

CLINICAL IMPLICATIONS OF INDOMETHACIN
SUPERFUSED OVER THE CAPILLARIES OF FROGS
WITH ACTIVATED WHITE BLOOD CELLS

by

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ABSTRACT

Treatment for inflammation is controversial. The purpose of this study was to investigate the effect of indomethacin on capillary permeability in animals with signs of systemic inflammation. We hypothesized that the permeability of an individual capillary would be lower after a shear stress challenge in the presence of indomethacin when evidence of systemic infection was present in the animal. Frogs (n=13) were pithed and the mesentery was exposed, hydraulic conductivity (L_p) was assessed at 30 cm H₂O using the modified Landis technique after an abrupt change in shear stress. Two capillaries from each frog were used; one was a control and one with indomethacin superfused over the tissue. The frogs showed a systemic infection (nitro blue tetrazolium activation) but individual capillaries had no evidence of rolling or sticking white cells. There was a significant decrease in L_p (P=0.002) when comparing the control and treatment vessels. The results of the analysis indicate capillary L_p assessed in mesentery of infected frogs, decreased when exposed to shear stress and indomethacin. The data imply that gaps between endothelial cells may get smaller when indomethacin is introduced into the system decreasing the flow of fluids out of the capillary.

CHAPTER 1

INTRODUCTION

Inflammation was first described by Roman medical writer, Aulus Cornelius Celsus in the first century AD (Cotran, Kumar, & Collins, 1999). Inflammation is a rapid activation of biochemical and cellular mechanisms that is relatively nonspecific (Trask, Rote, & Huether, 2006). The cardinal signs of inflammation are redness, heat, swelling and pain. Dilation of the small blood vessels in the injury area causes the redness also known as erythema. Increased blood flow through the area results in the perception of heat. Accumulation of fluid outside the blood vessel is what causes edema. The distortion of the tissue by the swelling and chemical mediators, such as bradykinin, serotonin and prostaglandins cause the pain felt during inflammation. The goal of inflammation is ultimately to rid the organism of both the initial cause of cell injury and the consequences of the injury (Cotran, Kumar, & Collins, 1999). German pathologist, Rudolf Virchow, noted in the Nineteenth century that a fifth consequence of inflammation is the loss of function to the inflamed area (Trask, Rote, & Huether, 2006). Even small areas of inflammation are painful with a corresponding loss of function. If the area of inflammation is large, pain and injury to tissue function can be an overwhelming consequence (Trask, Rote, & Huether, 2006). “Three major events occur during the inflammatory response: the blood supply to the affected area is increased substantially, capillary permeability is increased, and leucocytes migrate from the capillary vessels into

the surrounding interstitial spaces to the site of inflammation or injury” (Rankin, 2004, p. 3).

Leukocytes (white blood cells [WBCs]) are one of the cellular components of blood, they defend the body against organisms that cause infection and they remove debris in the circulation (McCance, 2006). Leukocytes are described according to structure and function. The categories of leukocytes are granulocytes, agranulocytes and lymphocytes. Granulocytes include neutrophils, basophils and eosinophils and are described as phagocytes (“cell eating”). Agranulocytes, are monocytes and macrophages also described as phagocytes; lymphocytes are described as immunocytes (create immunity) (McCance, 2006). Mast cells are also located in the blood; they contain an abundant mixture of biochemical mediators, including histamine, chemotactic factors, and cytokines (McCance, 2006). In response to a stimulus, such as infection or inflammation, mast cells release quickly and the effects are seen immediately (Trask, Rote, & Huether, 2006).

Treatment for inflammation is controversial (Rote, 2006). Do we let the body handle the inflammation on its own or do we “help” by introducing outside treatments? “The inflammatory response is activated to protect the body from further injury, prevent infection of the injured tissue, and promote healing” (Trask, Rote, & Huether, 2006, p. 175). Inflammation occurs at the vascular level and produces microscopic changes that include blood vessel dilation, increased vascular permeability, and leakage of fluid out of the vessel, the adherence and migration of white blood cells to the inner walls of the vessel and to the site of injury (Trask, Rote, & Huether, 2006). The permeability of a

capillary determines how much inflammation is going to occur with a certain injury.

Controlling capillary permeability is the key to controlling inflammation (Porter, Jones, & Winland-Brown, 2007; Cotran, Kumar, & Collins, 1999).

Purpose

The purpose of this study was to investigate the effect of indomethacin on capillary permeability in cells with signs of systemic inflammation. The aim of this study was to conduct a secondary data analysis gathered from the research of Donna A. Williams, PhD, analyze it, and report findings of such research. Dr. Williams' research focuses on capillary permeability, specifically, how a change in shear stress ($\Delta\tau$) and the introduction of non-steroidal anti-inflammatory drug (NSAID), indomethacin, changes L_p of the capillary. Her research included two data sets of capillaries, one from "healthy" and the other from "infected" frogs. The data set for this study was from the "infected" frogs. Dr. Williams determined that frogs were infected if they had activated white cells, which indicated a systemic inflammatory process.

Background and Significance of Study

Taking medications in the absence of an illness or need seems strange; however, as a registered nurse this investigator has observed this time and time again. People will take an NSAID in the absence of inflammation "to keep the inflammation away".

Looking at the effects of medications at a cellular level will increase the knowledge of how and when to use medications. Dr. Williams is one of the few bench scientists who

use drug agents when studying live capillaries. The focus of this paper was to assess reactions at the cellular level then start understanding systemic reactions.

As a critical care nurse for the past 10 years, this author has seen many reactions people have to inflammation and the multiple ways they and their doctors treat these reactions. “Diseases in the intensive care unit are a most common reason for death in middle age and younger, and a bad outcome can often be related to disturbances of blood circulatory control systems, also including mechanisms controlling tissue and plasma volume” (Grande, 2007, p. 1). Inflammation is the process by which the body’s [WBC’s] and chemicals protect us from infection and foreign substances such as bacteria and viruses (Etzioni, 2009).

Inflammation is often treated with a classification of medications called NSAID’s. In 1971, the mechanism of action of a nonselective inhibitor of cyclooxygenase (COX) was described. Cyclooxygenase is an enzyme that participates in prostaglandin synthesis from arachidonic acid. Prostaglandins are hormone-like molecules that have a wide variety of effects, some of which lead to pain, fever, and inflammation. In 1990, two different COX enzymes were discovered, known as COX-1 and COX-2 (Solomon, 2009b). COX-1 is present in most tissues and functions primarily to maintain the lining of the stomach and is involved in kidney and platelet function (Solomon, 2009b). COX-2 is found mainly at the site of inflammation and so inhibiting COX-2 accounts for the anti-inflammatory effect of an NSAID (Solomon, 2009b). Inhibiting COX-1 is undesirable due to the adverse effects of causing ulcers, bleeding and kidney problems (Solomon, 2009a).

Indomethacin an NSAID was discovered in 1963 and was approved by the Food and Drug Administration (FDA) in 1965. Indomethacin medication was chosen for this study because of its affect on COX 1 and 2. Indomethacin is not used much today clinically, due to newer NSAIDs being produced. Osteoarthritis, rheumatoid arthritis, ankylosing spondylitis and gout are a few diseases that indomethacin is still used to treat today. Gout is caused by inflammation when needle-like crystals are deposited in connective tissue and/or in the fluid that cushions a joint (called synovial fluid) (Becker, 2009). These crystals are made of uric acid, a substance produced when the body breaks down purines found in human tissue and many foods such as beer, yeast, organ meat, legumes, mushrooms, spinach, asparagus and cauliflower. Most uric acid is carried through the bloodstream to the kidneys, which eliminate it from the body through the urine. However, if the body produces too much uric acid or if the kidneys cannot adequately eliminate it, uric acid builds up in the blood (Becker, 2009). Taking indomethacin helps decrease the inflammatory process of gout, making it easier for people to continue with activities of daily living (Man, Cheung, Cameron & Rainer, 2007; Boss, 2007). Unfortunately, people continue to take this medication after the inflammatory process is remediated, increasing their chance for unwanted adverse effects (Solomon, 2009a). The inhibition of prostaglandins in the stomach by indomethacin may cause an adverse effect of the development of peptic ulcers in some individuals. This study discusses the need for inflammatory medications but it also notes that they should not be used when the inflammatory process is not present.

Statement of Problem and Research Question

According to Williams (1999), shear stress on vascular function has been studied extensively except at the level of the individual capillary. Researcher Williams wanted to evaluate L_p of an “inflamed capillary” in response to $\Delta\tau$ after exposing the capillary to indomethacin. The secondary analysis by this investigator is from the research completed by Williams. Data were analyzed to answer the following hypothesis.

Hypothesis

L_p of an individual capillary will be lower after a shear stress challenge in the presence of indomethacin when evidence of systemic infection is present in the animal.

Definitions

- **Indomethacin:** Developed in the 1960’s, indomethacin is a NSAID that inhibits the enzyme cyclooxygenase necessary for the formation of prostaglandins. It also inhibits the motility of polymorphonuclear leukocytes. Indomethacin has also been used clinically to delay premature labor, reduce amniotic fluid in polyhydramnios and to treat patent ductus arteriosus. Indomethacin inhibits both COX-1 and COX-2, thus inhibiting the production of prostaglandins in the stomach and intestines which protects the mucous lining of the gastrointestinal tract therefore causing ulcers (Solomon, 2009).
- **Capillary Permeability:** The property of blood capillary walls that allows for the selective exchange of substances. Small lipid-soluble molecules such as carbon dioxide and oxygen move freely by diffusion. Water and water-soluble molecules cannot pass

through the endothelial walls and are dependent on microscopic pores. These pores show narrow areas (tight junctions) which may limit large molecular movement (Guyton & Hall, 1996).

- **White Blood Cell:** A cellular component of the blood that lacks hemoglobin, has a nucleus, is capable of motility, and defends the body against infection and disease by ingesting foreign materials and cellular debris, by destroying infectious agents and cancer cells, or by producing antibodies (Guyton & Hall, 1996).
- **Inflammation:** The vasodilatation of the local blood vessels with consequent excess local blood flow increases permeability of the capillaries with leakage of large quantities of fluid in the interstitial space, causes clotting of the fluid in the interstitial spaces because of excessive amounts of fibrinogen and other proteins leaking from the capillaries, migration of large numbers of granulocytes and monocytes into the tissue, and swelling of the tissue cells (Guyton & Hall, 1996)
- **Shear Stress:** A force tending to cause deformation of a material by slippage along a plane or planes parallel to the imposed stress (Mosby, 2006).

Assumptions

The assumptions of this study were: The geometry of the frog capillary is a cylinder (Michel, Mason, Curry, Tooke, & Hunter, 1974). Indomethacin that is being superfused over the frog mesentery will reach the capillary beds. Pithing of the frogs did not alter the capillary response or flow. The blood flow of the capillary is not altered when the frog mesentery is exteriorized.

Limitations

There were several limitations identified in the study. The first limitation was that frog mesenteric capillaries are similar to other continuous capillaries but they are different when compared to mammalian vessels. Frog capillaries are larger with diameters close to 20 μm because there are approximately six endothelial cells per cross section compared to 1-2 in mammalian cells. “The large size makes these vessels suitable for microperfusion experiments” (Curry, 1986, p. 371). According to Curry (1986), frog capillaries have “transcapillary pathways that are experimentally testable” (p.377). Frog mesentery capillaries also have gaps that average 150 nm long and occur about every 2-4 μm around the endothelial cell periphery (Adamson, Liu, Fry, Rubin, & Curry, 1998).

The second limitation was that the frogs used were wild-caught. There was no control over what the frogs had been exposed to prior to capture. These frogs were examined for signs and symptoms of infection and all capillaries used were devoid of rolling or sticking white cells. Capillary inspection for white cells has been used for years to determine if inflammation is present locally (at the capillary itself). Activation of white cells using a solution of nitro blue tetrazolium (NBT) was completed to determine if systemic infection was present in frogs being studied.

The experimental protocol was performed with solution that was cooled to 15 degrees Celsius, which is the temperature that the frogs are kept prior to use. This limits the data gathered from this study to be compared to other capillary studies being performed at this temperature.

The final limitation of this study was that clinical practice cannot be based directly on the basic science of this particular study; rather the science provides a place to start. “The belief of the scientific community is that we have an obligation to apply the full power of science to solving the toughest problems facing humanity, even if they are potentially contentious. And as scientists and clinicians, we must do whatever we can to relieve pain and suffering. However, when entering these kinds of domains, scientists have a duty to be extremely sensitive to the potential implications and uses of the results of their work” (Leshner, 2004, p. 1)

CHAPTER 2

REVIEW OF THE LITERATURE

The review of literature addresses several topics; inflammation, indomethacin, capillary permeability, shear stress, and prostaglandins. The literature regarding inflammation included a discussion of pathophysiology, symptoms and treatments. In addition descriptions of indomethacin, capillary permeability, shear stress and prostaglandins are explored.

Inflammation

Inflammation can be defined as the bodies' normal response to injury or infection. According to Trask, Rote, & Huether (2006) three major events occur during the inflammatory response: increased blood supply to the affected area, increased capillary permeability and migration of leukocytes into the interstitial space surrounding the site of inflammation. In 1961, Majno & Palade demonstrated that during acute inflammation, vascular permeability in the venular capillaries increases and is associated with the development of gaps between the endothelial cells (Michel, 1998).

The body's response to inflammation has three purposes to: neutralize and destroy invading and harmful agents, limit the spread of harmful agents to other tissue, and prepare damaged tissue for repair (Peterson, Symes, & Springer, 1995). Inflammation is a protective mechanism that begins the healing process.

There are many causes of inflammation. The inflammatory response will be evoked by any injury to tissue. Injury can be exogenous, outside the body, or

endogenous, inside the body. Exogenous forms of injury may result from trauma, surgery, burns or chemical injury to skin. Endogenous injuries may result from tissue ischemia such as pulmonary embolism or myocardial infarction.

Inflammation and infection often get confused because they usually exist together. Normally, inflammation coexists with an infection, however not all inflammation involves an infectious agent.

Inflammation can be acute or chronic condition. Acute inflammation lasts less than two weeks and involves a distinct set of events. Chronic inflammation is more diffuse and extends over a long period of time. However, the inflammatory response is the same, regardless of cause, and includes chemical responses, vascular responses, and cellular responses (Peterson, Symes, & Springer, 1995).

Chemical Response

When tissue is injured, chemicals are released by activated granulocytes, lymphocytes and macrophages. The mast cell is the most important activator in the inflammatory response. Located in the loose connective tissue close to blood vessels, mast cells are cellular bags of granules. Mast cells degranulate and release their contents into the extracellular space, activating the inflammatory response (Figure 1).

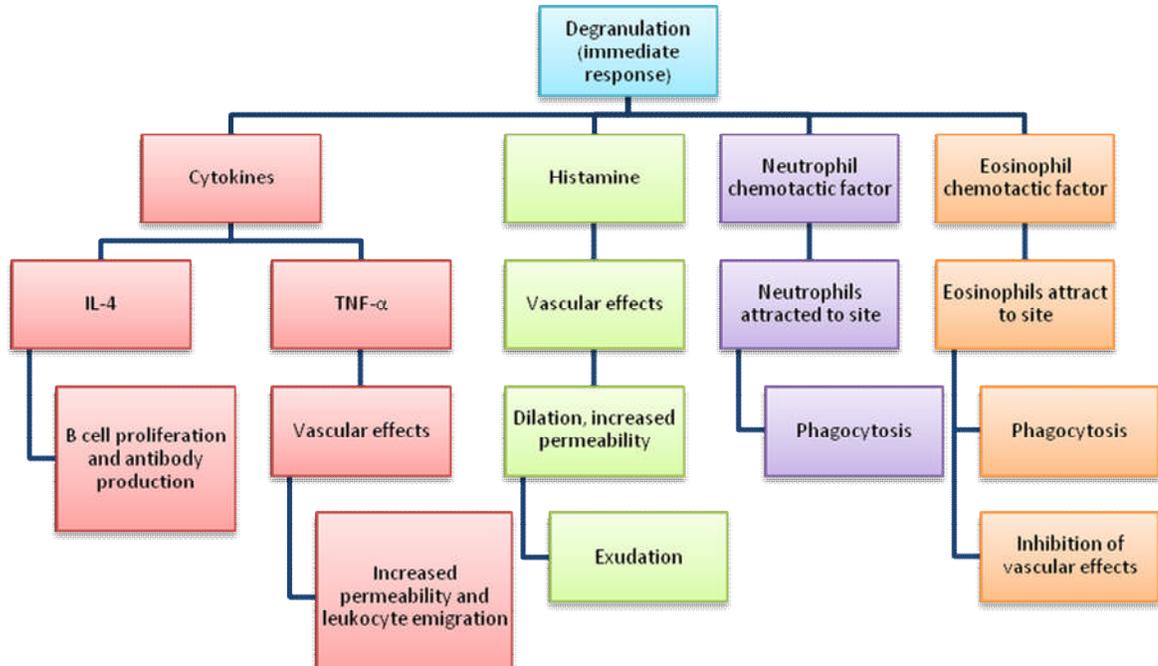


Figure 1. Effects of Degranulation by Mast Cells

Adapted from “Alterations of Immunity and Inflammation” by N.Rote, 2006, Pathophysiology: The Biologic Basis for Disease in Adults and Children, p. 250. Copyright 2006 by Mosby, Inc.

The following chemicals are released histamine, prostaglandins, leukotrienes, cytokines, oxygen radicals and enzymes. Histamine increases vascular dilation and permeability, allowing fluid to escape from the blood vessels.

Mast cells also synthesize mediators such as prostaglandins, leukotrienes and cytokines (Figure 2).

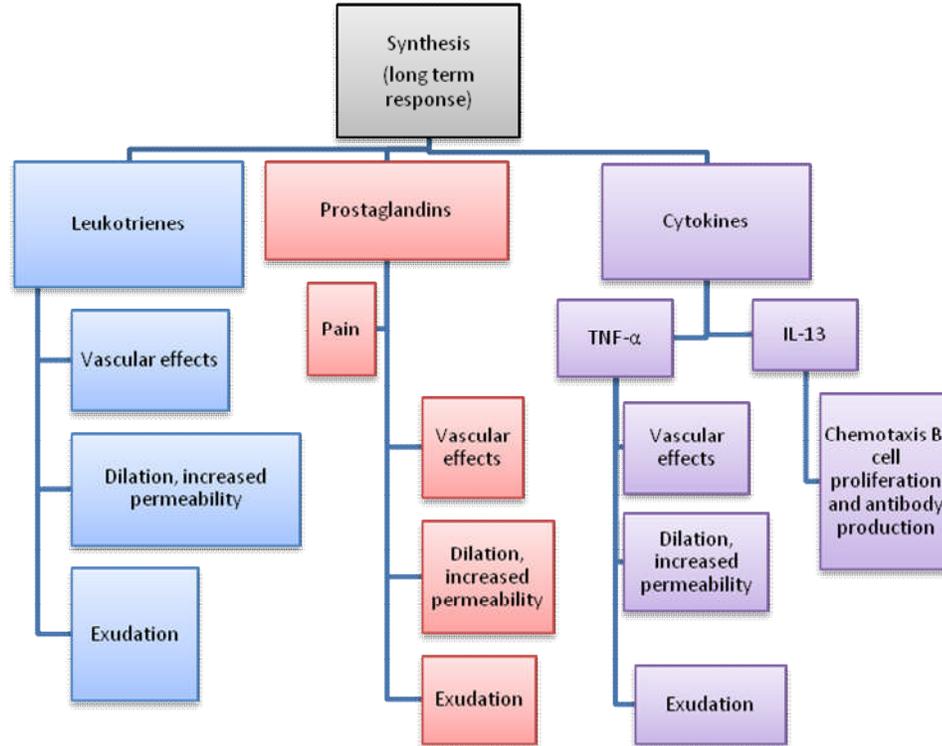


Figure 2. Effects of Synthesis by Mast Cells

Adapted from "Alterations of Immunity and Inflammation" by N.Rote, 2006, Pathophysiology: The Biologic Basis for Disease in Adults and Children, p. 250. Copyright 2006 by Mosby, Inc.

Prostaglandins arise from the cyclooxygenase pathway, causing vasodilatation, increasing permeability and platelet aggregation. The release of prostaglandins also causes pain by enhancing the sensitivity of pain endings. The role of prostaglandins in inflammatory process has been studied by multiple researchers. Studies of rheumatoid arthritis (McCoy, Wicks, & Audoly, 2002), sepsis induced cardiomyopathy (Mebazaa, DeKeulenaer, Paqueron, Andries, Ratajezak & Lanone, 2001), chronic pancreatitis (Sun, Reding, Bain, Heikenwalder, Bimmler, & Graf, 2007), chronic rhinosinusitis (Perez-Novo, Claeys, Van Cauwenberge, & Bachert, 2006), and pain from inflammation (Beloeil, Gentili, Benhamou, & Mazoit, 2009) (Bar, Natura, Telleria-Diaz, Teschner,

Vogel & Vasquez, 2004) all concluded that the production of prostaglandins is increased before any of these inflammatory states occur.

Leukotrienes cause smooth muscle contractility, bronchospasm, and an increase in vascular permeability. Cytokines are proteins involved in cell to cell communication, coordinating antibodies and T-cell immune interactions and amplifying immune reactivity (Mosby, 2006). Enzymes and oxidizing agents attack lipids and proteins, causing cell membrane destruction and digestion of intercellular matrix and protein structures, which in turn increases permeability (Peterson, Symes, & Springer, 1995).

Vascular Response

Due to tissue damage after injury, there is an immediate vasoconstriction resulting in tissue hypoxia and acidosis (Peterson, Symes, & Springer, 1995). Following vasoconstriction redness, pain, and heat are present and increases blood flow to the injured area. This increase in blood flow to the area raises the pressure in the vessels pushing fluid out into the surrounding tissues, causing swelling and edema (Rankin, 2004). Due to the chemicals being released, venous capillaries become more permeable causing even more fluid to leak into surrounding tissues, causing increased pain and impaired function (Peterson, Symes, & Springer, 1995). Fibrin is deposited into the lymph system, causing a blockage, which delays the spread of toxins. These vascular changes become beneficial because toxins are diluted by fluid leaking out of the vessels with the remaining blood becoming thick and circulation to the area slows (Rote, 2006).

Cellular Response

Normally leukocytes travel down the center of blood vessels, rarely interacting with vessel walls. However, during inflammation, leukocytes line the walls of the vessels, (referred to as “margination”,) so they are more able to move out of the vessel and to the site of inflammation (Peterson, Symes, & Springer, 1995). The terms rolling and sticking white cells results from the leukocyte activation cascade. The cascade involves, capture, rolling, slow rolling, firm adhesion, and transmigration of the leukocyte (Rankin, 2004). The leukocyte activation cascade is the gold standard for determining inflamed cells (Rankin, 2004).

Healing and Repair

Healing is the complete restoration of structure and function (Peterson, Symes, & Springer, 1995). Complete resolution is only possible if the tissue is capable of regeneration. If tissue is unable to regenerate, then tissue is repaired with connective tissue resulting in a scar. Bone is capable of regeneration, while myocardial tissue cannot regenerate (Peterson, Symes, & Springer, 1995).

Indomethacin

Indomethacin is a potent inhibitor of prostaglandin synthesis. Prostaglandins sensitize afferent nerves and potentiate the action of bradykinin in inducing pain in animal models (Solomon, 2009b). Prostaglandins are known to be among the mediators of inflammation. Indomethacin is an inhibitor of prostaglandin synthesis. Its mode of action may be due to a decrease of prostaglandins in peripheral tissues. Indomethacin has

been an effective anti-inflammatory agent, providing relief of symptoms but not altering the progressive course of the underlying diseases (Hawkins & Rahn, 1997). Woo, Man, Lam & Rainer (2005) showed that indomethacin was safe and an effective treatment for pain from blunt injuries. Post cardiac surgery patients were identified to have a decreased length of stay when indomethacin was used in conjunction with morphine (Reimer-Kent, 2003). Indomethacin is rapidly and almost completely absorbed in the gastrointestinal tract following oral consumption (Flower, Moncada, & Vane, 1985). Peak concentration in plasma is within two hours of fasting, somewhat delayed if taken with food. Indomethacin is 90% bound to plasma proteins (Solomon, 2009b).

Indomethacin is an amphiphilic agent that is hard to retain inside liposomes as it penetrates lipid bilayers quickly (Flower, Moncada, & Vane, 1985). Hernandez, Estelrich, Montero, & Valls (1989) studied the effects of indomethacin on monolayers and described criteria for selecting the correct lipid composition to encapsulate the drug to lessen leakage that causes intestinal side effects. Iwai, et al. (2009) reported, the administration of indomethacin caused deep damage in the mucosa of rats, where the epithelial cells were totally denuded and severe edema was noted after one day.

Mion, Cuber, Minaire, & Chayvialle (1994) showed that intravascular perfusion of higher concentrations of indomethacin (2.5mM) increases the intestinal permeability with increased inflammation changes in histology.

Capillary Permeability

“Capillary endothelium of most vertebrate tissues and organs consist of a single layer of flattened cells bound together within an acellular basal lamina, presumably secreted by the cells.” (Renkin, 1977, p. 735) Materials may penetrate this structure by passing directly through the cells and penetrating without entering the cells (by extracellular channels) or through the junctions between them (Renkin, 1977). Figure 3 is a diagram of capillary transport pathways for an unfenestrated capillary.

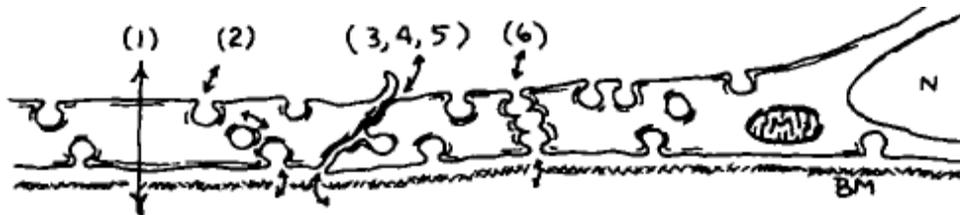


Figure 3. Diagram of Transport Pathways in Capillary Endothelium

Unfenestrated capillaries, found in skin, skeletal muscle, frog mesentery, intestinal smooth muscle, heart, and lung. The lumen is at the top, and the basement membrane (BM) is indicated by stippling. N = nucleus; M = a mitochondrion, just to show that these are present. Presumed transport pathways are as follows. (1) Cellular, directly through endothelial cell membranes and cytoplasm (water, small nonpolar solutes, lipid-soluble solutes). (2) Vesicular, small cytoplasmic vesicles believed to shuttle back and forth between opposing cell surfaces and to exchange fluid and solutes by equilibrating contents at each surface. Though all components of plasma and interstitial fluid except those too large to enter the vesicles may be exchanged. This pathway may be quantitatively significant only for plasma proteins and other large molecules. Macropinocytosis may also contribute to endothelial transport of macromolecules. (3) Lateral diffusion in cell membranes through junctional complexes may provide a pathway for water-insoluble lipids. (4) Narrow, "small pore" junctions may provide pathways for diffusion and ultrafiltration exchange of water and lipid-insoluble solutes up to the size of small plasma proteins. (5) Wide, "large pore" or "leak" junctions permit exchange of plasma proteins and other large molecules as well. The structure of the junctions appears to vary, not only between different tissues and organs, but from arterial to venous regions of the microvascular bed in the same organ. (6) Transitory open channels formed by the confluence of chains of micropinocytotic vesicles may provide an additional extracellular transport pathway. Depending on the size of the vesicular junctions, its characteristics might resemble either the "small pore" or "large pore" pathway. Adapted from "Multiple pathways of capillary permeability" by E.M. Renkin, 1977, *Circulation Research*, 41, p.736. Copyright 1977 by the American Heart Association. Reprinted with permission. (Appendix A).

The exchange of fluid between plasma and interstitium is determined by the hydrostatic and oncotic pressures in each compartment (Rose, 2009b). Starling's hypothesis states that fluid filtration through a capillary membrane is dependent on the balance between the pressure of blood on the membrane and the osmotic pressure of the membranes (Huether, 2006). The law says the passage of fluid out of a capillary depends on the hydrostatic and osmotic pressures of the blood and the same pressures of tissue fluid. The net effect of the opposing pressures determines the direction and rate of flow (Huether, 2006). Starling's law is expressed as $\text{Net filtration} = L_p S \times (\Delta \text{hydraulic pressure} - \Delta \text{oncotic pressure})$ (Rose, 2009b). Edema development requires an alteration in one or more of Starling's forces being an increase in net filtration (Rose, 2009b). Edema is due to the changes in venous pressures not arterial pressures in the capillary. The precapillary sphincter constricts when arterial pressure increases, minimizing the increase in capillary hydrostatic pressure and preventing edema development (Rose, 2009b).

The first measurements of capillary permeability were made by Landis in 1926 on single capillaries of frog mesentery. The Landis technique measures transvascular filtration rate by measuring the movement of red blood cells in a micropipette-occluded capillary. Modifications were made to this technique by Michel, Mason, Curry, Tooke, & Hunter in 1974. The modified Landis technique was used by Dr. Williams to perform the research done for this data analysis.

Clough, Michel, & Phillips (1988) reported that an increase in permeability to fluid and macromolecules is one of the best known consequences of tissue injury.

Increased permeability is due to the formation of gaps in the glycocalyx, which then increases the leakage of fluid into the interstitial space. Adamson (1990) showed that frog mesenteric capillaries exposed to a protease treatment, resulted in a moderate increase of L_p . A decrease in cationic ferritin was noted in the pronase perfused capillaries, showing large changes to the glycocalyx structure that resulted in the outflow of molecules in the capillary. Capillaries become more permeable due to an increase in their L_p , usually after an exposure of some type of stimulus. Clough, Michel & Phillips (1988) concluded that “the increase permeability of inflamed microvessels is associated with the appearance of gaps between the endothelial cells” (p.112). Williams & Huxley (1993) concluded that increase in capillary permeability was also dependent on where the capillary was in the network and when the measurement was done. This research has directed many other researches to look at factors that might cause the appearance of these gaps. The application of heat (Clough, Michel & Phillips, 1988), changing shear stress (Williams, 1999, 2003, 2006; Davies, 1995; Kim, Harris, & Tarbell, 2005; Sill, Chang, Artman, Frangos, Hollis & Tarbell, 1995), endothelial cell shape and size (Adamson, 1993), are all ways researchers have been able to change capillary permeability. “Capillary permeability increases if there is alteration, damage, or death of the endothelial cell or a change in the intercellular space, which renders the endothelial cell and basement membrane, a less effective barrier to the passage of larger molecules” (Fishel, Are, & Barbul, 2003, p. s505). The insult of endotoxin exposure, ischemia-reperfusion, vessel injury with platelet deposition, or vessel injury is what begins the damage to endothelial cells (Fishel, Are, & Barbul, 2003).

Majno & Palade (1961) stated that “in acute inflammation or following injection of histamine, bradykinin, or 5-HT, vascular permeability increases in the venules and venular capillaries and is associated with the development of gaps between the endothelial cells”. Majno, Shea & Leventhal (1969) suggested that the gaps were formed by the contraction of one cell away from another. “When permeability is increased by exposure of the tissues to increasing doses of agents such as bradykinin and histamine, it appears that the gaps which have formed do not enlarge further but that the number of gaps between the endothelial cells increases” (Michel, 1988, p.21). “Tight junctions between adjacent epithelial cells create a physiological intracellular barrier which maintains distinct tissue spaces and separate the apical from the lateral plasma membranes. Tight junctions are important structures in controlling the diffusion of molecules such as toxic compounds and drugs through the intestine” (Usami, Muraki, Iwamoto, Ohata, Matcushita & Miki, 2001, p 351).

In 1995, Neal & Michel showed that permeability of frog microvessels increased when perfused with the ionophore A23187. This ionophore was used because it mimics the action of histamine and bradykinin by increasing the concentration of calcium within the endothelial cells. Kajimura & Michel (1999) showed that permeability of potassium in frog mesenteric capillaries increases with increased flow. This increase then can be inhibited by infusing specific pharmacological agents showing that the relation between potassium and flow is a biological phenomenon and not an artifact of measurement. The study demonstrated that indomethacin failed to influence the potassium and flow

relationship. Indomethacin was used because prostacyclin is a mediator of flow-dependent response in microvascular beds.

Shear Stress

Increasing blood flow through arteries that resulted in vasodilatation was first noticed in 1933 (Davies, Spaan, & Krams, 2005). It was over 50 years later that shear stress was noted to be an important factor in the response of the endothelium to changes in varying viscosity. Increasing blood flow to a tissue not only raises the rate of delivery of solutes, but also increases their transport through the endothelium into the tissues (Kajimura, Head & Michel, 1998). Shear stress increases capillary permeability by a mechanochemical transduction across the cell membrane (Haidekker, L'Heureux & Frangos, 2000).

Sill et al. (1995) demonstrated that shear stress increased L_p of cultured endothelial monolayers. Shear stress induced an increase in L_p observed by Sill et al. (1995) was a result of a physiological response of the cells rather than any mechanical damage to the monolayer. Shear response requires cellular activity. Jo, Dull, Hollis, & Tarbell (1991) demonstrated an increase in permeability in cultured endothelial albumin due to a shear stress challenge. Kim, Harris, & Tarbell (2005), showed that increase in L_p by shear stress disappeared within 20-30 minutes after removal of stimulus and that L_p was greater in capillaries than terminal arterioles.

The change in shear stress on a capillary has been shown to correlate positively in the L_p of the capillary (Williams, 1999; Williams, 2003; Williams, 2007). These studies show that capillary permeability changes when a change in flow ($\Delta\tau$) is present in the

intact capillary. Capillaries respond according to the amount of shear stress, resulting in a change in L_p . The rise in L_p allows for increased flow from the capillary. Williams (2003) concluded that changes in fluid shear stress can alter capillary filtration coefficient.

Research by Frangos, Eskin, McIntire & Ives (1985) showed that endothelial cells grown in culture release prostacyclin in response to shear stress. The rate of prostacyclin production from endothelial cells exposed to a pulsatile shear stress was twice that of cells exposed to steady shear stress and sixteen times greater than that of cells in stationary culture. A study completed by Alshihabi, Chang, Frangos & Tarbell (1996), also showed that prostaglandins are released in response to a shear stress challenge in vascular smooth muscle. These studies all have been done on cells in culture. Tsao, Lewis, Alpert & Cooke (1995) concluded that endothelial lining is altered by shear stress due to stimulation of nitric oxide and lesser by prostacyclin effects. Waters (1996) demonstrated that endothelial cell permeability was related to an increase in shear stress and not related to the blocking of nitric oxide or cyclooxygenase. Secondary analysis of data by Dr. Williams and Staigmiller (2010) from Dr. William's NIH funded project #R01 HL63125 showed that L_p of capillaries located in frogs free from infection did not differ from control or indomethacin-superfused capillaries, when subjected to a shear stress challenge.

CHAPTER 3

METHODS

Data for this study were obtained from Donna Williams PhD; National Institutes of Health (NIH) funded project #R01 HL63125 “Shear stress and cellular control of capillary filtration” The Methods and Materials section will describe Dr. Williams’ method of obtaining data. The methods and protocol were approved by the Animal Care and Use Committees at the University of Missouri and Montana State University. Data were used from Dr. Williams’ study to examine the effects of indomethacin on a frog capillary, in which the whole frog showed a systemic inflammatory process. The information gathered applies to the influence of NSAID’s on human capillaries. The purpose of this study was to investigate indomethacin’s effect on capillary permeability in animals with signs of systemic inflammation.

Following training by Dr. Williams, a video was used for (Figure 4) each of the capillaries undergoing the experimental protocol by Dr. Williams. A total of 19 videos with two capillaries (pre and post intervention) on each video were watched. The videos were selected randomly; the health or states of the capillaries were unknown. A ruler was made from yellow tape with marks of 0.5 μm apart. The ruler was used directly on the video monitor to perform this project. Diameter was measured with the tape measure in three places spaced 100 μm apart and averaged. The tape was then placed on the monitor following the capillary wall from corner to corner of the screen. A human red blood cell was chosen to be measured and the investigator moved forward on the video frame by

frame and the cell was timed every $0.5 \mu\text{m}$ times four to see how much time it took the cell to move this distance. This was completed on three cells. The number of cells counted for each capillary depended on how many were visible and if it was possible to track its movement. After gathering data from the videos of the experiment, the data were placed into an excel worksheet and calculations were completed for multiple results including pre and post cannulation diameter, mean velocity, shear rate, and plasma shear stress, types of white blood cells (WBC's), balance pressure and finally capillary L_p . These results were then compared to the results obtained by Dr. Williams.



Figure 4. A Frog Capillary Being Cannulated with a Micropipette, Captured on Video
Photo courtesy of D. Williams

Materials and Methods (from Williams, 2007)

Male North American leopard frogs (*Rana pipiens*, JM Hazen, Alburg, VT; CD Sullivan, Nashville, TN) were maintained in a controlled environment (15°C, 12-h light:12-h dark). All frogs were exposed to gentamicin (1.3 mg ml⁻¹, bath water, 24 h) upon arrival at the animal care facility and were housed (6 to 10 frogs/container) with free access to fresh, antibiotic-free water and a dry area for at least 1 week before the experiments. Frogs were fasted for 4 days prior to experiments. The Animal Care and Use Committee at the University of Missouri–Columbia approved all animal housing and handling procedures.

Intravital Video Microscopy

Animal Preparation

Each frog was pithed cerebrally and cotton was placed in the cranial cavity. A right lateral incision was made through the skin and abdominal wall. Blood samples were collected for measurement of protein concentration (Bio-Rad, Richmond, CA), hematocrit (micro-capillary reader, IEC, Needham Heights, MA), hemoglobin (Drabkins Reagent, Sigma, St. Louis, MO), red blood cell counts, and white blood cell counts (bright line hemacytometer, Hausser Scientific, Horsham, PA). A loop of intestine was exteriorized and coaxed gently around a polished quartz pillar to maintain blood perfusion to the tissue. The mesentery and intestine remained moist and cool (14 to 16°C) by air-equilibrated frog Ringer's solution flowing continuously across the tissue.

Temperature was monitored with a thermocouple wire (type-T, Digi-Sense®, Cole Parmer, Vernon Hills, IL) anchored under the tissue.

Capillary Identification

Transilluminated sections of mesentery were inspected for capillaries using an inverted, compound microscope (UM 10× long working distance objective, 0.22 NA, Diavert, Leitz). Microvascular segments with approximately 500 to 1000 μm between branch points were defined as individual capillaries. Capillaries were devoid of vascular smooth muscle cells, contained flowing frog red blood cells (fRBC) and had no rolling or sticking WBC. At the branching ends of each capillary the direction of fRBC flow was noted and each capillary was categorized (Chambers and Zweifach, 1944). TC (true capillaries with divergent flow at one end and convergent flow at the other end) and VC (venular capillaries with convergent flow at both ends) were used.

Video Imaging

The image of each capillary was displayed on a video monitor (PM-125, Ikegami Tsushinki Co., JN) with a miniature high resolution CCD camera (TM-7CN, PULNiX America Inc., Sunnyvale, CA) coupled to the microscope. The camera was equipped with an electronic shutter controller (SC-745) operating at a speed of 1/10,000 s, which allowed either fRBC or human red blood cells (hRBC) to be visualized clearly. Experiments were video recorded in real time (Panasonic AG-6300, Matsushita Electric Industries, JN) with a video timer (0.01 s, VTG-33, For-A, JN) superimposed onto the image of the capillary (final magnification, 500×). Clips of video recorded data were

digitized (media converter DVMC-DA2, Sony) and archived on an external hard drive (LaCie, Ltd.). The scale used for all measurements was calibrated to a stage micrometer (0.01 mm, Meiji Techno, JN).

Glass Microtools

Micropipettes, occluders, and restraining rods were created with a pipette puller (Model PB-7, Narishige, Tokyo, JN) from acid-rinsed, borosilicate glass (1.5 mm, TW150-4, World Precision Instruments, WPI, Sarasota, FL). Each micropipette was ground to a single bevel (12 to 20 μm tip OD) using a Narishige grinding wheel (Model EG-4, 0.3 μm abrasive film, Thomas Scientific, Swedesboro, NJ). A sealed, acrylic chamber (WPI) held each micropipette in position and a water manometer was attached to the chamber to maintain pressure and flow. Three micromanipulators (Prior, Stoelting Co., Wood Dale, IL) were used to adjust the glass microtools in the $X-Y-Z$ planes.

Solutions

Frog Ringer's was prepared fresh daily from 5 \times concentrated stock solution (pH 7.4 at 15°C) and contained (in mM) NaCl (111.0), KCl (2.4), MgSO_4 (1.0), CaCl_2 (1.1), glucose (5.0), NaHCO_3 (2.0), and *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES)/Na-HEPES (5.0).

Dialyzed (6000 to 8000 MWCO, Spectra/Por membrane, Spectrum, Houston, TX) bovine serum albumin (BSA, 10 mg ml^{-1} ; A-4378, Sigma Chemical Co., St. Louis, MO) dissolved in frog Ringer's was used as the control solution. Fresh stock solution of pronase E from *Streptomyces griseus* (1.0 mg ml^{-1} ; 4 U mg^{-1} , P-5147, Sigma Chemical

Co.) was made fresh daily and diluted before each experiment in 10 mg ml^{-1} BSA/frog Ringer's to a final concentration of 0.1 mg ml^{-1} . hRBC were obtained from a finger prick, washed 3 times in Ringer's, and added to each pipette solution (2 to 3% vol/vol hematocrit). All solutions were maintained on ice until use and visible bubbles were removed when each micropipette was back-filled with solution.

Mechanical Stimulus

Abrupt $\Delta\tau$ and τ Time Curves. Each capillary was cannulated at $8 \text{ cm H}_2\text{O}$ (5.9 mm Hg , *in situ* TC pressure) and low flow was established for 2 min (Steady State 1). Next, manometer pressure was changed abruptly to $30 \text{ cm H}_2\text{O}$ (22.1 mm Hg) to produce the mechanical stimulus, $\Delta\tau$. The higher flow was maintained for 2 min (Steady State 2) at which point the capillary was occluded to measure volume flux/surface area (J_v / S) also at $30 \text{ cm H}_2\text{O}$. It was assumed that filtration during occlusion reflected filtration at Steady State 2.

A time curve for τ was generated for each capillary from measures of the free-flowing hRBC. Plateaus for Steady State 1 and Steady State 2 were identified by visual inspection from these curves. The magnitude of the mechanical stimulus ($\Delta\tau$) was calculated from the plateau values as: $\Delta\tau = \tau_{\text{steady state 2}} - \tau_{\text{steady state 1}}$

The intent of each experiment always was to introduce a square wave stimulus; however, deviations from intent do occur with work *in situ*. As such, the actual stimuli were categorized into square wave, overshoot, or undershoot patterns. As described previously (Williams, 2003), a square wave designation was assigned when τ at 15 s was

within $2.0 \text{ dynes cm}^{-2}$ of the mean value for Steady State 2. Capillaries were excluded from the $\Delta\tau / L_p$ analysis due to technical problems, which included overshoot or undershoot stimulus patterns and/or interruption of Steady State 2 by a clogged pipette.

Estimates of Capillary Fluid τ From Measures of RBC Velocity (v), Capillary Diameter, and Apparent Viscosity

v . The method used to measure v of RBC has been described in detail (Williams, D. 1999, 2003). Briefly, instantaneous RBC velocity (v^i) was measured directly on the video monitor in the frame-by-frame mode as the distance traveled by a single hRBC in time (dx/dt , $\mu\text{m s}^{-1}$).

$$\underline{v}^i = dx/dt$$

A correction factor (CF; Michel et al., 1974) was calculated from hRBC radius (R , μm) and each capillary radius (r , μm , measured simultaneously with v^i) as:

$$\text{CF} = 2(1 - [(R)^2/2(r)^2])$$

v was then calculated from v^i and CF assuming a centered RBC within the vessel.

Capillary Diameter. Diameter was measured from video recording at 3 sites spaced $\approx 100 \mu\text{m}$ apart along the capillary and averaged.

Apparent Viscosity. η^{apparent} was estimated from protein concentration (frog plasma, before, and BSA/Ringer's, after cannulation; Chick and Lubrzynska, 1914) as, $\eta^{\text{apparent}}(\text{poise}) = (5.7 \times 10^{-5}[\text{protein, mg ml}^{-1}]^{37^\circ}) + 0.0089$, and corrected to 15°C using the ratio between viscosity of water at 37 and 15°C (Weast, 1970). Use of protein concentration provided an estimate for the lower limit of capillary fluid viscosity.

$\underline{\tau}$. Shear rate was calculated from v and r as: Shear Rate (s^{-1}) = $4v/r$

τ was then calculated as: $\tau(\text{dynes cm}^{-2}) = (\text{Shear Rate})(\eta^{\text{apparent}})$.

Calculation of Volume Flux (J_v) Per Surface Area (S)
and Hydraulic Conductivity (L_p) From
Measures of Capillary Diameter and J_v

J_v . The modified Landis technique (Landis, 1927; Michel et al., 1974) was selected to measure J_v to ensure that the downstream end of each capillary remained intact prior to and during the mechanical $\Delta\tau$ stimulus. After the $\Delta\tau$ protocol a glass occluder, which had been positioned over the downstream end of the capillary perpendicular to the longitudinal axis of the microvessel, was lowered gently onto the capillary to occlude it and trap the hRBC. Movement of the trapped hRBC acted as an index of fluid flux across the capillary wall. Visual inspection verified successful occlusion. Time course data were obtained from serial occlusions, all at 30 cm H₂ O, moving the occluder progressively upstream along the capillary towards the micropipette, with one J_v measurement per occlusion and a minimum of 12 μm between each occlusion site.

Capillary Diameter. Diameter was measured 3 times at 3 time points during occlusion of the capillary to verify that, within the limits of detection and resolution of the video system (2 μm), S remained constant. Capillary volume and S were calculated from capillary radius (r , cm). A cylindrical capillary geometry was assumed.

J_v/S . J_v/S was calculated from hRBC velocity measured during the occlusion ($dx/dt^{\text{occlusion}}$, cm s^{-1}), capillary length measured between the occluder and each marker cell at the onset of the dx/dt measurement (x_o , cm), and the volume to surface area ratio ($r/2$, cm).

$$J_v/S(\text{cm s}^{-1}) = (dx/dt^{\text{occlusion}}) (1/x_o) (r/2)$$

$dx/dt^{\text{occlusion}}$ and x_o were measured on 3 hRBC (spaced $\approx 50 \mu\text{m}$ apart) at 3 time points (2.0, 2.3, and 2.6 s). Thus, 9 individual values of J_v/S were obtained from the first occlusion to decrease the standard deviation of the mean and increase precision.

L_p . Assuming a linear relationship between J_v/S and capillary pressure (P_c) as per the Starling equation, the slope obtained from regression analysis of J_v/S and P_c was used to estimate capillary filtration coefficient, L_p ($\text{cm s}^{-1} \text{cm H}_2\text{O}^{-1}$).

$$J_v/S = L_p[(P_c - P_i) - \sigma(\pi_c - \pi_i)]$$

where $(P_c - P_i)$ and $(\pi_c - \pi_i)$ are the differences in hydrostatic (P) and oncotic (π) pressures between the capillary lumen (c) and interstitium (i) of the mesentery and sigma (σ) is the reflection coefficient of the capillary barrier to protein. With an exteriorized mesentery, interstitial pressure is negligible and with fresh, protein-free frog Ringer's solution superfusing the tissue continuously, oncotic pressure differences are difficult to estimate. Calculating interstitial pressure from mesenteric protein concentration altered values for L_p by $0.1 \times 10^{-7} \text{ cm s}^{-1} \text{cm H}_2\text{O}^{-1}$, a difference that was within the error of the method. A procedure we have used here and in the past, which conservatively underestimates each value for L_p (Williams, 1999, 2003).

Each of the 9 measurements of J_v / S obtained during the first occlusion was calculated to L_p at 30 cm H₂O and averaged for a given capillary.

(Williams, 2007. pp. 49-50).

Experimental Protocol

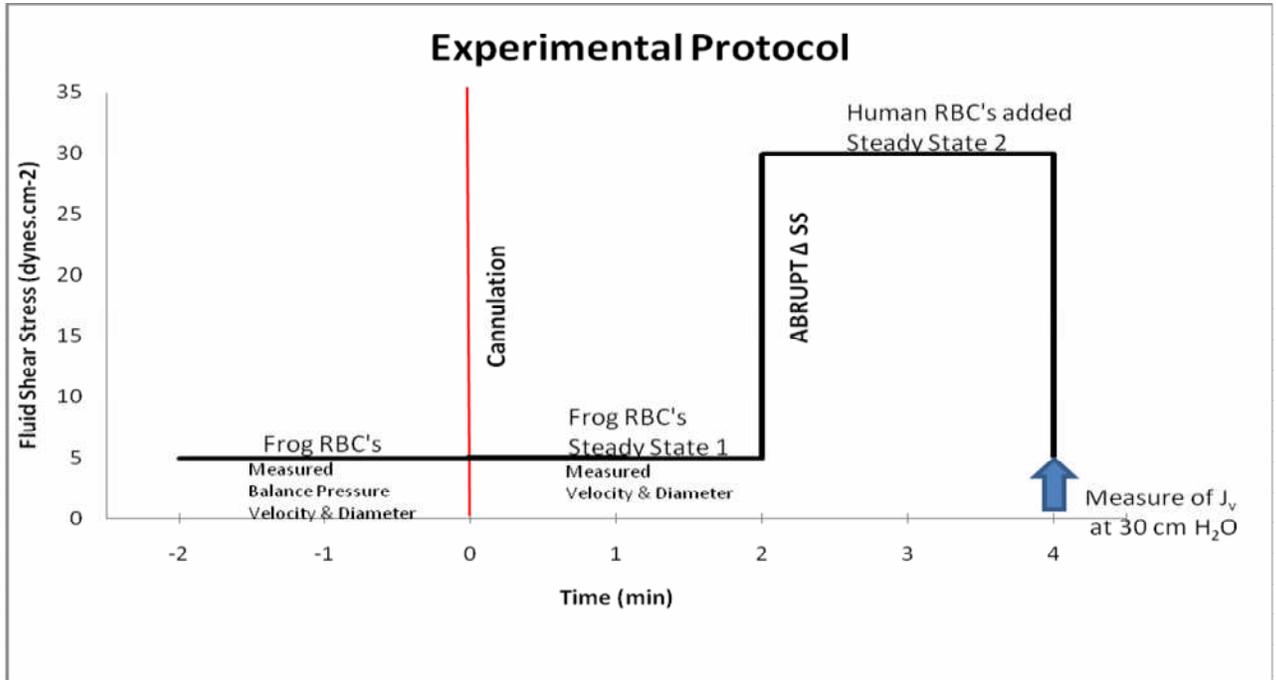
By long standing traditional criteria, capillaries are to have sticking or rolling white cells to indicate local inflammation (Landis, 1927). None of the capillaries used were found to have sticking or rolling white cells, but the frogs (n=10, 3 outliers), 29.4g wt, 20.2 cm length, 65.2 bpm pithed) were determined to be infected frogs from the NBT reduction test. To show activated white cells, 12 microliters of 0.9% NBT solution was added to 12 microliters of frog blood. This solution was mixed thoroughly, incubated for 20 minutes at 37 degrees Celsius, and then set out at room temperature for 20 minutes. The activated white cells take up the NBT and go through an oxidative burst that is seen under a light microscope (Alexiou et al., 2004). The frogs that showed a systemic reaction to some immune response (positive NBT test) were used for this project.

An experimental protocol was used to evaluate L_p of a capillary with and without indomethacin. "Variability due to differences in technical skill was minimized with the same individual (DAW) performing all cannulations and experimental protocols. To minimize measurement error and bias, a second individual (MHF) measured RBC velocity, capillary diameter, and J_v " (Williams, D. 2007. pp 50). Each frog capillary was cannulated and L_p was measured.

Protocol for Capillaries Located in
Frogs that have Systemic Reaction

During this protocol (Figure 5), the capillaries were measured with and without exposure to indomethacin. Velocity of frog RBC's, diameter, shear rate, plasma and shear stress were measured prior to cannulation. Cannulation occurred and the blood was allowed to flow for two minutes to establish steady state one. At two minutes, an abrupt change in shear stress was performed. "For the ABRUPT protocol, pipette pressure was set at 30cm H₂O and maintained during the cannulation. Steady State 2 and J_v/S measurement. The higher pressure relative to average pressure in frog mesenteric VC (8cm H₂O) introduced a rapid (ABRUPT) change in fluid velocity/shear stress" (Williams, 2003, pp 151). The capillary was then left at Steady State 2 for two minutes. At the end of two minutes the capillary was blocked and J_v measured by measuring the flow of the human marker RBC's (1-3%) in the capillary. The second capillary went through the same protocol with the additional aspect of 10⁻⁵ M indomethacin superfused over the tissue before assessment of L_p . The solution of 10⁻⁵M indomethacin in a frog Ringer's solution was prepared by MHF by taking 10 milligrams of indomethacin and 5 milligrams of sodium carbonate and adding that to 10 milliliters of distilled water. The mixture was then put in a sonicator for about seven minutes to dissolve. Two milliliters of the solution was added to 500 milliliters of frog Ringer's solution. This was used as the superfusion solution (outside the capillaries) for 5 to 7 minutes before cannulation.

Figure 5. Experimental Protocol



Statistical Analyses

Data were tested for normality and outliers were identified from an outlier box plot. Data are reported as mean \pm SE. Paired t-test was used to test for differences between means. A *P*-value of 0.05 was set prior to experiment to show significance.

CHAPTER 4

RESULTS

Data from two capillaries per frog in 13 animals was analyzed. Three capillaries, per animal, produced outlier values for L_p (ranging from 9.7 to 81.1×10^{-7}). These outliers were excluded.

White Cells

The frogs used from this data analysis were deemed “infected” due to the activated white cells that were seen by the NBT test. Figure 6 shows white cell concentrations calculated for each frog; the majority of WBC’s was medium sized cells called eosinophils.

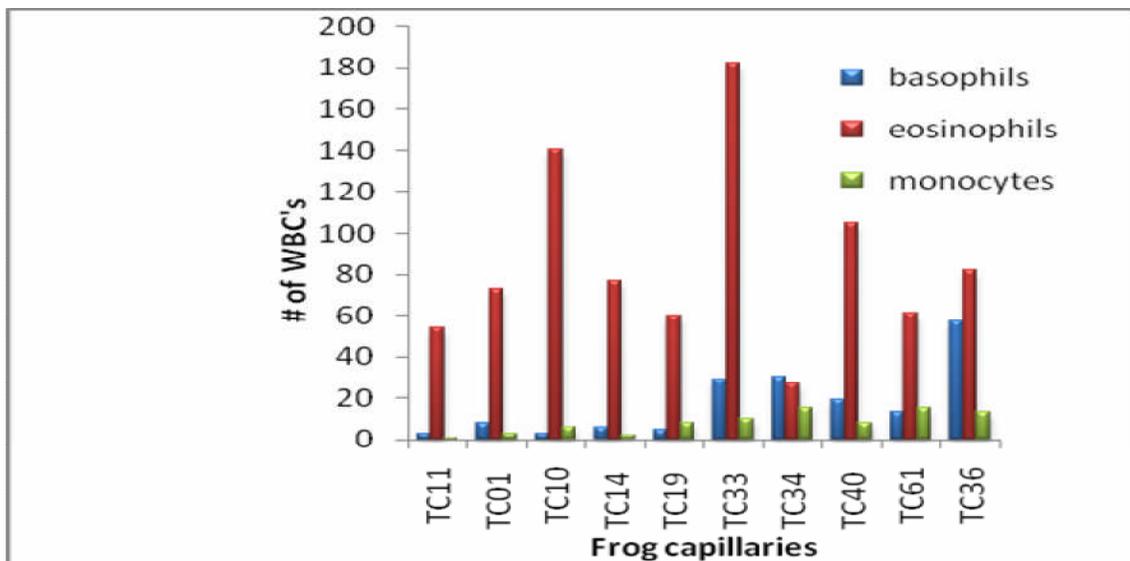


Figure 6. Frog WBC's Separated into Basophils, Eosinophils and Monocytes

Capillary Diameter (μm)

The Control (*C*) capillary diameters averaged $12.9 \pm 1.9 \mu\text{m}$ (range 9.3 – 15) precannulation and $14.8 \pm 2.8 \mu\text{m}$ (range 10 – 18.7) postcannulation. The indomethacin treated (*I*) capillary diameters averaged $13.4 \pm 2.5 \mu\text{m}$ (range 9.3 - 17.8) precannulation and $13.8 \pm 3.1 \mu\text{m}$ (range 10.4 – 18.7) postcannulation. A paired t-test of *C* and *I* diameters precannulation resulted in a p-value of 0.43. A postcannulation paired t-test of *C* and *I* resulted in a p-value of 0.74.

Mean Velocity ($\mu\text{m/s}$)

The mean velocity of each *C* and *I* capillary pre and postcannulation was measured. The mean of *C* pre cannulation was $1027.4 \pm 545 \mu\text{m/s}$ and post cannulation was $4546 \pm 1574 \mu\text{m/s}$. The mean of *I* pre cannulation was $871 \pm 614 \mu\text{m/s}$ and post cannulation was $5212 \pm 1928 \mu\text{m/s}$. Significance was noted in a paired t-test between pre and post cannulation *C* capillaries, with a resulting p-value of 0.0001. Pre and post cannulation of *I* resulted in p-value of 0.00003.

Shear Rate (s^{-1})

The shear rate of each *C* and *I* capillary pre and postcannulation was measured. The mean of *C* pre cannulation was $641.7 \pm 431 \text{ s}^{-1}$ and post cannulation was $2488.1 \pm 935 \text{ s}^{-1}$. The mean of *I* pre cannulation was $534.2 \pm 389 \text{ s}^{-1}$ and post cannulation was $3070.6 \pm 1096 \text{ s}^{-1}$. Significance was noted in a paired t-test between pre and post

cannulation of *C* capillaries, with a resulting p-value of 0.0005. Pre and post cannulation of *I* resulted in p-value of 0.00002.

Plasma Shear Stress

The plasma shear stress of each *C* and *I* capillary pre and postcannulation was measured. The mean of *C* pre cannulation was 10.7 ± 7.2 dynes·cm⁻² and post cannulation was 38.8 ± 14.6 dynes·cm⁻². The mean of *I* pre cannulation was 8.85 ± 6.5 dynes·cm⁻² and post cannulation was 47.9 ± 17.1 dynes·cm⁻². Significance was noted in a paired t-test between pre and post cannulation of *C* capillaries, with a resulting p-value of 0.0007. Pre and post cannulation of *I* resulted in p-value of 0.00002.

Change in Shear Stress Stimulus

The change in shear stress was measured for each *C* and *I* capillaries. The mean of *C* was 34.9 ± 11.7 dynes·cm⁻² (range 23 – 58.5). The mean of *I* was 44.5 ± 19 dynes·cm⁻² (range 10 – 64.9). A paired t-test resulted in a p-value of 0.32.

Tube Hematocrit (%)

Tube hematocrit was measured and compared. The mean of *C* was 8.6 ± 5.9 (range 3.1-20). The mean for *I* was 11.8 ± 2.6 (range 5.3-19.1). A paired t-test of *C* and *I* resulted in $p = 0.13$.

Balance Pressure

Balance pressure of each capillary was measured and compared. The mean of *C* was 10.9 ± 3.4 (range 5.5 – 15.9). The mean for *I* was 7.8 ± 3.2 (range 1.4 – 13.5). A paired t-test of *C* and *I* resulted in $p = 0.08$.

L_p

Figure 7 presents individual values of L_p of *C* and *I* plotted as a function of the square wave Δt presented to each capillary.

The L_p mean of *C* was $10.9 \pm 5.3 \times 10^{-7}$ cm/s/cm H₂O (median 10.8×10^{-7} cm/s/cm H₂O) (Figure 8). The range was 3.2 to 18.2×10^{-7} cm/s/cm H₂O. The L_p mean of *I* was $4.7 \pm 2 \times 10^{-7}$ cm/s/cm H₂O (median 4.4×10^{-7} cm/s/cm H₂O) (Figure 8). The range was 2.0 to 8.0×10^{-7} cm/s/cm H₂O. A paired t-test of L_p resulted $p = 0.002$.

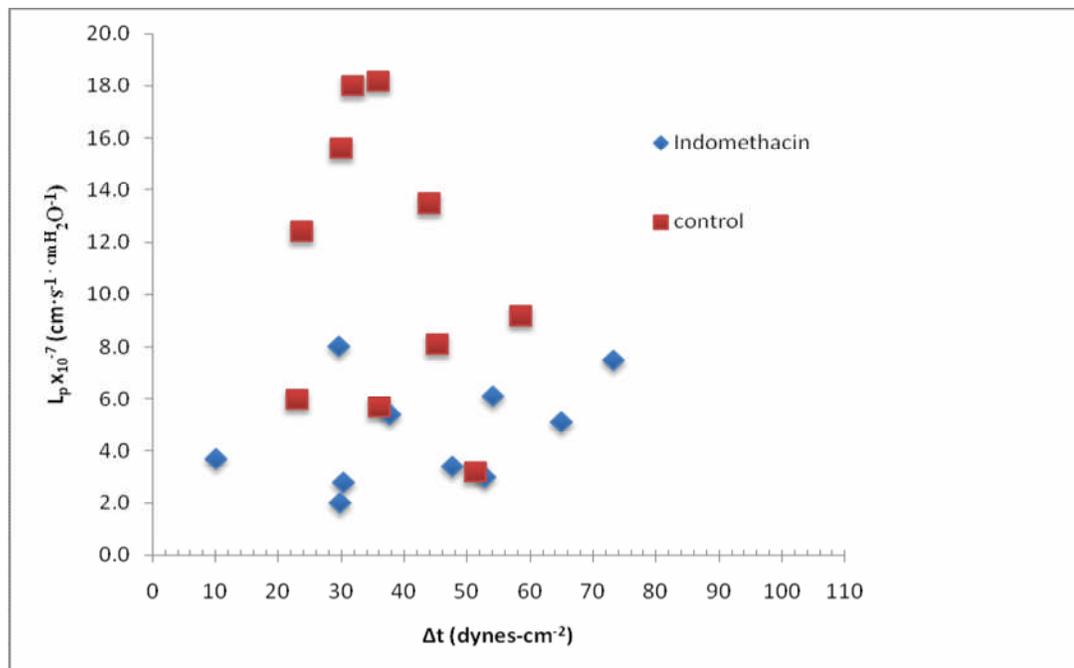


Figure 7. Hydraulic Conductivity (L_p) Plotted as a Function of the Magnitude of an Abrupt, Square Wave Change in Shear Stress (Δt) for Control and Indomethacin - Treated Capillaries.

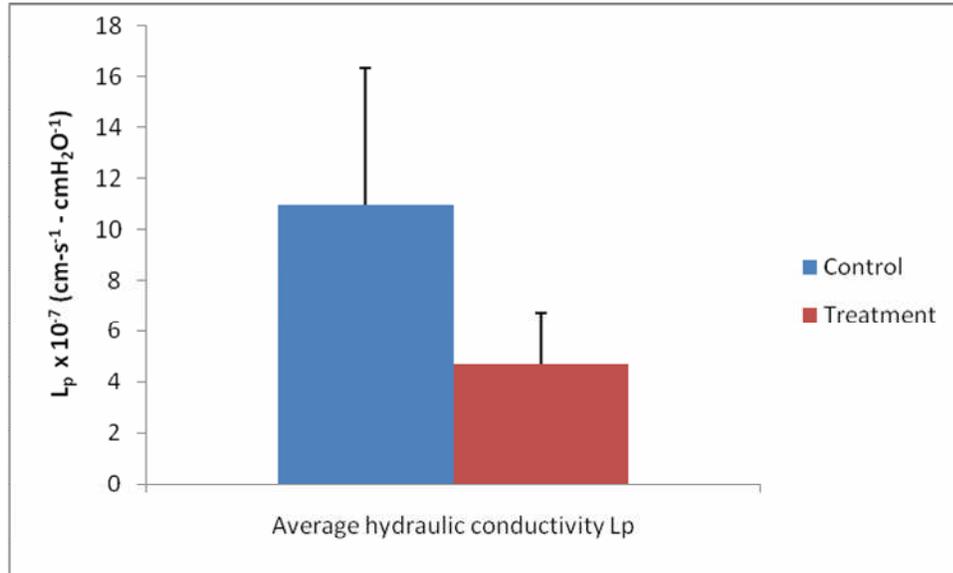


Figure 8. Average Hydraulic Conductivity (L_p) Assessed Using Experimental Protocol. Data are mean \pm SE

CHAPTER 5

DISCUSSION

Evaluation of Findings

The purpose of this study was to investigate the effect of indomethacin on capillary permeability in animal subjects with signs of systemic inflammation. The PI analyzed data from Dr Williams study funded by NIH. The secondary analysis of Dr. Williams' research data was blinded and randomized. Data from capillaries located in frogs with activated white cells were analyzed by the investigator. The results of the analysis indicate capillary L_p assessed in mesentery of infected frogs, decreased when exposed to shear stress and indomethacin. The data imply that gaps between endothelial cells may get smaller when indomethacin is introduced into the system decreasing the flow of fluids out of the capillary. The data gathered revealed the majority of WBC's found in these frogs were eosinophils. This study cannot be generalized to anything other than North American leopard frogs at this time, but it provides a base for exploration of other reactions that indomethacin and NSAIDs may have on the microvascular system.

A study completed by another investigator where the frogs were deemed healthy concluded that indomethacin did not decrease L_p of capillaries in healthy frogs (Staigmiller, 2010). When comparing these two data analyses, a conclusion can be reasoned that indomethacin has no effect unless inflammation is present.

Discussion of Findings as Related to Literature

Many people tend to self-diagnose, basing their symptoms on past experiences. The following example of Mrs. Jones represents a common scenerio repeated frequently in many health care providers offices. “Mrs Jones” was diagnosed with gout in the past and treated with indomethacin for pain in her great toe. Now, she begins to experience pain in her knee, and attempted to self-medicate with indomethacin. Upon futher examinationby her health care provider it was clear that “Mrs. Jones” does not have gout. Her diagnosis is osteomyletis resulting from a fall two weeks earlier. The subjective and objective findings for inflammation and infection are similar and can fool even the most skilled clinicans. It is essential for health care providers to discern the differences between inflammation and infection because the treatment for inflammation is very different from the treatment for infection.

Inflammation is an essential process in the normal defense against various pathogens inflammation promotes tissue healing. Leukocytes are the principal cellular mediators of inflammation. Inflammation is characterized histologically by the accumulation of leukocytes in the affected tissue due to migration of circulating leukocytes out of the vasculature, a process that is actively mediated and precisely controlled by leukocytes, the cytokines they produce, and the vascular endothelium (Cotran, Kumar, & Collins, 1999). Excessive or uncontrolled inflammatory responses can lead to the pathologic inflammation seen in many rheumatologic and chronic inflammatory disorders (Etzioni, 2009). Migration of circulating leukocytes out of the vasculature to sites of inflammation is an orchestrated process mediated by adhesion

molecules on both leukocytes and vascular endothelial cells. These molecules are normally expressed and activated in a precisely controlled manner. The movement of leukocytes from the bloodstream into sites of inflammation may be divided into specific steps: rolling, activation and arrest, and transmigration (Huether, 2006). Several well-established anti-inflammatory agents, such as NSAIDs act by inhibiting the expression of these molecules. New pharmacologic agents are in development and the beneficial therapeutic effects must be balanced against negative physiological consequences, such as bleeding in the gastrointestinal tract and increased susceptibility to infections (Etzioni, 2009).

Infectious diseases are the number one cause of death in the world (Rote & Huether, 2006). Even with the availability of many potent antibiotics, people are still dying of treatable infections. Many microorganisms find the human body to be a hospitable site in which to grow and thrive. Much of this symbiotic association between the body and organisms is maintained by the immune and inflammatory systems. If either or both of these systems are compromised, many microorganisms will leave their local sites causing infection in other places of the body (Rote & Huether, 2006). There are four separate stages associated with infection: colonization, invasion, multiplication and spread (Table 1) (Rote & Huether, 2006).

Table 1. Stages of Infection

Stage	Mechanism	Consequence
Colonization	Pathogens present on or in body without tissue invasion	Source of cross infection to others
Invasion	Resists host defenses; attaches to host cells through adhesion molecules and receptors	Opportunity for cell injury, alterations in function, or cell death
Multiplication	Uses host nutrients and environment, or cell organelles, for reproduction	Tissue damage, cell alterations, or cell death and disease symptoms
Spread	Migrates locally or through bloodstream and lymphatics	Local or systemic manifestations of disease through cell injury or effect of toxins

Adapted from "Infection" by Rote & Huether, 2006, *Pathophysiology: The Biologic Basis for Disease in Adults and Children*, p.295. Copyright 2006 by Mosby Inc.

White blood cells constitute the body's primary defense system against foreign organisms, tissues, and other substances. A WBC count for adult humans is 4.5-11.0 cells/ μ L (Schnell, Van Leeuwen, & Kranpitz, 2003). It is important for the clinician to determine whether an increased WBC count is the result of a pathologic condition or physiologic stress. Stressful situations can increase epinephrine, which may also increase WBC count rapidly. A total WBC count indicates the degree of response to a pathologic process but for a more complete evaluation of the pathologic process is provided by the differential count (Rote & Huether, 2006). The differential count identifies the number of neutrophils, eosinophils, basophils, monocytes and lymphocytes. Neutrophils are the body's first line of defense as phagocytes arrive at the site of injury within six to twelve hours (Schnell, Van Leeuwen, & Kranpitz, 2003).

Eosinophil's function is phagocytosis of antigen-antibody complexes and response to allergy-inducing substances and parasites. Eosinophils contain granules that

contain histamine used to kill foreign cells and proteolytic substances that damage parasitic worms (Schnell, Van Leeuwen, & Kranpitz, 2003). The data analysis from Dr William's study revealed activated WBC's in the frogs; the majority of the cells were eosinophils. Further research would investigate the reasons for the activated WBC's, which needs to be assessed to identify reasons for the elevated counts.

For inflammation to resolve there must be adequate control of the inflammatory process, basic nutrient availability, and appropriate growth factors (Peterson, Symes, & Springer, 1995). Other factors that affect resolution of inflammation include age, presence of chronic illnesses, and intervention.

Intervention by external pharmacologic agents such as prostaglandin inhibitors (NSAIDs) impacts the natural process of inflammation. They inhibit prostaglandin synthesis by suppressing enzymes active in the production of prostaglandins and by slowing leukocyte motility (Peterson, Symes, & Springer, 1995). Twenty or so different NSAIDs are available in the United States and many are available without a prescription. Approximately 60 million NSAID prescriptions are written each year. More than 17,000,000 Americans use NSAIDs on a daily basis, making it one of the most widely used classifications of drugs in the world. This number is expected to increase as the population ages and experiences painful degenerative and inflammatory conditions (Solomon, 2009c).

Certainly with rising costs of health care, providers must be cognizant of the cost of medications. Indomethacin is one of the less expensive NSAIDs. The cost of 30 indomethacin (25mg) capsules is \$14.99 (Drugstore.com, 2010). If all 17 million

Americans were taking only indomethacin, this would be a cost of more than 25 million dollars per month. Adding to this cost is the treatment of the adverse effects caused by the use of NSAIDs. A study by Sturkenboom et al. (2002) concluded that iatrogenic costs associated with NSAID therapy adds 58% to the the cost of NSAID treatment, mainly due to the adverse gastrointestinal effects from the NSAID's and ensuing treatment required to remediate the gastrosintesional effects.

As the aging population numbers increase so will the use of NSAIDs resulting in an increased number of adverse effects from their use. An estimate of five to seven percent of hospital admissions is from the adverse effects of NSAIDs ranging from gastrointestinal, nervous, renal and allergic reactions (Solomon, 2009c). Healthy gastric and duodenal mucosa use COX –1 to produce protective prostaglandins. Non-selective NSAIDs block both COX-1 and COX-2, which impairs gastric prostaglandin production (Feldman, 2009). NSAIDs are derived from carboxylic acid and do not ionize in the stomach and then can be absorbed across the gastric mucosa (Feldman, 2009). Once in the pH neutral mucosa, the medication becomes trapped in the epithelial cells, where damage can occur (Feldman, 2009). Most complications are seen within the first three months of therapy, and to lessen the likely hood of gastrointestinal complications is to administer NSAIDs for a shorter period of time. Researchers and providers are hoping with the development of new COX-2 inhibitors that gastrointestinal and renal complications will lessen (Solomon, 2009c).

One area in which the use of NSAIDs is controversial is orthopedic injuries and surgery. Bone repair is a complex process initiated by injury and an inflammatory

response. Prostaglandins mediate inflammation, influence the balance of bone formation and reabsorption, and are essential for bone repair. NSAIDs inhibit cyclooxygenase, which are essential for prostaglandin production (Bandolier, 2004). Many orthopedic physicians discourage the use of NSAIDs prior to any orthopedic surgery or during the bone healing process. (e.g.: fractures) (Bandolier, 2004).

“Renal prostaglandins promote the secretion of renin, impair sodium reabsorption in the loop of Henle and cortical collecting tubule and partially antagonize the effect of antidiuretic hormone to increase water reabsorption in the collecting tubules” (Rose, 2009a, p. 1). If this prostaglandin synthesis occurs in a patient that is in the state of volume depletion and takes NSAIDs, renal complications may occur. Complications include hyperkalemia, hyponatremia and edema but are reversible with discontinued therapy.

Millions of Americans self medicate their pain and inflammation with NSAIDs. Toxicity and acute poisonings have become more common as the aging population uses more NSAIDs. It is imperative for health care providers to provide a thorough evaluation of a patient before prescribing any NSAID. For example, patients with known heart disease should not take any over-the-counter medication, including NSAIDS without first talking with their health care provider.

Bench Science

Bench science is carefully controlled research conducted in a laboratory setting, often using nonhuman subjects, focused on furthering understanding of cellular and molecular mechanisms (Leshner, 2004). To improve human health, research findings

must be translated into practical applications for the clinical setting. “Bench to bedside” translation of research requires generation of new data by basic science researchers who consult with health care clinicians so that the findings from science can be used to improve patient’s health and disease management.

Cellular data discovered in the laboratory would be useless without timely interpretation and application to the disease processes and the human patients in the clinical setting. Conversely, the development of interventions to promote a response or to facilitate management of an acute or chronic illness in humans without a scientific basis is unsound clinical practice and in many situations, unethical. Translating research into clinical practice traditionally has been viewed as the domain of medicine. However, it is important to note that, bench science belongs to all health care disciplines, and it provides a firm foundation for evidenced based nursing practice and advanced practice nursing interventions.

This investigator was able to cannulate one frog capillary in the lab under the supervision of researcher, Dr. Donna Williams. The actual hand on manipulation of the capillary with a micropipette when viewed with an inverted light microscope was quite exhilarating to this investigator. This investigator grew enormously in understanding of the experiment and its implications that followed the experience of manipulating the frog capillary.

Taking the information from the bench to the bedside is vital to advance understanding of the mechanisms underlying disease and to improve the quality of patient care. Science must be at the basis of any provider’s knowledge. Nurse practitioners are

assuming a vital role in providing primary care for people across the lifespan, their knowledge of science will provide them with increased accountability, competence credibility and integrity.

Recommendations for Further Research

This research was limited to indomethacin and frog capillaries. There is a need for additional in vivo research using other medications and frog capillaries. The information obtained in this study should not be applied directly to humans, without additional research. The anatomy and physiology of humans and frogs is completely different so directly applying the knowledge from these data would be completely unethical. Bench science is a jumping off point for further investigations into how and why medications work in humans. Further studies are needed to identify the impact on inflammation. Additional NSAIDs should be tested to evaluate if effects are similar or not.

Conclusion

Evidence based practice in all health care fields, applies the best evidence obtained from bench science or scientific methods of research to health care decision making. Not only do clinicians evaluate the quality of the evidence but they also weigh the risks with the benefits of the treatment. There are five basic steps in evidence based practice: develop a clinical question, find the best evidence in current research, assess the research for validity, apply the finding to clinical practice, and evaluate the outcomes (Sackett, Straus, Richardson, Rosenberg & Haynes, 2000). The education that nurses receive allows them to see associations that others cannot (Schwartz, 2007).

A clinician would not change practice based solely on this study, however this study does provide important information. Peer review needs to be done. After decades of use and research on NSAIDs, it is now recommended by the American Heart Association that health care providers prescribe NSAIDs to patients as the lowest dose as possible for the shortest period of time (D'Arcy, 2009). The Federal Drug Administration has also placed a black box warning for prescription and over-the-counter NSAID medications indicating there is an increased risk of stroke, myocardial infarctions, and gastrointestinal bleeding, associated with NSAID use. Schwatz (2007) stated “when a new drug is being developed, consulting with nurses who are experts in symptom management can play a major role in understanding what types of side effects are likely to affect adherence to drug regimens. Similarly, when an industrial engineer is in the process of creating a mask for sleep apnea, it is nurse scientists who have the best, research-based perspective on what elements of the design might stop a patient from actually using the device, whatever its biophysical advantages” (¶ 21).

Be willing to unlearn what you have learned. Study results can change clinical practice quickly and as health care professionals, advanced practice nurses are encouraged to be lifelong learners. Our current practice is constantly changing as we learn more about the function of the body through bench science. “Translational research is an iterative process. It takes a team. No one can be in a lab, a clinic and in the community all at the same time” (Schwartz, 2007, ¶ 22).

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APPENDICES

APPENDIX A

PERMISSION LETTER TO USE FIGURE 3



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Melissa Priebe
Montana State University
Jmjn1@msn.com

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APPENDIX B

TABLES OF RAW DATA

Table 2. Diameter (μm)

Pre-can w/o indo (C)	Post-can w/o indo (C)	Pre-can w/ indo (I)	Post-can w/indo (I)
12.0	13.0	9.3	10.7
11.5	12.0	17.8	18.7
15.0	16.0	13.5	15.0
11.3	11.3	10.7	10.7
10.5	10.0	10.0	10.4
15.0	17.9	13.2	18.0
13.0	14.0	13.0	16.0
9.3	18.7	14.5	14.8
13.8	15.7	15.0	12.0
11.3	13.0	13.6	12.0
Mean (\pm SE) 12.9 \pm 1.9	Mean (\pm SE) 14.8 \pm 2.8	Mean (\pm SE) 13.4 \pm 2.5	Mean (\pm SE) 13.8 \pm 3.1
Pre C and post C	p-value = 0.06		
Pre I and post I	p-value = 0.29		

Table 3. Mean Velocity ($\mu\text{m/s}$)

Pre-can w/o indo (C)	Post-can w/o indo (C)	Pre-can w/ indo (I)	Post-can w/indo (I)
769.8	6041.4	382.8	5038.5
307.4	6774.1	1609.0	7574.2
420.4	6098.0	1750.0	7257.1
1913.0	2686.9	1868.7	4487.0
1067.5	2544.1	893.4	3420.7
1156.3	5233.8	123.9	4760.1
572.4	3185.9	683.4	5280.1
1476.6	3624.3	789.3	3961.1
402.7	5916.3	1441.3	7248.2
343.0	4049.7	544.3	1432.0
Mean (\pm SE) 1027.4 \pm 545	Mean (\pm SE) 4546.0 \pm 1574	Mean (\pm SE) 871.4 \pm 614	Mean (\pm SE) 5212.2 \pm 1928
Pre C and post C	p-value = 0.0001		
Pre I and post I	p-value = 0.00003		

Table 4. Shear Rate (s-1)

Pre-can w/o indo (C)	Post-can w/o indo (C)	Pre-can w/ indo (I)	Post-can w/indo (I)
513.2	3717.8	328.1	3778.9
212.6	4516.1	723.1	3246.1
224.2	3049.0	1037.5	3870.5
1350.4	1896.6	1401.5	3365.5
808.6	2035.3	714.7	2641.1
628.8	2345.8	77.2	2115.6
352.3	1820.5	420.6	2640.1
1265.7	1553.3	438.0	2165.9
230.7	3021.1	768.7	4832.1
242.1	2492.1	326.6	954.6
Mean (\pm SE) 641.7 \pm 431	Mean (\pm SE) 2488.1 \pm 935	Mean (\pm SE) 534.2 \pm 389	Mean (\pm SE) 3070.6 \pm 1096
Pre C and post C	p-value = 0.0005		
Pre I and post I	p-value = 0.00002		

Table 5. Plasma Shear Stress

Pre-can w/o indo (C)	Post-can w/o indo (C)	Pre-can w/ indo (I)	Post-can w/indo (I)
8.4	58.8	5.3	59.0
3.4	70.5	11.4	50.6
3.8	47.6	17.4	60.4
22.6	29.6	23.4	52.5
13.2	31.8	11.6	41.2
10.7	36.6	1.3	33.0
5.8	28.4	6.9	41.2
21.1	24.2	7.3	33.8
4.1	47.1	13.8	75.4
3.8	38.9	5.2	14.9
Mean (\pm SE) 10.7 \pm 7.2	Mean (\pm SE) 38.8 \pm 14	Mean (\pm SE) 8.85 \pm 6.6	Mean (\pm SE) 47.9 \pm 17
Pre C and post C	p-value = 0.0007		
Pre I and post I	p-value = 0.00002		

Table 6. Tube Hematocrit (%)

w/o indo (<i>C</i>)	w/indo (<i>I</i>)
8.3	8.5
9.5	8.8
6.0	8.5
4.6	5.3
20.0	
7.0	13.8
3.1	19.1
11.0	15.1
4.3	8.0
16.7	12.2
Mean (\pm SE) 8.6 \pm 5.9	Mean (\pm SE) 11.8 \pm 2.6
	p-value = 0.13

Table 7. Change in SS Stimulus

w/o indo (<i>C</i>)	w/ indo (<i>I</i>)
51.3	52.7
58.5	64.9
44.0	54.1
23.7	47.6
30.0	37.6
31.8	29.5
36.0	29.7
23.0	30.4
45.3	73.2
35.8	10.0
Mean (\pm SE) 34.9 \pm 10.9	Mean (\pm SE) 44.5 \pm 30.2
	p-value = 0.32

Table 8. Balance Pressure (cm H₂O)

w/o indo (<i>C</i>)	w/ indo (<i>I</i>)
6	6.1
9.3	9.3
7.7	8.5
15.9	8.9
6.7	1.4
11.4	7.1
13.7	13.5
11.8	5.3
5.5	6.8
9.6	10.5
Mean (\pm SE)	Mean (\pm SE)
10.9 \pm 2.5	7.8 \pm 3.1
	p - value = 0.08

Table 9. Hydraulic Conductivity (cm·s⁻¹·cmH₂O⁻¹)

w/o indo (<i>C</i>)	w/ indo (<i>I</i>)
3.2	3.0
9.2	5.1
13.5	6.1
12.4	3.4
15.6	5.4
18.0	8.0
5.7	2.0
6.0	2.8
8.1	7.5
18.2	3.7
Mean (\pm SE)	Mean (\pm SE)
10.9 \pm 10.6	4.7 \pm 0.5
	p -value = 0.002