribute to mediator release and inflammation in the joint capsule.

Interleukin 6 concentrations significantly increased ($P=0.011$) throughout the study period in inflamed joints. Peak II6 values obtained in this study compare well with values obtained in synovial fluid from horses with naturally occurring acute severe joint disorders. This increased II6 release may have stemmed from resident synovial cells, chondrocytes, or from the influx of neutrophils. Previous in vitro studies have reported interleukin 6 release from synoviocytes in response to inflammatory stimuli. In addition, II1β is a direct stimulus for II6 release.

The increased PGE$_2$ concentrations observed in II1β-inflamed joints is consistent with increased PGE$_2$ concentrations obtained in clinical joint disease in people. and
Prostaglandin release can be induced by cellular damage, vascular distention or stress, and IL-1. Individual tissues produce a single eicosanoid, and PGE$_2$ is commonly released from articular inflammation. Equine synoviocytes and chondrocytes have been shown to secrete PGE$_2$ rather than PGE$_1$. PGE$_2$ has a direct effect on synoviocytes to release mediators. Variability in control joint PGE$_2$ concentrations at baseline was probably the result of surgical manipulation because of the variability in baseline concentrations, expression of PGE$_2$ as a ratio of baseline values allowed to stabilize variances and to gain a better understanding of patterns of release of PGE$_2$.

The amounts of substance P released was significantly greater in inflamed joints. However, there was no significant difference between innervated or denervated joints on substance P release, as might have been expected. The acute denervation in our model may not have influenced the concentration of substance P at the nerve ending, because substance P is synthesized by the cell body in the dorsal root ganglion, and is carried by axonal transport to the nerve ending where it is stored until release.

Interleukin-1B induced a significant neutrophilic vasculitis in inflamed joints. This response emphasizes the rapidity with which the joint reacts to inflammatory stimulation. Clinically, pain, effusion and a leukocytic response are observed within hours after exposure to a inflammatory stimuli such as bacteria or trauma. Activation of cell surface adhesion molecules may regulate leukocyte migration and chemotaxis to the joint. Resident articular endothelial and synovial cells can upregulate the expression of adhesion molecule in response to pro-inflammatory cytokines such as interleukin 1.$^{34}$ At least three distinct adhesion molecules have been described in articular tissue: E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1). E-selectin is
probably involved in the initial adhesion of circulating resting neutrophils, and E-selectin was preferentially expressed on small superficial venules in synovium. Our findings further identify a significant neutrophilic vasculitis in the most superficial vessels of the synovium and document the rapidity of the response.34

Our results indicated a degradative response of articular cartilage in innervated joints exposed to II1β, whereas no effect on synthesis was observed. A significant decrease in degradation of newly synthesized proteoglycan was observed in inflamed denervated joints. In vitro incubation of equine cartilage explants to II1 showed increased degradation and decreased proteoglycan synthesis,24 or decreased synthesis only without effect on degradation.35 However, these latter studies used different labelling times, or did not account for amount incorporated. Measurement of mRNA transcription of degradative enzyme may be more accurate to detect an effect of II1. In one report, the degradative response of articular cartilage following exposure to II1β preceded the synthetic effects of the cytokine. Our findings of a short term effect of II1β on cartilage degradation in vitro is consistent with rapid recovery of cartilage following removal of II1.35 The protective effect of denervation on degradation of cartilage supports previous findings of a synergistic response of innervation to enhance the inflammatory response, through neuropeptide release or contributions of postganglionic nerve terminals in the joint capsule to promote mediator release.11,29

In this model, interleukin-1 was chosen to induce articular inflammation, as it is a reported pro-inflammatory cytokine which has been used in several models of articular inflammation. Interleukin-1 activity is increased in clinical cases of inflammatory arthritis of horses,36,37 and increased II1 concentrations (ELISA) have been also been reported.28 Human recombinant II1β was used instead of the α isoform, based on previous findings that human recombinant II1β was three to ten times more potent than II1α to stimulate
equine synoviocyte PGE$_2$ production. Interleukin-$1\beta$ has been shown to increase human PGE$_2$ release by chondrocytes.

This study is the first to report the effects of in vivo intraarticular administration of IL-1$\beta$ in equine joints. Interleukin-1 has been documented as an important mediator in articular inflammation, and has been isolated from synovial fluid of horses with various forms or articular disease. Incubation of equine cartilage with IL-1 in vitro results in a significant inhibition of matrix synthesis and increases matrix degradation. Our study confirms that IL-1 can induce a significant inflammatory response in vivo, akin to naturally occurring articular inflammation. Intra-articular injection of recombinant human IL$\alpha$ or IL$\beta$ in rabbit stifle joints resulted in a significant dose-dependent increase in intraarticular inflammatory cells by 4 hours after injection. The mean number of leukocytes present at 4 hrs was $2.4 \times 10^7$ cells in the joint wash, and this number continued to increase to a mean of $23.7 \pm 8.0$ cells by 24 hours. Direct comparison with our study is difficult because synovial fluid was obtained by lavage of the joint space with 1 ml of saline, and nucleated cell counts were obtained on the resultant fluid volume. However, these results support our findings of an inflammatory response following intraarticular IL$\beta$ injection.

We have reported the cytokine data as concentrations and as a relative value because the ELISA kits have not been specifically validated for cytokine analysis in the horse. However, homology between human and equine IL-1 was demonstrated by hybridization of a human IL-$1\beta$ cDNA probe with RNA extracted from IL-1 producing equine blood adherent monocytes as determined by RNA immunoblotting. This assay indicates a high degree of homology between the human and the equine proteins. Homology has also been reported between human and murine IL-$1\beta$, indicating preservation of a functional sequence across species. Furthermore, characterization of equine IL-1 has been partially done, and showed a high similarity between the human and equine molecules. The EIA used in this study to
quantify PGE$_2$ has been described for use in inflamed synovial fluid in horses.$^{41}$ Although the manufacturer reports up to 50% cross reactivity to PGE$_1$, PGE$_2$ has been shown to be preferentially released by equine chondrocytes and synoviocytes in response to a variety of stimuli.$^{42}$

In conclusion, results of this study illustrate a rapid but mild inflammatory response induced by interleukin-1$\beta$ in equine joints, that is characterized by increased interleukin 6 and PGE$_2$ release, and a significant neutrophilic vasculitis. Despite short term exposure to this inflammatory cytokine, induction of cartilage matrix degradation was evident. Acute denervation did not influence this inflammatory response, but was protective against the cartilage degradative response. This model could serve as a basis for targeting different inflammatory mechanisms in acute articular inflammation, namely decrease inflammatory cell trafficking, cytokine release and protection of cartilage matrix against an early degradative response.
Footnotes

a Cardiomax II, Columbus Instruments, Columbus, OH.
b Honeywell VRI2, Electronics for Medicine Inc, Pleasantville, NY
c Masterflex variable speed pump, Cole Palmer Int, Chicago, IL
d Model 7D Polygraph, Grass instruments, Quincy, MA
e Cellular Products Inc., Buffalo, NY
f Sherwood medical, St-Louis, MO
g Coulter S+IV, Coulter Electronics, Hialeah, FL
h Gam Rad, San Juan Capistrano, CA
i Advanced Magnetics, Cambridge, MA
j TiterZyme PGE2 EIA kit, PerSeptive Diagnostics, Cambridge, MA
k Micro-BCA protein assay, Pierce Chemical Co, Rockford, IL
l Sigma Chemicals, St-Louis, MO
m Polyclonal antibody to substance P, Biogenex Laboratories, San Ramon, CA
n StrepAvigen Multilink immunodetection System, Biogenex Laboratories, San Ramon, CA
Figure 6.1. Illustration of the isolated pump-perfused auto-oxygenated isolated joint model used in the study.
Figure 6.1. Illustration of the isolated pump-perfused auto-oxygenated isolated joint model used in the study.
Figure 6.2. Synovial fluid total nucleated cell count across time among groups.

(Group 1 - ■ Control; Group 2 - ● Control-Denervated; Group 3 - ▲ Inflamed; Group 4 - ♦ Inflamed-denervated) *indicates a significant difference between control joints (Groups 3 and 4) and inflamed joints (Groups 1 and 2) (P=0.001).
Figure 6.2
Figure 6.3 Synovial fluid total nucleated cell differential (%) over time among groups.

There was a significant increase in % neutrophils and a significant decrease in % large and small monocleuar cells across time. There was no difference detected among groups.
Figure 6.3

Groups

Baseline  240 min  300 min  330 min

Synovial fluid differential (%)
Figure 6.4. Synovial fluid interleukin 1B concentration ratios across time and among groups. * indicates a significant difference compared to baseline (P=0.001). ¥ indicates a significant difference between groups 3 and 4 (P=0.02).
Figure 6.4
Figure 6.5. Synovial fluid interleukin 6 concentration ratios across time and among groups. *indicates a significant difference between control joints (Groups 1 and 2) and inflamed joints (Groups 3 and 4) (P=0.01).
Figure 6.5
Figure 6.6. PGE$_2$ ratio over baseline across time and among groups. (Group 1 - ■ Control; Group 2 - ● Control-Denervated; Group 3 - ▲ Inflamed; Group 4 - ♦ Inflamed-Denervated)

* indicates a significant difference in Group 4 (Inflamed-Denervated) (P=0.038).
Figure 6.6
Figure 6.7. Total substance P (pg) in synovial fluid collection from 240-300 minutes (■) and 300 to 330 minutes (■).* Indicates a significant difference between control joints (Groups 1 and 2) and inflamed joints (groups 3 and 4) (P=0.025).
Figure 6.7
Figure 6.8 Synovial membrane histology specimens (100X) illustrating A-a control isolated joint from group 1 and B-an inflamed isolated joint from group 3. Inflamed isolated joints (Groups 3 and 4) showed a significant neutrophilic vasculitis (P=0.01).
Figure 6.9 Synovial membrane substance P and nerve specific enolase immunostaining.

There was no difference among groups as to the number of positively stained fibers.
<table>
<thead>
<tr>
<th>Extent of Edema</th>
<th>Hemorrhage</th>
</tr>
</thead>
<tbody>
<tr>
<td>0- None</td>
<td>0- none</td>
</tr>
<tr>
<td>1- Less than 25% of intima/subintima</td>
<td>1- Less than 25% of specimen involved</td>
</tr>
<tr>
<td>2- 25-50% of intima/subintima</td>
<td>2- 25-50% of specimen involved</td>
</tr>
<tr>
<td>3- 50-75% of intima/subintima</td>
<td>3- 50-75% of specimen involved</td>
</tr>
<tr>
<td>4- Greater than 75% of intima/subintima</td>
<td>4- 100% of specimen involved</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Neutrophilic vasculitis</th>
<th>Type of infiltrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0- None</td>
<td>1- Vessels only</td>
</tr>
<tr>
<td>1- Scattered intraluminal neutrophil clusters, minimal or none intramural or extravascular</td>
<td>2- Vessels/subintima</td>
</tr>
<tr>
<td>2- Widespread intraluminal neutrophils, minimal or no intramural</td>
<td>3- Vessels/subintima/intima</td>
</tr>
<tr>
<td>3- &lt; 50% of vessels have intramural clusters; slight perivascular infiltrate</td>
<td></td>
</tr>
<tr>
<td>4- &gt; 50% of vessels have intramural clusters; slight perivascular infiltrate</td>
<td></td>
</tr>
<tr>
<td>5- Almost all vessels have intramural and perivascular infiltrates</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.1 Score description for evaluation of synovial membrane histology
<table>
<thead>
<tr>
<th>Group 1 (Control-Innervated)</th>
<th>Concentration</th>
<th>Content</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
</tr>
<tr>
<td>Baseline</td>
<td>&lt;1.6 ± 0.25</td>
<td></td>
<td>0.50 ± 0.15</td>
</tr>
<tr>
<td>240 minutes</td>
<td>&lt;1.6 ± 0.43</td>
<td></td>
<td>0.92 ± 0.27</td>
</tr>
<tr>
<td>300 minutes</td>
<td>&lt;1.6 ± 0.29</td>
<td>3.25 ± 0.65</td>
<td>0.69 ± 0.18</td>
</tr>
<tr>
<td>330 minutes</td>
<td>&lt;1.6 ± 0.34</td>
<td>2.71 ± 0.61</td>
<td>0.75 ± 0.21</td>
</tr>
<tr>
<td>Total content</td>
<td>5.96 ± 1.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2 (Control-Denervated)</td>
<td></td>
<td></td>
<td>0.45 ± 0.21</td>
</tr>
<tr>
<td>Baseline</td>
<td>&lt;1.6 ± 0.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>240 minutes</td>
<td>&lt;1.6 ± 0.16</td>
<td></td>
<td>0.21 ± 0.11</td>
</tr>
<tr>
<td>300 minutes</td>
<td>&lt;1.6 ± 0.16</td>
<td>1.06 ± 0.48</td>
<td>0.31 ± 0.15</td>
</tr>
<tr>
<td>330 minutes</td>
<td>&lt;1.6 ± 0.18</td>
<td>0.54 ± 0.20</td>
<td>0.42 ± 0.15</td>
</tr>
<tr>
<td>Total content</td>
<td>1.59 ± 0.58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 3 (Inflamed-Innervated)</td>
<td></td>
<td></td>
<td>1.10 ± 0.46</td>
</tr>
<tr>
<td>Baseline</td>
<td>&lt;1.6 ± 0.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>240 minutes</td>
<td>28.23 ±13.26*</td>
<td>19.07 ±8.96*</td>
<td></td>
</tr>
<tr>
<td>300 minutes</td>
<td>16.99 ±7.25*¥</td>
<td>120.24 ±37.26*¥</td>
<td>15.44 ±5.58*¥</td>
</tr>
<tr>
<td>330 minutes</td>
<td>10.67 ±5.10*¥</td>
<td>44.36 ±15.11*¥</td>
<td>8.89 ±4.25*¥</td>
</tr>
<tr>
<td>Total content</td>
<td>164.60 ±46.71*¥</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 4 (Inflamed-Denervated)</td>
<td></td>
<td></td>
<td>0.46 ± 0.11</td>
</tr>
<tr>
<td>Baseline</td>
<td>&lt;1.6 ± 0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>240 minutes</td>
<td>12.74 ±3.43*</td>
<td>8.61 ±2.31*</td>
<td></td>
</tr>
<tr>
<td>300 minutes</td>
<td>3.65 ±0.75*</td>
<td>75.05 ±18.23*</td>
<td>3.32 ±0.68*</td>
</tr>
<tr>
<td>330 minutes</td>
<td>2.37 ±0.61*</td>
<td>32.14 ±12.34*</td>
<td>1.97 ±0.51*</td>
</tr>
<tr>
<td>Total content</td>
<td>107.19 ±28.18*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6.2 Synovial fluid IL18 concentration, content and ratio among groups and across time. Ratio calculated using Group 1 as reference.

* indicates inflamed joints (Groups 3 and 4) significantly different from controls (Group 1 and 2) (P<0.0001)
¥ indicates inflamed innervated joints (group 3) significantly greater than inflamed-denervated joints (Group 4) (P=0.04)
<table>
<thead>
<tr>
<th>Group 1 (Control-Innervated)</th>
<th>Concentration</th>
<th>Content</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>Mean±SE</td>
<td>Mean±SE</td>
<td></td>
</tr>
<tr>
<td>240 minutes</td>
<td>&lt;3.2±0.00</td>
<td>0.00±0.00</td>
<td></td>
</tr>
<tr>
<td>300 minutes</td>
<td>&lt;3.2±0.38</td>
<td>0.32±0.12</td>
<td></td>
</tr>
<tr>
<td>330 minutes</td>
<td>5.68±2.64</td>
<td>7.78±2.61</td>
<td>1.78±0.82</td>
</tr>
<tr>
<td>Total content</td>
<td>21.05±6.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2 (Control-Denervated)</td>
<td>Mean±SE</td>
<td>Mean±SE</td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>&lt;3.2±0.01</td>
<td>0.00±0.00</td>
<td></td>
</tr>
<tr>
<td>240 minutes</td>
<td>&lt;3.2±0.84</td>
<td>0.33±0.26</td>
<td></td>
</tr>
<tr>
<td>300 minutes</td>
<td>3.30±2.81</td>
<td>7.02±5.84</td>
<td>1.03±0.88</td>
</tr>
<tr>
<td>330 minutes</td>
<td>2.23±1.79</td>
<td>0.96±0.41</td>
<td>0.70±0.56</td>
</tr>
<tr>
<td>Total content</td>
<td>7.98±5.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 3 (Inflamed-Innervated)</td>
<td>Mean±SE</td>
<td>Mean±SE</td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>&lt;3.2±0.20</td>
<td>0.08±0.06</td>
<td></td>
</tr>
<tr>
<td>240 minutes</td>
<td>28.46±23.88*</td>
<td>8.89*±7.46</td>
<td></td>
</tr>
<tr>
<td>300 minutes</td>
<td>43.47±34.65*</td>
<td>213.15±108.81*</td>
<td>13.58*±10.83</td>
</tr>
<tr>
<td>330 minutes</td>
<td>43.73±31.61*</td>
<td>250.69±149.5*</td>
<td>13.66*±9.88</td>
</tr>
<tr>
<td>Total content</td>
<td>463.84±256.15*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 4 (Inflamed-Denervated)</td>
<td>Mean±SE</td>
<td>Mean±SE</td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>&lt;3.2±0.43</td>
<td>0.17±0.14</td>
<td></td>
</tr>
<tr>
<td>240 minutes</td>
<td>46.01±25.71*</td>
<td>14.38*±8.04</td>
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</tr>
<tr>
<td>300 minutes</td>
<td>59.81±30.52*</td>
<td>551.66±329.02*</td>
<td>18.69*±9.54</td>
</tr>
<tr>
<td>330 minutes</td>
<td>68.79±33.60*</td>
<td>584.99±357.27*</td>
<td>21.49*±10.50</td>
</tr>
<tr>
<td>Total content</td>
<td>1136.56±684.78*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6.3 Synovial fluid IL6 concentration, content and ratio among groups and across time. Ratio calculated using Group 1 as reference.
* indicates inflamed joints (Groups 3 and 4) significantly different from controls (Group 1 and 2) (P=0.01)
<table>
<thead>
<tr>
<th>Group</th>
<th>18 hrs</th>
<th>72 hrs</th>
<th>144 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Isolated</td>
<td>Ratio</td>
</tr>
<tr>
<td>Group 1 (Control-Innervated)</td>
<td>8.14 ±2.32</td>
<td>7.49 ±2.28</td>
<td>0.97 ±0.16</td>
</tr>
<tr>
<td>Group 2 (Control-Denervated)</td>
<td>7.38 ±1.43</td>
<td>6.47 ±1.63</td>
<td>1.00 ±0.23</td>
</tr>
<tr>
<td>Group 3 (Inflamed-Innervated)</td>
<td>23.78 ±8.98</td>
<td>23.68 ±7.08</td>
<td>1.21 ±0.20</td>
</tr>
<tr>
<td>Group 4 (Inflamed-Denervated)</td>
<td>10.74 ±2.51</td>
<td>13.18 ±9.45</td>
<td>1.20 ±0.16</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>18 hrs</th>
<th>72 hrs</th>
<th>144 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Isolated</td>
<td>Ratio</td>
</tr>
<tr>
<td>Group 1 (Control-Innervated)</td>
<td>0.20 ±0.02</td>
<td>0.22 ±0.03</td>
<td>1.14 ±0.14</td>
</tr>
<tr>
<td>Group 2 (Control-Denervated)</td>
<td>0.24 ±0.04</td>
<td>0.19 ±0.04</td>
<td>0.87 ±0.15</td>
</tr>
<tr>
<td>Group 3 (Inflamed-Innervated)</td>
<td>0.31 ±0.04</td>
<td>0.26 ±0.04</td>
<td>0.93 ±0.13</td>
</tr>
<tr>
<td>Group 4 (Inflamed-Denervated)</td>
<td>0.27 ±0.04</td>
<td>0.23 ±0.04</td>
<td>0.99 ±0.09</td>
</tr>
</tbody>
</table>

Table 6.4 Proteoglycan synthesis (CPM/µg protein) and content (µg/µg protein) and respective ratio of isolated to contralateral control joint among groups and at 18 hours, 72 hours and 144 hours time in culture. There was no significant difference among groups or across time.
<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Control-innervated)</td>
<td>(Control-Denervated)</td>
<td>(Inflamed-innervated)</td>
<td>(Inflamed-Denervated)</td>
</tr>
<tr>
<td><strong>Total glycosaminoglycan</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 hrs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>60.28 ±12.76</td>
<td>101.03 ±19.62</td>
<td>132.72 ±34.89</td>
<td>73.50 ±10.12</td>
</tr>
<tr>
<td>Isolated</td>
<td>57.07 ±8.44</td>
<td>82.21 ±12.06</td>
<td>119.37 ±25.63</td>
<td>92.75 ±11.98</td>
</tr>
<tr>
<td>Ratio</td>
<td>1.20 ±0.33</td>
<td>0.67 ±0.07</td>
<td>1.02 ±0.16</td>
<td>1.34 ±0.15</td>
</tr>
<tr>
<td>72 hrs</td>
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</tr>
<tr>
<td>Control</td>
<td>22.13 ±6.98</td>
<td>34.41 ±12.34</td>
<td>34.11 ±6.97</td>
<td>33.70 ±4.45</td>
</tr>
<tr>
<td>Isolated</td>
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<td>38.81 ±13.99</td>
<td>43.37 ±10.48</td>
<td>45.22 ±10.07</td>
</tr>
<tr>
<td>Ratio</td>
<td>1.14 ±0.29</td>
<td>1.23 ±0.28</td>
<td>1.35 ±0.24</td>
<td>1.49 ±0.42</td>
</tr>
<tr>
<td>144 hrs</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>45.21 ±10.47</td>
<td>63.63 ±14.61</td>
<td>61.14 ±16.28</td>
<td>34.50 ±8.79</td>
</tr>
<tr>
<td>Isolated</td>
<td>48.49 ±11.79</td>
<td>45.73 ±12.05</td>
<td>58.97 ±17.20</td>
<td>45.01 ±9.92</td>
</tr>
<tr>
<td>Ratio</td>
<td>1.13 ±0.16</td>
<td>0.73 ±0.13</td>
<td>1.05 ±0.22</td>
<td>1.18 ±0.19</td>
</tr>
<tr>
<td><strong>Chondroitin sulfate</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>18 hrs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>38.86 ±11.58</td>
<td>68.79 ±12.77</td>
<td>103.48 ±30.19</td>
<td>53.44 ±9.62</td>
</tr>
<tr>
<td>Isolated</td>
<td>35.06 ±9.33</td>
<td>55.71 ±6.96</td>
<td>69.71 ±24.85</td>
<td>63.21 ±7.23</td>
</tr>
<tr>
<td>Ratio</td>
<td>1.13 ±0.50</td>
<td>0.88 ±0.09</td>
<td>0.97 ±0.19</td>
<td>1.00 ±0.44</td>
</tr>
<tr>
<td>72 hrs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.15 ±2.82</td>
<td>23.25 ±28.79</td>
<td>22.16 ±7.37</td>
<td>18.54 ±3.79</td>
</tr>
<tr>
<td>Isolated</td>
<td>7.20 ±2.07</td>
<td>23.20 ±19.79</td>
<td>28.02 ±11.69</td>
<td>31.06 ±7.29</td>
</tr>
<tr>
<td>Ratio</td>
<td>0.73 ±0.30</td>
<td>1.21 ±0.38</td>
<td>2.16 ±1.04</td>
<td>2.24 ±0.73</td>
</tr>
<tr>
<td>144 hrs</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>24.59 ±6.50</td>
<td>39.12 ±11.03</td>
<td>43.68 ±13.01</td>
<td>53.44 ±8.79</td>
</tr>
<tr>
<td>Isolated</td>
<td>29.03 ±6.91</td>
<td>26.09 ±10.31</td>
<td>36.19 ±14.57</td>
<td>32.88 ±8.17</td>
</tr>
<tr>
<td>Ratio</td>
<td>1.26 ±0.35</td>
<td>0.84 ±0.14</td>
<td>0.75 ±0.26</td>
<td>0.97 ±0.76</td>
</tr>
<tr>
<td><strong>Keratan sulfate</strong></td>
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<td></td>
</tr>
<tr>
<td>18 hrs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9.50 ±3.00</td>
<td>7.80 ±1.97</td>
<td>13.80 ±4.98</td>
<td>6.33 ±3.25</td>
</tr>
<tr>
<td>Isolated</td>
<td>11.58 ±4.34</td>
<td>8.71 ±3.91</td>
<td>14.19 ±5.30</td>
<td>14.20 ±6.88</td>
</tr>
<tr>
<td>Ratio</td>
<td>1.54 ±0.63</td>
<td>0.98 ±0.17</td>
<td>0.78 ±0.28</td>
<td>0.87 ±1.57</td>
</tr>
<tr>
<td>72 hrs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9.49 ±2.01</td>
<td>2.98 ±1.98</td>
<td>10.84 ±4.60</td>
<td>10.80 ±5.29</td>
</tr>
<tr>
<td>Isolated</td>
<td>6.11 ±2.81</td>
<td>6.76 ±3.14</td>
<td>10.59 ±5.16</td>
<td>7.69 ±4.53</td>
</tr>
<tr>
<td>Ratio</td>
<td>2.04 ±1.72</td>
<td>1.28 ±0.78</td>
<td>0.81 ±0.31</td>
<td>0.62 ±0.22</td>
</tr>
<tr>
<td>144 hrs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8.04 ±2.87</td>
<td>10.23 ±4.43</td>
<td>13.25 ±6.19</td>
<td>3.70 ±2.58</td>
</tr>
<tr>
<td>Isolated</td>
<td>7.89 ±5.57</td>
<td>8.99 ±5.57</td>
<td>17.59 ±7.52</td>
<td>4.23 ±1.98</td>
</tr>
<tr>
<td>Ratio</td>
<td>0.71 ±0.48</td>
<td>0.54 ±0.20</td>
<td>0.99 ±0.34</td>
<td>0.72 ±0.22</td>
</tr>
</tbody>
</table>

Table 6.5 Cartilage total glycosaminoglycan, chondroitin and keratan sulfate concentrations among groups at culture time 18, 72 and 144 hours. There was no significant difference among groups or across time.
### Table 6.6 Newly synthesized proteoglycan released in 24, 48 and 72 hr media among groups expressed as amount released (CPM), ratio of isolated to contralateral control joints, and % of total cartilage content.

*♦ indicates a significant decrease in release in inflamed denervated joints (P=0.03)*

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Control-Innervated)</td>
<td>(Control-Denervated)</td>
<td>(Inflamed-Innervated)</td>
<td>(Inflamed-Denervated)</td>
</tr>
<tr>
<td>24 hrs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amount released (CPM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>72261</td>
<td>21066</td>
<td>75567</td>
<td>29460</td>
</tr>
<tr>
<td>Isolated</td>
<td>84129</td>
<td>35207</td>
<td>73173</td>
<td>29735</td>
</tr>
<tr>
<td>Ratio</td>
<td>1.02</td>
<td>0.26</td>
<td>1.34</td>
<td>0.50</td>
</tr>
<tr>
<td>% released</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.40</td>
<td>0.08</td>
<td>0.41</td>
<td>0.07</td>
</tr>
<tr>
<td>Isolated</td>
<td>0.40</td>
<td>0.07</td>
<td>0.39</td>
<td>0.05</td>
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<tr>
<td>Ratio % released</td>
<td>1.50</td>
<td>1.07</td>
<td>1.00</td>
<td>0.13</td>
</tr>
<tr>
<td>48 hrs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amount released (CPM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>28737</td>
<td>8970</td>
<td>38246.59</td>
<td>21252.09</td>
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<td>Ratio</td>
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<td>0.12</td>
<td>1.90</td>
<td>0.68</td>
</tr>
<tr>
<td>% released</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.15</td>
<td>0.02</td>
<td>0.18</td>
<td>0.05</td>
</tr>
<tr>
<td>Isolated</td>
<td>0.16</td>
<td>0.02</td>
<td>0.25</td>
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<tr>
<td>Ratio % released</td>
<td>1.13</td>
<td>0.14</td>
<td>1.80</td>
<td>0.45</td>
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<tr>
<td>Amount released (CPM)</td>
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<td></td>
<td></td>
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</tr>
<tr>
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<td>22657</td>
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<td>1.27</td>
<td>0.35</td>
</tr>
<tr>
<td>% released</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.14</td>
<td>0.04</td>
<td>0.12</td>
<td>0.03</td>
</tr>
<tr>
<td>Isolated</td>
<td>0.16</td>
<td>0.04</td>
<td>0.13</td>
<td>0.03</td>
</tr>
<tr>
<td>Ratio % released</td>
<td>1.24</td>
<td>0.22</td>
<td>1.07</td>
<td>0.14</td>
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### Degradation of endogenous proteoglycan

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (Control-Innervated)</th>
<th>Group 2 (Control-Denervated)</th>
<th>Group 3 (Inflamed-Innervated)</th>
<th>Group 4 (Inflamed-Denervated)</th>
</tr>
</thead>
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<tr>
<td><strong>24 hrs</strong></td>
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<td></td>
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<tr>
<td>Amount released (µg)</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>Control</td>
<td>46.19</td>
<td>2.17</td>
<td>50.78</td>
<td>7.03</td>
</tr>
<tr>
<td>Isolated</td>
<td>48.73</td>
<td>4.61</td>
<td>58.94</td>
<td>12.26</td>
</tr>
<tr>
<td>Ratio</td>
<td>1.07</td>
<td>0.12</td>
<td>1.15</td>
<td>0.11</td>
</tr>
<tr>
<td>% released</td>
<td>0.22</td>
<td>0.03</td>
<td>0.25</td>
<td>0.02</td>
</tr>
<tr>
<td>Ratio % released</td>
<td>1.02</td>
<td>0.11</td>
<td>1.10</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>48 hrs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amount released (µg)</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>Control</td>
<td>50.94</td>
<td>1.78</td>
<td>52.86</td>
<td>5.42</td>
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<tr>
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<td>69.30</td>
<td>15.64</td>
</tr>
<tr>
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<td>1.20</td>
<td>0.05</td>
<td>1.26</td>
<td>0.16</td>
</tr>
<tr>
<td>% released</td>
<td>0.45</td>
<td>9.86</td>
<td>0.27</td>
<td>0.02</td>
</tr>
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<td>17.22</td>
<td>0.32</td>
<td>0.02</td>
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<td>0.10</td>
<td>1.19</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>72 hrs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amount released (µg)</td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>Control</td>
<td>58.90</td>
<td>10.07</td>
<td>53.71</td>
<td>5.04</td>
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<td>2.16</td>
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<td>0.09</td>
</tr>
<tr>
<td>% released</td>
<td>0.26</td>
<td>0.02</td>
<td>0.27</td>
<td>0.01</td>
</tr>
<tr>
<td>Isolated</td>
<td>0.22</td>
<td>0.02</td>
<td>0.24</td>
<td>0.04</td>
</tr>
<tr>
<td>Ratio % released</td>
<td>0.90</td>
<td>0.12</td>
<td>0.87</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Table 6.7 Endogenous proteoglycan released in 24, 48 and 72 hr media among groups expressed as amount released (µg) ratio of isolated to contralateral control joints, and % of total cartilage content.

There was no difference among groups or across time.
<table>
<thead>
<tr>
<th></th>
<th>Edema</th>
<th>Hemorrhage</th>
<th>Neutrophilic Vasculitis</th>
<th>Type of Infiltrate</th>
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</thead>
<tbody>
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<td></td>
<td>Control</td>
<td>Isolated</td>
<td>Control</td>
<td>Isolated</td>
</tr>
<tr>
<td>Fibrous synovium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1 (Control-Innervated)</td>
<td>0.17 ±0.11</td>
<td>1.22 ±0.55</td>
<td>0.00 ±0.00</td>
<td>0.44 ±0.20</td>
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<td>Group 2 (Control-Denervated)</td>
<td>1.47 ±0.62</td>
<td>1.39 ±0.62</td>
<td>0.13 ±0.13</td>
<td>0.33 ±0.11</td>
</tr>
<tr>
<td>Group 3 (Inflamed-Innervated)</td>
<td>1.50 ±0.62</td>
<td>0.56 ±0.20</td>
<td>0.00 ±0.00</td>
<td>0.26 ±0.06</td>
</tr>
<tr>
<td>Group 4 (Inflamed-Denervated)</td>
<td>0.38 ±0.29</td>
<td>0.90 ±0.40</td>
<td>0.00 ±0.00</td>
<td>0.24 ±0.06</td>
</tr>
<tr>
<td>Villous synovium</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1 (Control-Innervated)</td>
<td>1.06 ±0.47</td>
<td>0.93 ±0.46</td>
<td>0.00 ±0.13</td>
<td>0.67 ±0.17</td>
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<tr>
<td>Group 2 (Control-Denervated)</td>
<td>0.80 ±0.20</td>
<td>0.72 ±0.35</td>
<td>0.00 ±0.28</td>
<td>0.40 ±0.12</td>
</tr>
<tr>
<td>Group 3 (Inflamed-Innervated)</td>
<td>1.28 ±0.29</td>
<td>1.44 ±0.43</td>
<td>0.00 ±0.44</td>
<td>0.20 ±0.13</td>
</tr>
<tr>
<td>Group 4 (Inflamed-Denervated)</td>
<td>0.67 ±0.36</td>
<td>1.33 ±0.29</td>
<td>0.05 ±0.05</td>
<td>0.67 ±0.34</td>
</tr>
</tbody>
</table>

Table 6.8 Histopathology scores (mean ± SE) among groups, sites, and comparing contralateral control to isolated joints.
¥ indicates significant difference between villous and fibrous synovium.
© indicates significant difference between isolated (Groups 1-4) and contralateral control joints.
† indicates significant difference between Groups 3 and 4 and Groups 1 and 2.
References


SUMMARY

Articular inflammation is a common finding in equine joints disorders. As in other organs, several aspects of the joint system, including blood flow, transsynovial permeability and fluid exchanges, cellular infiltration and mediator release may be involved in the inflammatory process and contribute to joint morbidity. With inflammation, tissues within the joint contribute to mediator release and stimulate degradation of the cartilage matrix. Perfused oxygenated extracorporeal isolated models have been thoroughly described for organs such as heart, lung and intestine, but have never been created for the joint organ. The studies reported here describe an isolated pump-perfused auto-oxygenated extracorporeal isolated joint model developed in the equine. Furthermore, these studies characterize local Starling's forces in the isolated joint at isogravimetric state and alterations in Starling's forces in response to hemodynamic manipulations. Subsequently, these studies report on local hemodynamic, fluid exchanges, metabolic responses, alterations in permeability to small and large molecules, inflammatory mediator release, cellular events and articular cartilage metabolic alterations in experimentally induced acute articular inflammation in the isolated, and furthermore innervated or denervated, joint.

Two preliminary studies were performed in the development of the isolated joint model. The first studies was essential to gain a better understanding of the local biomechanical behavior of the fluid filled closed joint cavity. In this study, the investigation of pressure volume curves in the joint revealed a sigmoid relationship, with low compliance at normal subatmospheric pressures, and gradually increasing compliance at supraatmospheric pressures of up to 30 mmHg. Thereafter, compliance of the joint again
decreased exponentially. This study also revealed an important function of synovial fluid to increase joint compliance and hysteresis therefore preventing collapse of the joint cavity and allow fluid exchanges at subatmospheric pressures. Furthermore this study emphasized the importance of joint angle on pressure-volume relationships. In the second preliminary study, a colored microsphere technique was validated for determination of blood flow to the synovial membrane and joint capsule. Furthermore, that study documented a significant decrease in synovial membrane blood flow at intraarticular pressures of 30 mmHg or greater.

The development and physiologic responses of the isolated model were subsequently described. The extracorporeal pump perfused isolated joint unit developed in this model was successfully be maintained for 6 hours. Using this system, isogravimetric state was defined at a circuit arterial pressure of 133 mmHg and venous pressure of 10.5 mm Hg. Starling’s forces were defined for this system. Arterial and venous pressure manipulations revealed that increases in arterial pressure significantly raised transsynovial flow and synovial fluid production, when IAP was maintained at atmospheric pressure. A similar but much less important response was observed with increased venous pressure.

Acute inflammation was successfully induced in the isolated innervated or denervated joint preparation, using interleukin-1B. Acute inflammation induced a significant increase in oxygen consumption and extraction ratio in response to increased metabolic demand. Acute inflammation also increased synovial membrane permeability to albumin, while innervation was associated with a higher permeability to large molecules (MW 144,000). Inflammation also significantly increased synovial fluid production and the permeability surface area product of the synovial membrane.

Acute inflammation was associated with a significant cellular response characterized by neutrophilic leukocytosis in synovial fluid and a neutrophilic vasculitis in the synovial membrane. A significant increase in interleukin-6, prostaglandin E$_2$ and substance P was
detected in synovial fluid collected from inflamed joints. Inflammation induced a significant degradative response in articular cartilage, even after this short term exposure to an inflammatory mediator.

The isolated joint model described in these studies provided new insights in our understanding of articular physiology, allowing study of all pathophysiologic processes involved in articular inflammation. This model should be useful to further our knowledge of the response of joint to various disease processes, and should be very suitable to study the pharmacokinetics and pharmacodynamics of systemic or intraarticular therapies.
APPENDIX A

DYNAMIC VASCULAR AND TRANS-SYNOVIAL FORCES
OF THE ISOLATED JOINT

Introduction

It has been widely presumed that Starling's forces govern trans-synovial fluid flow as in other connective tissue spaces. Starling's hypothesis predicts that the rate of transcapillary fluid filtration ($J_V$) is a linear function of capillary pressure ($P_{cap}$) and interstitial colloid osmotic pressure and a negative linear function of plasma colloid osmotic pressure and interstitial pressure ($P_i$). Due to the presence of a large third space (joint cavity), a simple two-compartment model of fluid exchange with the interstitium does not necessarily apply. Many sophisticated and elaborate studies have been performed to indirectly measure or theoretically calculate the primary coefficients modifying the fluid flow from plasma to synovial fluid (osmotic coefficient ($\sigma_d$) and filtration coefficients of the capillary ($K_c$) and synovium ($K_s$)). An isolated, rabbit perfused hindlimb preparation has been used to describe trans-synovial flow, but pressure changes and blood flow were not representative of only the joint and trans-synovial flow was in the absorptive phase. Presumption and theoretical calculation of hemodynamic, microvascular, and trans-synovial parameters, therefore, were required due to a lack of an isolated joint model. Isolated organ preparations have facilitated these measurements in the heart, lung, intestine, and hoof laminae. An isolated joint model would more accurately permit simultaneous measurement of dependent changes in blood flow, synovial fluid production.
and composition, and interstitial fluid accumulation as expected to occur with physiologic manipulation, or pathology such as acute joint inflammation or degenerative joint disease.

Few studies have evaluated physiologic parameters of the joint separately, including lymphatic flow. Clearance studies from the synovial cavity following intraarticular injection of $^{133}$Xenon or $^{123}$I and $^{131}$-I labeled albumin can estimate blood flow, but are limited by radioactivity, diffusion, and the inflammatory response. It has been demonstrated that an intra-articular injection of even balanced electrolyte solution produces transient, but significant, synovitis that alters blood flow. Metabolic parameters in synovial fluid ($pCO_2$, pH, and lactate concentration) have been measured to indirectly estimate blood flow relative to oxygen demand, and estimate joint ischemia, but they do not provide direct and simultaneous measurements of fluid exchange. In virtually all studies, measured values of filtration or trans-synovial fluid flow were either theoretically calculated, inherently inaccurate, or limited by the number of physiologic parameters that could be evaluated. Dynamic evaluations of the vascular/fluid component(s) of joints is critical to understanding joint physiology, pathophysiology and anticipation of responses to medications. Therefore, the purpose of this study was to develop an autooxygenated, pump-perfused, isolated joint model and use this model to determine the hemodynamic and trans-synovial forces and characteristic responses of joint tissue to controlled physiologic (pressure) manipulations.

The horse was selected for use in an isolated joint preparation due to anatomical considerations, including a well-developed singular digit (McIII) with a simple neurovascular network and lack of musculature. The joint selected (metacarpophalangeal [MCP] joint) is large enough to allow collection of ample quantities of synovium and synovial fluid for multiple analyses. Also, the MCP joint is the second most common joint affected with arthritis in horses and therefore would have future clinical relevance to the
study of naturally-occurring arthritis. Equine osteoarthritis is naturally-occurring, histologically similar to human osteoarthritis, and has been extensively studied due to the impact of osteoarthritis on the equine racing industry. Many studies of equine osteoarthritic cartilage (natural or induced) have confirmed similar behavior of equine chondrocytes and articular tissues as in man and other species. Substance P innervation and receptor localization in the equine joint are similar to man. Comparatively in the horse, joint disorders are also the most common cause of lameness and loss of athletic use.

**Materials and methods**

**Horses**

Seven normal horses (aged 1-5 years [mature, but young]; 306-556 kg body weight; Thoroughbred-type), normal as determined by physical and lameness examination, CBC and serum chemistry profile, were obtained. Inclusion criteria for the study dictated that both MCP joints were palpably normal with a normal range of motion (~ 90°), normal radiographic evaluation (4 standard views), and normal synovial fluid analysis. Gross evaluation of articular cartilage at the termination of the study had to be normal, or the horse was eliminated from the study and replaced. All experiments were conducted in accordance with the Animal Care and Use Guidelines of The Ohio State University.

**Anesthesia and Systemic Hemodynamic Measurements**

Only one horse was studied on a given day because of the complexity of the preparation. Horses were sedated with xylazine (0.5 mg/kg, iv), and anesthesia was induced with guaifenisin (~25 mg/kg) followed by sodium thiopental (2 mg/kg, iv). Anesthesia was maintained with sodium pentobarbital (5-15 mg/kg/hr, iv, to effect) and
controlled mechanical ventilation with 100% oxygen. Horses were positioned in lateral recumbency, and anesthesia monitored by continuous display of direct systemic arterial blood pressure measurement, hourly measurement of cardiac output by a thermodilution technique, and intermittent arterial pH and blood gas measurements. Mean systemic arterial blood pressure was maintained at ≥ 70 mm Hg, and ventilation was adjusted to maintain $P_aCO_2 \leq 55$ mmHg. Heparin (50,000 units IV) was administered every 90 minutes.

**Joint isolation procedure and Instrumentation**

An MCP joint of one forelimb was randomly selected. Before joint isolation, an arterial cannula (PE 240) was placed in the median artery proximal to the anticipated site of joint isolation. Blood was diverted from the cannulated median artery, circulated through a calibrated peristaltic pump, and infused via the cannulated medial palmar artery to the isolated joint preparation. (Fig 1) Venous outflow was collected from the medial palmar and lateral and medial palmar digital veins, directed into a reservoir and reinfused into the horse via the median vein using a peristaltic pump. Weight of the preparation was continuously measured using an FT03 transducer connected to a physiograph for continuous display. Circuit arterial pressure ($P_{a,cir}$) was measured and initially set at 150 mmHg by adjustment of flow in the arterial pump, and circuit venous pressure ($P_{v,cir}$) was adjusted using a clamp and initially maintained at 10 mmHg. Pressure transducers were placed at the level of the joint preparation. Final isolation of the MCP joint was achieved by transecting all soft tissue and sectioning bone at the level of the distal metacarpus and dislocation of the proximal interphalangeal joint. (Fig 1). A distended lymphatic vessel visible around the catheterized lateral palmar artery was cannulated with a fine 26 g catheter for collection of lymph. The catheter was capped between collections. A 22 g intraarticular catheter was placed in the dorsal pouch of the MCP joint and attached to a shielded
photoelectric cell drop counter. The drop counter was connected to the physiograph for continuous display. The synovial fluid was continuously drained and collected to maintain atmospheric intraarticular pressure (IAP) and determine trans-synovial fluid flow ($Q_s$). In this system, 16.5 drops was approximately 1 ml of fluid. Synovial tissue blood flow was measured by the colored microsphere technique at three separate times; before joint isolation, after 15 minutes of equilibration in the isogravimetric state (baseline) and after $P_{acir}$ manipulations and return to an isogravimetric state. Body temperature of the joint preparation was maintained with a heat lamp.

**Colored Microsphere Technique**

Synovial blood flow in the isolated joint preparation was measured at three time points in the experiment using a colored microsphere technique. Blue, red or yellow colored microspheres were injected into the arterial inflow line and a reference arterial blood sample was withdrawn from the medial palmar digital artery at a rate of 4.94 ml/min for 1 minute. (Fig 2) Tissue specimens and reference blood samples were digested in KOH and filtered through an 8 μm pore size polyester filter. Dimethylformamide was added to release the dye from the microspheres. Dye concentration was measured by spectrophotometry using a matrix inversion technique for resolution of composite spectra when multiple colors are used. Blood flow was expressed as ml/min/g of tissue.

**Baseline Vascular Measurements and Calculations**

The isogravimetric state (no gain or loss of weight in the isolated preparation) was maintained for 25 minutes before collecting baseline data (5 minutes); circuit arterial blood flow ($Q_{acir}$;ml/min), venous blood flow ($Q_{vcir}$;ml/min), lymphatic flow ($Q_l$;ul/min), synovial flow ($Q_s$;drops/min), and interstitial fluid accumulation (preparation weight).
Synovial fluid was collected during this 5 minute baseline period as a representative sample for the previous 30 min manipulation and flow expressed as number of drops per minute over this 5 minutes. Capillary pressure ($P_{\text{cap}}$) was determined using the venous occlusion technique during the isogravimetric state, and pre- and postcapillary resistance and pre- and post capillary resistance ratios were calculated.\(^2\) Total vascular resistance, precapillary resistance and postcapillary resistance were determined by dividing the arterial-venous, arterial-capillary, and capillary-venous pressure gradients, respectively, by blood flow.\(^2\)

Lymph, plasma and synovial fluid was collected and placed in a capillary tube for total protein and albumin determination and calculation of oncotic pressure, osmotic reflection coefficient and transitional microvascular pressures.

Calculations were made for total vascular resistance ($R_t$) of the isolated joint preparation ($R_t = P_{a_{\text{cir}}} - P_{v_{\text{cir}}} / Q_{a_{\text{cir}}}$); vascular compliance (% change in preparation weight during the immediate elevation in the vascular phase of the weight gain curve/ change in pressure (assuming the filtrate has a unit density and that the change in pressure equals the difference between two sequential $P_{a_{\text{cir}}}$ or $P_{v_{\text{cir}}}$), and tissue compliance (% change in preparation weight during the last 5 minutes of each pressure manipulation trial / change in pressure).\(^2\)

**Measurement of synovial fluid, plasma and lymph total protein concentration**

Total protein concentration was determined using a Dye binding assay\(^h\) using bovine serum albumin as a standard. Synovial, serum and lymph albumin fractions were measured by electrophoresis. Protein (total and albumin) was used for calculation estimates of oncotic pressures for plasma ($\pi_p$), synovial fluid ($\pi_s$), and lymph ($\pi_l$); osmotic reflection coefficient ($\sigma_d$); net filtration pressure; and transitional microvascular pressure.
Fluid oncotic pressure ($\pi$) was estimated by the calculation formula

$$\pi = 1.4C + 0.22C^2 + 0.005C^3,$$

where $C$ is the concentration of the protein in the fluid.\(^2\)

Fluid osmotic reflection coefficient was calculated as $1 - C_s/C_p$ where $C_s$ is the concentration (g/100ml) of protein in the synovial fluid and $C_p$ is the concentration of protein in the plasma collected at the same time period. Calculation was made using the steady-state relationship between synovial fluid and plasma protein concentration ratio ($C_s/C_p$) and synovial fluid flow in a similar fashion as has been done with lymph flow.\(^2\)

Synovial fluid flow was increased with by increasing $P_{v_{cir}}$ until $C_s/C_p$ reached a steady-state, filtration-independent phase.\(^3,18\) The $\sigma_d$ was estimated by using $1-C_s/C_p$, where $C_s/C_p$ was filtration-independent.\(^2,3,18\)

Net filtration pressure was calculated as the difference between the outward and inward capillary forces in the Starling equation [net filtration pressure = $(P_{cap} + \pi_s) - (P_i + \pi_p)$].\(^21\)

Interstitial fluid pressure ($P_i$) was zero because IAP was zero and the equation was reduced to [net filtration pressure = $P_{cap} + \pi_s - \pi_p$].

The calculation of net filtration pressure was modified as follows to account for tissue characteristics since $\sigma_d$ could be calculated in our MCP preparation [net filtration pressure

$$= (P_{cap} - P_i) - \sigma_d (\pi_p - \pi_s).$$\(^1,18,20,21\)

Transitional microvascular pressure for synovial fluid flow ($TMP_s$) was calculated using the formula $TMP_s = \pi_p - \pi_s + P_s$ where $\pi_p$ is the oncotic pressure in plasma, $\pi_s$ is the oncotic pressure in synovial fluid and $P_s$ is the hydrostatic pressure of the synovial fluid.\(^53\)

Hydrostatic synovial fluid pressure was zero in the preparation since synovial fluid was removed and maintained at atmospheric pressure, therefore the formula was reduced to $TMP_s = \pi_p - \pi_s$. Transitional microvascular pressure

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pressure estimates the theoretical pressure at which filtration and absorption forces are in balance. A $TMP$ for lymph flow ($TMP_i$) was estimated similarly. Lymph hydrostatic pressure was zero and lymph flow was zero.

**Pressure Manipulations**

After baseline measurements including tissue blood flow (microspheres) and establishment of the isogravimetric state, $P_{a_{cir}}$ was adjusted to 100, 150, 200 and 250 mmHg for 30 minutes and circuit arterial pressure ($P_{a_{cir}}$), circuit arterial ($Q_{a_{cir}}$) and venous ($Q_{v_{cir}}$) flow, cardiac output (CO), systemic arterial pressure ($P_a$; systolic and mean), synovial fluid production ($Q_s$), lymph production ($Q_l$) and weight changes (vascular and tissue compliance) were recorded during the last 5 minutes of the 30 minute period at each pressure. $P_{a_{cir}}$ was returned to the isogravimetric state for 30 minutes before venous pressure manipulations ($P_{v_{cir}}$). Tissue blood flow (microsphere) and capillary pressure ($P_{cap}$) were recorded during the last 5 minutes of the isogravimetric condition. Capillary pressure was measured using the venous occlusion method. $P_{v_{cir}}$ was adjusted to 20 and 40 mmHg and measurements made as described above. Total time of the isolated joint preparation was a mean of 3.5 hours. The total anesthesia time was a mean of 4.5 hours. The MCP joint was injected with Evans blue-albumin for gross inspection of the articular cartilage after euthanasia and recording the above data.

**Gross evaluation of the joint**

Horses were humanely euthanized with an overdose of sodium pentobarbital (iv). The MCP joint was opened and the cartilage visually inspected for any evidence of osteoarthritic cartilage (surface fibrillation, erosion or fraying). The Evans-blue dye
enhanced the visibility of these lesions. Horses with any evidence of osteoarthritis were eliminated from the study.

Synovium was harvested for microsphere analysis immediately after inspection of the cartilage. The joint capsule and synovial lining were collected from the dorsal and palmar joint pouches. The synovial membrane was dissected from the fibrous layer of the joint capsule and separated into 0.5-2.0 g specimens that were processed as described for microsphere analysis.

**Statistical Analysis**

A non-parametric repeated-measures (time) analysis (Friedman test) was used to determine differences among the variables measured for each pressure manipulation. A LSD post-test specifically located the differences among pressure manipulations. Significance was set at p< 0.05.

**Results**

Data reported are as the mean +/- SEM from horses (n=7) that met all the inclusion criteria. (Table 1) No horses were eliminated from the study.

**Anesthesia and Systemic Hemodynamics**

All horses met the anesthesia inclusion criteria (mean systemic arterial blood pressure ≥ 70 mm Hg, $P_{a}O_{2}$ ≥150 mm Hg and $P_{a}CO_{2}$ ≤ 55 mmHg) for the duration of the study. (Table 2) Mean systemic arterial pressure ranged from 106-136 mmHg and systolic arterial pressure ranged from 142-164 mmHg and did not significantly change during the 4.5 hours of study (p = 0.56 and 0.84, respectively). Cardiac output significantly (p=0.005) decreased from 0.077 L/min/kg at the beginning of anesthesia to 0.045 L/min/kg
at the end of the study. Arterial oxygen tension ($PaO_2$) was highest at the beginning of anesthesia, lowest at the end of the anesthesia and ranged from 359.5-461.3 mmHg ($p=0.05$). Correspondingly, arterial carbon dioxide tension ($PaCO_2$) ranged from 37.4-54.8 mmHg and significantly increased with the duration of anesthesia ($p=0.01$).

Tissue Perfusion

Synovial membrane blood flow before joint isolation was a mean of 0.073 ml/min/g and did not significantly change at isogravimetric conditions immediately after joint isolation (mean 0.16 ml/min/g) or at isogravimetric conditions near the termination of the study (mean 0.049 ml/min/g) ($p=0.26$). (Table 2) The palmar sites tended to have greater tissue blood flow (mean 0.261 ml/min/g) than the dorsal sites (mean 0.058 ml/min/g) only immediately after isolation ($p=0.083$).

Baseline and Isogravimetric Characteristics

Total blood flow and arterial pressure in the palmar artery before joint isolation (i.e., to the distal limb) was 68 ml/min and 149 mmHg, respectively. Venous pressure taken from the distal palmar vein was 20.0 mHg. (Table 2; Fig 2)

Blood flow and pressures for the joint ($Q_{a_{cir}}$, $P_{a_{cir}}$ and $P_{v_{cir}}$) were 37.1 ml/min, 133.6 mmHg and 10.5 mmHg, respectively after joint isolation and during isogravimetric conditions. (Table 2) Capillary pressure was 22.6 mmHg and total vascular resistance was 13.5 mmHg/ml. The precapillary contribution to the total resistance was 73% (9.55 mmHg) and the postcapillary (venous) contribution was 5.42% (0.71 mmHg). (Table 3) At isogravimetric conditions, $P_{cap}$ significantly correlated with the isogravimetric venous pressure ($P_{v_{cir}}$) ($p<0.05$). (Fig 3)
Lymph flow was zero and trans-synovial fluid flow was 5.28 drops/min or approximately 300 ul/min during the isogravimetric state for the joint. (Table 3) The osmotic reflection coefficient was based on synovial fluid and was 0.22 for total protein and 0.18 for albumin. Lymph was only obtained from one horse and did not increase with $P_{vcir}$. Lymph flow was not obtained due to immobilization of the joint preparation, maintenance of an IAP of zero and preferential trans-synovial fluid flow. The oncotic pressure exerted by synovial fluid (7.82 mmHg) was significantly greater than lymph (2.82 mmHg; $p=0.02$) and less than plasma (13.3 mmHg). This resulted in a lower TMP for synovial fluid flow (mean 5.48 mmHg) than for lymph flow (10.7 mmHg; $n=1$).

*Isolated Joint Vascular and Trans-synovial Forces and Characteristic Response to Pressure Manipulation*

Isolated joint blood flow ($Q_{a_{cir}}$) significantly increased with $P_{a_{cir}}$ (range 100-250 mmHg; $p=0.002$); peak 46.83 ml/min at $P_{a_{cir}}$ 250 mmHg) and did not change with increased $P_{v_{cir}}$. ($P=0.67$; Table 2; Fig 4)

Measured joint vascular pressures ($P_{a_{cir}}$ and $P_{v_{cir}}$) closely approximated the predetermined set value for the pressure manipulations (i.e., arterial pressures of 100, 150, 200, 250 and venous pressures of 20 and 40). (Table 2) Venous pressure of the joint at the isogravimetric state ($P_{v_{cir}}=10.5$ mm Hg) was significantly lower than venous pressure of the entire distal limb (pre-preparation baseline; $P_{v_{cir}}=20.0$ mmHg). $P_{v_{cir}}$ did not significantly change as $P_{a_{cir}}$ was increased (range 7.0 - 7.8 mmHg; $p=0.38$). $P_{a_{cir}}$ did not significantly change as $P_{v_{cir}}$ was increased (range 164 - 167.2 mmHg; $p=0.62$). (Table 2) Joint vascular resistance ($R_t$) did not significantly change during arterial or venous pressure manipulations ($p=0.22$).
Trans-synovial fluid flow ($Q_s$) did not significantly increase with increasing $P_{a_{c_{ir}}}$ up to 200 mmHg (mean 4.08 drops/min [approximately 250 µl/min]; Table 2; Fig 5). At $P_{a_{c_{ir}}}$ of 250 mmHg, trans-synovial fluid flow significantly increased (28.6 drops/min; approximately 1.73 ml/min) and returned to the previous flow when the joint preparation was returned to isogravimetric conditions (5.28 drops/min) (p=0.047). Synovial fluid production did not significantly increase with increased $P_{v_{c_{ir}}}$ (p = 0.24).

During the arterial pressure manipulations from isogravimetric conditions, the weight of the isolated joint significantly changed [as measured after the vascular phase of the weight gain curve] (p=0.028). (Fig 6) Weight significantly increased from $P_{a_{c_{ir}}}$ 100 mmHg to $P_{a_{c_{ir}}}$ 250 mmHg. (p=0.03). Weight did not significantly increase with increased $P_{v_{c_{ir}}}$. (Fig 6) Tissue compliance significantly increased as arterial pressure increased. (P=0.05; Table 2; Fig 7)

Vascular compliance was low (overall mean 0.005) in the isolated joint during arterial pressure manipulations, but changed in direct concordance with changes in $P_{a_{c_{ir}}}$ As $P_{a_{c_{ir}}}$ increased from 100-250 mmHg, vascular compliance increased from 0.003 to 0.01. (P = 0.01; Table 2; Fig 8) The vascular compliance returned to 0.002 when the joint preparation was returned to isogravimetric conditions (121 mmHg). (Table 2; Fig 8) A marked increase in vascular compliance occurred from 0.002 to 0.08 during venous occlusion and increased $P_{v_{c_{ir}}}$, and remained increased at $P_{v_{c_{ir}}}$ 40 (p=0.0003).

**Discussion**

Our study is the first to describe a pump-perfused joint model that isolates the articular structures for physiologic investigation. This model successfully maintained physiologic function and synovial tissue perfusion for 4.5 hours. The quantity of tissue and fluid production was adequate for multiple sampling. Expected responses to
physiologic pressure manipulations occurred and added to our existing knowledge on the interrelation of vascular and codependent trans-synovial fluid shifts. For example, filtration pressure for synovial fluid production was mainly influenced by capillary pressure because of the low osmotic reflection coefficient and relatively high permeability to proteins compared to other tissue beds.

The mild changes in systemic hemodynamics observed during the extended periods of general anesthesia were predictable.\textsuperscript{8,17,51,55} The time-related changes in CO, $\text{PaO}_2$ and $\text{PaCO}_2$ has been observed in horses under long term general anesthetic agents\textsuperscript{17} and may be related to alterations in autonomic tone (CO) and positional related responses in animals of large body size.\textsuperscript{8,51} Controlled ventilation depresses cardiac output by decreasing venous return to the heart.\textsuperscript{25} Importantly, for anesthetized horses, all values were well above our minimum criteria and within the reported acceptable ranges; CO 30-60 L/min/kg, $\text{PaO}_2$ 100-350 mmHg, and $\text{PaCO}_2$ 40-60 mmHg.\textsuperscript{24}

Synovium blood flow of the MCP joints (0.073 ml/min/g) was similar to that reported for normal equine carpal joint synovium (0.1 ml/min/g) using a similar technique and did not significantly change throughout the duration of our study.\textsuperscript{22} Total blood flow to the equine digit has been similarly reported at 0.079 ml/min/g.\textsuperscript{2} Evidence of normal tissue perfusion was required to document minimal influence of the manipulations to create the preparation, and the duration of anesthesia. Normal tissue blood flow suggests minimal disturbances in vascular tone, vascular shunting and/or microvascular thrombus formation.

Our total limb flow was 68 ml/min and included flow to the digit and the MCP joint. The isolated joint demonstrated both hemodynamic similarities and differences to other isolated musculoskeletal organs, such as the digit and complete limb preparations.\textsuperscript{2,10,13,46} The MCP joint blood flow was 37.1 ml/min at the $P_{\text{air}}$ of 133
mmHg during the isogravimetric state in lateral recumbency. When our joint arterial pressure was reduced to 100 mmHg, MCP joint blood flow decreased to 11 ml/min. Based on these findings, MCP joint blood flow was 54% of distal limb flow at an arterial blood pressure of 133 mmHg and decreased to 16% of total limb flow at an arterial blood pressure of 100 mmHg. An increase IAP would be expected to further decrease MCP joint blood flow, at least synovial flow, but this was not measured in our study. In a study of the isolated equine digit in a standing vertical position, total digit flow was 7.9 ml/min/100g and $P_{acir}$ was controlled at 100 mmHg. The total digit preparation weights were not reported in the study. A comparison of our physiologic MCP joint values to those obtained for the isolated equine digit are listed in Table 3. Our measured isogravimetric joint circuit pressure ($P_{acir} = 133$ mmHg) was within the physiologic range (80-150 mmHg) and was slightly less than the total limb pressure (149 mmHg). Other reports have measured equine distal digit pressures at ~ 100 mmHg. Based on these reports, pressure manipulations were selected from between 100 mmHg to 250 mmHg in order to assess changes within and beyond the physiologic range. Our measured isogravimetric venous pressure ($P_{vcir} = 10.5$ mmHg) and $P_{cap}$ (22.6 mmHg) for the MCP joint was less than the $P_{vcir}$ (30 mmHg) of the vertical digit. The most obvious explanation for this difference is that our horses and our joint preparation were positioned in lateral recumbency. The high $P_{cap}$ in the digit is countered by a high tissue pressure ($P_t$) and is recognized as being higher than analogous tissue pressures in dogs and humans. Our isogravimetric pressures identified for the joint approximate values in the canine limb.

Osmotic reflection coefficient using lymph could not be regularly measured in our study because lymph flow was absent at normal venous pressures. This was predictable since the limb was in lateral recumbency (no increase in tissue pressure as during
weightbearing). Lymph flow does occur from the equine limb, although in small quantity, when the animal is weightbearing and moving.\textsuperscript{5} Other studies have confirmed that lymph flow from normal joints is minimal.\textsuperscript{34,47} Lymph was obtained in one case but could not be accurately used to calculate $\sigma_d$ as it was not obtained at filtration independent lymph flow rates. Higher values for $\sigma_d$ have been reported based on lymph values in other species' joints\textsuperscript{37} and the equine digit (0.66\textsuperscript{1} and -0.76). Our estimate of the osmotic reflection coefficient, an index of tissue permeability\textsuperscript{6} for the synovium (0.22), was much lower than for lymph (0.6 - 0.7) and reflected the higher total protein concentration in synovial fluid (range 2.6-4.7 g/dl) at high flows. The synovial cavity is the dominant interstitial space in the joint and $\sigma_d$ based on synovial fluid production more accurately reflects trans-synovial fluid forces. Other studies have reported higher $\sigma_d$ (0.7-0.91) for tissues such as skin, intestine, and lung.\textsuperscript{56,59}

Our findings, including a low $\sigma_d$ partially explain why trans-capillary filtration produced synovial flow (trans-synovial filtration), rather than lymph flow. During isogravimetric conditions, the hydrostatic pressure gradient producing flow across capillaries to the synovial fluid ($TMP_s$) was significantly less than for lymph ($TMP_l$). This suggests a relatively high hydraulic conductance (i.e. the change in flow/unit change in driving pressure/area)\textsuperscript{38} for synovial tissue. The greater permeability of synovium ($\sigma_d$), and subsequently the greater concentration of protein in synovial fluid, facilitates osmotically driven fluid flux and filtration to the joint cavity in preference to lymph and has been suggested by other authors.\textsuperscript{35} Our documentation of this combination of physiologic data (higher synovial osmotic pressure, lower $\sigma_d$ and lower hydrostatic pressure threshold

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for synovial fluid flow \( [TMP_s] \) explains our findings of active trans-synovial filtration without lymph flow, and threshold pattern of synovial fluid filtration with increased arterial pressure.\(^{34,39}\) A "yield phenomenon" has been described in studies of trans-synovial fluid absorption and occurred at intra-articular pressures of ~9 cm H\(_2\)O (~7.3 mmHg).\(^{34}\) The presence of a "yield or breaking pressure"\(^{34,39}\) for filtration was apparent in our data since flow increased only after arterial pressure was 200 mmHg. The exact threshold pressure for initiating lymph flow was not investigated. Morphologic changes, including increased surface area and reduced capillary depth partially explain this effect for absorption of synovial fluid.\(^{39}\) Trans-synovial filtration \( (Q_s; \) synovial fluid production) is dependent on the hydraulic conductance of the membrane and the surface area of the organ.\(^{56}\) In our study, the increased \( Q_s \) with increased \( P_a \) was probably due to greater tissue hydraulic conductance, but possibly also increased villous capillary surface area during increased arterial pressures. Based on our data, it is probable that the \( TMP \) of synovium \( (TMP_s = 5.48 \text{ mmHg}) \) was exceeded at arterial pressure of ~200 mmHg and drove fluid production. Since lymph flow did not increase, the \( TMP \) of lymph was probably not exceeded.

Although synovial fluid production did not significantly increase with increased venous pressure, a trend was apparent (Fig 5), and probably represented a corresponding increase in capillary hydrostatic pressure. Increases in capillary pressure correlated closely with increased venous pressure in our study. (Fig 3) The increased capillary pressure probably exceeded the \( TMP \) for synovium when venous pressures of 20 - 40 mmHg were produced. Lymph flow did not increase at these venous pressures suggesting that the \( TMP \) for lymph was not exceeded. Increases in venous pressure in rabbit stifles produced a greater increase in outflow of injected oil than when arterial pressure was increased \( (dQ_s/P_V > dQ_s/P_a; \) ratio 1.54).\(^{29}\) A similar ratio was not detected in our study, but greater
increases in venous pressure (> 40 mmHg) may have been required to produced larger increases in $Q_s$.

Synovial flow should be high when determining $\sigma_d$ so that protein concentration is not flow dependent. Filtration independence was probably achieved in our study for synovial fluid since the high circuit pressures induced high synovial fluid flow rates. Our study documented that lymph flow is unlikely to achieve high flow states in joints with zero IAP when vascular pressure increases. Lymph flow may increase when IAP increased. Our studies suggest that an IAP of > 11 mmHg should have exceeded the $\text{TMP}_l$, and increased lymph flow. Levick reported that an IAP of 18 cm H$_2$O (~13.8 mmHg) minimally increased lymph flow. Pathologic joints with increased IAP however, demonstrate greater lymphatic clearance based on scintigraphy.

Directional changes in MCP vascular forces in response to pressure manipulations produced expected physiologic responses. Steady increases in joint blood flow produced the corresponding increased arterial pressure. (Fig 4) Increasing $P_{acir}$ did not change $P_{vcir}$ and increasing $P_{vcir}$ did not change $P_{acir}$ (Table 2)

Our calculated total joint vascular resistance did not change significantly after each pressure manipulation, although the results were variable (Table 3). The precapillary component of vascular resistance composed the majority of the resistance (72%) similar to values reported for the equine digit (92%). In our study, the calculated pre- and post capillary vascular resistance did not total 100% of the vascular resistance ($R_t$). The difference in pre- and post-capillary vascular resistance and the total vascular resistance of the joint probably reflected the resistance to fluid flow from the capillary to the synovial fluid ($R_s$) and accounted for 21.6% of $R_t$. Our study recorded that filtration exists from the capillary bed to the synovial fluid (~300 ul/min) when IAP is zero. Theoretically, if IAP
was increased enough to stop fluid flow to the joint cavity (based on our calculations of \( TMP_s \), an IAP >11 mg Hg), then \( R_{pre} \) would increase its percent contribution to \( R_t \) as noted in digit preparations that do not have a steady fluid loss or lymph flow would increase. \(^{17}\) We did not measure pre- and post capillary resistance at each pressure manipulation. As synovial fluid production increased with vascular pressure manipulations, \( \%R_s \) may have changed in proportion of \( R_t \).

The immediate change in weight of the preparation after a pressure change suggests changes in vascular volume (ml/mmHg). The total length and area of the vascular bed is known to be proportional to the weight (in grams) of the isolated preparation. Furthermore, acute changes in weight (\( \Delta W \)) caused by pressure changes are due to changes in area and length of the vessels.\(^{23,33,46}\) Associated changes in pressure (\( \Delta P \)) and their relationship are represented by vessel compliance and can be estimated by \( \Delta W/\Delta P \).\(^{2,23,42,50,52}\) This estimate of vascular compliance (\( \Delta W/\Delta P \)) significantly increased as pressure in our studies increased suggesting that vessels become less stiff, (ie, more compliant). Vascular compliance (as estimated by weight changes in amputated perfused hindlimbs of rats) significantly increased with blood flow as in our study, however, an optimal flow was identified (0.25 ml/min) after which vascular compliance decreased.\(^{23}\) We did not identify an optimal flow for vascular compliance in the normal joint.

Our marked increase in vascular volume and compliance after increased venous pressure probably represents pooling of blood in venules. Arterioles are typically less compliant than venules, due to a myogenic response in the muscular layers of the arteriole wall.\(^{20,42}\) Because the method used to increase venous pressure also occluded (decreased) flow, accumulation of blood resulted in a large increase in vascular compliance. Studies using total limb preparations have demonstrated that increases in venous pressure by
venous occlusion produces the same change in limb weight as a 5-10X greater increase in arterial pressure. Increase in vascular load by increasing blood flow would be expected to increase vascular volume and possibly alter vessel vascular function (compliance). Our study documented this to occur in the isolated joint to a small degree (Fig 8). Because venous flows correspondingly increased and no venous occlusion was present (ie, $P_v$ did not change), obstruction to outflow did not occur and vascular pooling of blood was minimal. As with any estimate of vascular compliance that uses weight change for the calculation, it is possible that vessel compliance did not change but that total vascular volume increased as a result of opening new vascular beds, although studies suggest changes in volume occurs initially.

Tissue compliance was estimated from the weight change associated with the hemodynamic pressure manipulations. Preparation tissue weight (g) was a sensitive indicator of fluid accumulation in our MCP preparation and was used to determine when the joint was in the isogravimetric condition (no loss or gain of fluid). The weight changes accurately reflected tissue weight changes because synovial fluid was removed and $P_s$ maintained at zero. Hydrostatic pressure in the capillaries would be expected to increase with increased arterial pressure, and net fluid filtration would increase as documented in our study by an increase in synovial fluid flow. Fluid accumulated in the synovial tissue when MCP joint arterial blood pressures were between 200 and 250 mmHg and was detected as preparation weight gain. As tissue pressure increased and exceeded $TMP_s$, synovial fluid production from the joint also increased. The synovial tissue became more compliant (accepted more fluid for a given pressure change) as pressure increased. (Fig 7) Tissue compliance returned to baseline after the arterial pressures returned to normal (120 mmHg) and an isogravimetric condition was established, although joint weight did not completely return to baseline. (Fig 6) Adequate time (30 minutes) may not have been
provided for complete clearance of the accumulated tissue fluid and may explain why a significant increase in preparation weight was not seen with further increased venous pressures as expected. (Fig 6)

In summary, our study suggests that the isogravimetric MCP joint has no lymph flow and a net filtration of synovial fluid of ~ 300 ul/min at zero IAP. Resistance to synovial fluid flow is responsible for approximately 21% of the total vascular resistance. Arterial pressures > 200 mmHg are required to exceed the threshold arterial pressure for the joint and substantially increase synovial fluid flow. Trans-synovial flow occurs in preference to lymph flow due to the high hydraulic conductance and permeability of synovial tissue (low $\sigma_d$). Transitional microvascular pressures in excess of ~ 11 mmHg are needed to produce an increase in synovial fluid flow. Vascular compliance changes caused by increases in arterial pressure are minimal compared to those produced by increased venous pressure due to the greater elastance of arteries and the larger muscular arterial wall. Synovial tissue becomes significantly more compliant as tissue fluid accumulates with the various vascular pressure manipulations and supports the conclusion that hydraulic conductance is high in synovial tissue. In conclusion, this isolated joint preparation permitted the evaluation of codependent hemodynamic, microvascular, and trans-synovial flow responses to hemodynamic manipulations.
Footnotes

a-Masterflex variable speed pup, Cole Parmer Int, Chicago, IL
b-Cole Parmer Int, Chicago, IL
c-FTO3, Grass Instruments, Quincy, MA
d-Grass Model 70 Polygraph, Grass instrument Co, Quincy, MA
e-Spectramed P23XL transducers Oxnard, CA
f-VR-12, Honeywell Corp, Pleasantville, NY
g-Dye-Track, Triton Technology Inc, San Diego, CA
h-Bio-rad Laboratories Inc, Richmond, CA
**Figure A.1** Pump-perfused isolated metacarpophalangeal joint preparation.
Figure A.2 Equine forelimb and metacarpophalangeal joint before joint isolation demonstrating vascular sites for microsphere studies.
Figure A.3 Capillary pressure versus venous pressure during isogravimetric conditions. Capillary pressure ($P_{cap}$) was greater ($p<0.05$) at higher circuit venous pressure ($P_{cir}$) [$y = 1.46x + 10.8$; $r = 0.87$].
Figure A.4 Change in circuit arterial blood flow \(Q_{a\text{cir}}\) caused by manipulation of circuit arterial \(P_{a\text{cir}}\) and venous \(P_{v\text{cir}}\) pressure \((p < 0.009)\). The first bar from the left (a149) is before joint isolation, the second bar is after joint isolation at isogravimetric conditions. Different letter superscripts are significantly different \((p<0.05)\).
Figure A.5 Synovial fluid production (drops/min) caused by manipulation of circuit arterial ($P_{a_{cir}}$) and venous ($P_{v_{cir}}$) pressures ($p<0.021$). Different letter superscripts are significantly different ($p<0.05$).
Arterial (a) or Venous (v) Circuit Pressures (Pcir mm Hg)
Figure A.6 Preparation weight gain (g) caused by manipulation of circuit arterial ($P_a^{cir}$) and venous ($P_v^{cir}$) pressures ($p<0.04 P_a^{cir}; p < 0.57 P_v^{cir}$). Different letter superscripts are significantly different ($p<0.05$). Letters with a “t” superscript tended to be different ($p<0.1$).
**Figure A.7** Tissue compliance (ml/mmHg) changes caused by arterial ($P_{acir}$) ($p<0.05$) and venous $P_{vcir}$ pressure manipulation. Different letter superscripts are significantly different ($p<0.05$).
Tissue Compliance (ml/mmHg) vs. Arterial (a) or Venous (v) Circuit Pressures (P cir mmHg)

- Arterial (a) pressures at 150, 200, and 250 mmHg
- Venous (v) pressures at 20 and 40 mmHg

Legend:
- 'a' denotes significant difference at 150 mmHg
- 'b' denotes significant difference at 200 mmHg
- 'a, b' denotes significant differences at both 150 and 200 mmHg
Figure A.8 Vascular compliance (ml/mmHg) changes caused by arterial (Pacir) and venous ($Pv_{cir}$) pressure manipulation (p<0.01). Different letter superscripts are significantly different (p<0.05). $t^*$ = a trend (p<0.1) to be different from $Pv_{20}$. Letters marked with a “$t^*$” superscript tended to be different (p<0.1).
<table>
<thead>
<tr>
<th>BREED</th>
<th>AGE (yrs)</th>
<th>SEX</th>
<th>BODY WEIGHT (kg)</th>
</tr>
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<tr>
<td>QH-type</td>
<td>adult</td>
<td>MC</td>
<td>400</td>
</tr>
<tr>
<td>QH</td>
<td>1.5</td>
<td>MC</td>
<td>306</td>
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<td>MC</td>
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<td>F</td>
<td>402</td>
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<tr>
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<td>4.0</td>
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<td>355</td>
</tr>
<tr>
<td>TB</td>
<td>5.0</td>
<td>F</td>
<td>556</td>
</tr>
</tbody>
</table>

MC = male castrated; M = male; F = female; TB = Thoroughbred; QH = Quarter Horse

Table A.1 Signalment of horses used in the isolated metacarpophalangeal joint preparation (n=7).
Table A.2 Systemic hemodynamics and isolated joint vascular function (mean ± SEM) in 7 horses.
Reported values for measured variables:

<table>
<thead>
<tr>
<th>Measured Variables</th>
<th>Values Obtained</th>
<th>Reported values for equine digit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Joint Arterial Pressure (Pacir mmHg)</td>
<td>121.5 +/- 9.49</td>
<td>100.25 +/- 5.12</td>
</tr>
<tr>
<td>Joint Venous Pressure (Pvcir mmHg)</td>
<td>9.0 +/- 2.90</td>
<td>27.37 +/- 4.85</td>
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<tr>
<td>Joint Capillary Pressure (Pcap mmHg)</td>
<td>22.60 +/- 4.07</td>
<td>36.67 +/- 1.79</td>
</tr>
<tr>
<td>Joint Resistance (Rt mmHg/ml)</td>
<td>13.09 +/- 4.27</td>
<td>8.00 +/- 1.05</td>
</tr>
<tr>
<td>Joint Arterial Resistance (Rpre mmHg/ml)</td>
<td>9.55 +/- 3.66</td>
<td>7.43 +/- 1.03</td>
</tr>
<tr>
<td>Joint Venous Resistance (R post mmHg/ml)</td>
<td>0.71 +/- 0.38</td>
<td>0.657 +/- 0.066</td>
</tr>
<tr>
<td>Pre- to postresistance ratio</td>
<td>13.45</td>
<td>12.41 +/- 2.66</td>
</tr>
<tr>
<td>Contribution R pre (%)</td>
<td>72.96</td>
<td>92.88</td>
</tr>
<tr>
<td>Contribution R post (%)</td>
<td>5.42</td>
<td>8.21</td>
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<tr>
<td>Osmotic Reflection Coefficient</td>
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<td></td>
</tr>
<tr>
<td>-Total Protein</td>
<td>0.22 +/- 0</td>
<td></td>
</tr>
<tr>
<td>-Albumin</td>
<td>0.18 +/- 0</td>
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<tr>
<td>Oncotic Pressure Synovial Fluid (πs) mmHg</td>
<td>7.82 +/- 2.9</td>
<td></td>
</tr>
<tr>
<td>Oncotic Pressure Plasma (πp ) mmHg</td>
<td>13.31 +/- 3.7</td>
<td>19.09 +/- 0.84</td>
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<tr>
<td>Oncotic Pressure Tissue (πt) mmHg</td>
<td>2.82 +/- 0</td>
<td>6.59 +/- 1.54</td>
</tr>
<tr>
<td>Net Filtration Pressure [Pcap +πsπp]</td>
<td>17.1 +/- 4.05</td>
<td></td>
</tr>
<tr>
<td>Net Filtration Pressure [((Pcap)−d(πp−πs))]</td>
<td>21.4 +/- 4.05</td>
<td></td>
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<tr>
<td>Vascular Compliance (ml/mmHg)</td>
<td>0.003 +/- 0.001</td>
<td>0.012 +/- 0.002</td>
</tr>
<tr>
<td>Tissue Compliance (ml/mmHg)</td>
<td>0.025 +/- 0.007</td>
<td></td>
</tr>
</tbody>
</table>

Table A.3 Isolated metacarpophalangeal joint vascular and fluid values (mean ± SEM) at isogravimetric conditions.
References


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