FUCOIDIN EFFECT ON INFARCTION SIZE FOLLOWING MIDDLE CEREBRAL ARTERY OCCLUSION AND REPERFUSION

by

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ABSTRACT

Stroke is a significant health problem in the U.S. Cerebral ischemia and reperfusion initiates an inflammatory cascade, resulting in early leukocyte accumulation that leads to additional cell injury. During reperfusion, selectin adhesion molecules mediate the initial attachment of leukocytes to endothelium. Fucoidin blocks selectin interaction with their ligands and therefore decreases damaging leukocyte accumulation during postischemic reperfusion. Little is known about the effects of selectin inhibition after reperfused stroke. The purpose of this study was to determine the effects of selectin adhesion molecule blockade on cerebral infarction size and neurological function in an animal model of stroke. The filament method was used to induce stroke (4 hours) and reperfusion (24 hours) in control (n = 9) and fucoidin treated (n = 9) groups. The results of this study indicate that selectin blockade significantly reduced cerebral infarction size ($p < 0.001$) and improved neurological function ($p < 0.05$). These findings suggest that attenuating the leukocyte-mediated inflammatory response after stroke may be beneficial.
CHAPTER 1

INTRODUCTION

Stroke affects over a half a million Americans each year, making this disease one of our country's most serious health problems (American Heart Association [AHA] Heart and Stroke Statistical Update, 1999). Ischemic stroke is defined as the interruption or reduction of blood supply to the brain due to an intravascular thrombotic or embolic event (Adams, del Zoppo, & von Kummer, 1998). The majority of strokes (84%) are ischemic in origin, 53% being thrombotic and 31% being embolic (National Stroke Association [NSA] Stroke/Brain Attack Briefing, 1996). In humans, embolic stroke often involves the middle cerebral artery, resulting in focal tissue ischemia and ultimately cell death. Early restoration of blood flow, or reperfusion, is the desired treatment to limit brain cell death. Until recently, therapy for acute stroke was supportive in nature. Fortunately, acute stroke victims can now experience cerebral reperfusion due to the advent of thrombolytic therapy.

In 1995, the Federal Drug Administration approved tissue plasminogen activator (t-PA) for use in acute ischemic stroke. The approval was based on review of data collected in the National Institute of Neurological Disorders and Stroke Recombinant Tissue Plasminogen Activator Stroke Study. This double-blind, placebo-controlled, randomized study showed improved clinical outcomes at three months for those patients who received t-PA within three hours after onset of stroke symptoms (National Institute of Neurological Disorders and Stroke [NINDS] rt-PA Stroke Study Group, 1995). Data recently published from the Standard Treatment with Alteplase to Reverse Stroke
(STARS) study suggests similar favorable clinical outcomes at 30 days in patients who received t-PA for acute ischemic stroke. It has been recently reported that a sustained benefit of t-PA is seen at one year, with those patients who were given t-PA within three hours of stroke onset being more likely to have minimal or no deficits than the patients given placebo (NINDS rt-PA Stroke Study Group, 1999). These trials and other studies have made reperfusion therapy with t-PA the standard of care for the treatment of acute ischemic stroke. In addition, an increase in patient and healthcare provider educational programs, emphasizing the early signs and symptoms of stroke or “brain attack”, have resulted in an increase in the number of stroke victims seeking early care (Dornan, Kenton, & Gordon, 1999).

Ischemia-Reperfusion Injury

Paradoxically, the prompt return of blood flow to the ischemic brain may cause additional injury to blood vessels and surrounding cerebral tissue (Jean, Spellman, Nussbaum, & Low, 1998). Cerebral ischemia-reperfusion (I-R) initiates a complex inflammatory response involving the cerebral blood vessels and neuronal tissue. Several complex biochemical pathways, including the blood leukocyte response to injury, mediate I-R injury.

Inflammation and Leukocyte Activation

Proinflammatory mediators called cytokines are low-molecular weight glycoproteins that are released from injured parenchymal cells and vascular endothelium following I-R injury. Cytokines act as intercellular messengers that signal changes in leukocytes, other blood components, such as platelets, and the vascular endothelium
Leukocytes and vascular endothelium respond to these inflammatory signals, in part, by expressing surface adhesion molecules. This response promotes leukocyte adherence to the endothelium. Leukocytes may contribute to microvasculature damage and extension of infarction area in the brain via the release of toxic substances, such as oxygen radicals and proteases, and through direct trapping and plugging of the cerebral capillaries (Jean et al., 1998). It is now well accepted that leukocytes are major contributors to cerebral I-R injury (Kochanek & Hallenbeck, 1992).

Adhesion Molecules

Adhesion molecules are expressed on activated cells such as platelets, leukocytes and endothelium. The selectin family of adhesion molecules, including P-, L- and E-selectin, is responsible for the initial, rolling interaction of leukocytes to endothelium. Subsequent to selectin-mediated rolling, firm attachment of the leukocytes to the endothelium occurs via the integrin-immunoglobulin adhesion interactions. It is this firm attachment and subsequent transmigration across the endothelium, and release of toxic mediators, that may result in the extension of cerebral vascular damage.

Statement of the Problem

Stroke is the third leading cause of death in the United States, killing 160,000 people every year. In addition, it is the leading cause of serious long-term disability in this country. Over a half a million Americans experience a new or recurrent stroke; someone suffers a stroke every 53 seconds and a death from stroke occurs every 3.3 minutes (AHA Statistical Update, 1999). Stroke imposes a major economic burden on our healthcare
system. The National Stroke Association (1996) reports the estimated cost of stroke is approximately $30 billion each year, over half of which is attributed to direct medical costs, making this disease one of America’s most costly. The tremendous impact this insidious disease has upon our society makes it of utmost importance to study. Exploring new avenues for stroke treatment, prevention and rehabilitation is a priority for many scientists, physicians, and nurses.

The inflammatory response of the ischemic brain following stroke and the intravascular leukocyte response are difficult to study in the human brain. However, it has been more thoroughly explored in the animal model. Models for focal and global cerebral ischemia are available and are well-accepted models to examine the pathophysiologic mechanisms of stroke and reperfusion. Although much is known about inflammation and subsequent leukocyte involvement in cerebral ischemia-reperfusion as a result of animal research, there remains a lack of information regarding the effects and timing of anti-inflammatory, and specifically, anti-selectin treatments in cerebral I-R injury.

Statement of the Purpose

In order to examine the leukocyte contribution to cerebral I-R injury, the purpose of this study was to determine the effects of selectin adhesion molecule blockade on cerebral infarction size and neurological function following middle cerebral artery occlusion and reperfusion. The major hypothesis was that limiting leukocyte adhesion with fucoidin, an anti-selectin treatment, would reduce cerebral infarction size and improve neurologic function in treated animals compared to controls. Ultimately, the
information that was gained from this study may more clearly characterize the utility of anti-inflammatory therapy in the treatment of I-R injury in the brain.

Hypotheses

This study tested the following hypotheses:

1. Animals treated with fucoidin (FCN), the selectin adhesion molecule blocker, will have smaller cerebral infarcts versus non-treated control animals following middle cerebral artery occlusion (MCAO) and reperfusion.

2. Animals treated with FCN will exhibit improved neurological function versus non-treated control animals following middle cerebral artery occlusion (MCAO) and reperfusion.

Theoretical Framework

The theoretical framework for this study is based on the pathophysiology of ischemia and reperfusion, the role of leukocytes in the acute inflammatory response, and selectin adhesion molecule contribution to leukocyte mediated cerebral ischemia-reperfusion injury. The framework is presented as a model incorporating the events of cerebral ischemia-reperfusion, inflammation, cellular activation, leukocyte-endothelial adhesion and results of the adhesion and tissue injury (see Figure 1).

Pathophysiology of Cerebral Ischemia, Infarction and Reperfusion

Ischemia is defined as a reversible cellular injury that occurs when the tissue demand for oxygen exceeds the supply delivered. The imbalance in oxygen supply and
Figure 1. Model of Early Leukocyte-Mediated Cerebral Ischemia-Reperfusion Injury

Cerebral Ischemia and Reperfusion

Inflammatory Response

Leukocyte Activation

Endothelial Activation

Leukocyte-Endothelial Adhesion

Microvascular plugging

Toxic Mediator Release

Thrombosis Initiation

Leukocyte Migration

Additional Neural Cell and Vascular Injury
demand is produced by a reduction or cessation of blood flow that results in tissue hypoxia, decreased energy substrate and buildup of toxic metabolic wastes (West, 1986). In the brain, an acute ischemic event can rapidly progress to cerebral infarction, or stroke. Acute ischemic stroke results when an area of the brain loses blood supply because of vascular occlusion. Cerebral thrombi and cerebral emboli are the most common causes of occlusion, with atherosclerosis as the underlying process (McCance & Huether, 1998).

Interruption of cerebral blood flow (CBF) following occlusion results in an infarcted core with a surrounding territory of ischemic tissue termed the penumbra (Hossman, 1994). Cerebral blood flow in the penumbra is reduced, resulting in neural cells that function abnormally but still maintain some metabolic activity (Bratina et al., 1997). If CBF is not re-established, the penumbral region can progressively deteriorate, extending the area of infarction (Hossmann, 1994). In humans, the time frame in which cellular damage can be reversed, termed the “therapeutic window”, has been identified as three to six hours after the onset of occlusion (NINDS rt-PA Stroke Study Group, 1995). Normal cerebral blood flow in humans is approximately 53 ml per 100 g of brain tissue per minute (Bradberry, 1997). Reduction in cerebral blood flow to the range of 15-18 ml/100 g/min results in abnormal electrical activity. At a flow of 10 ml/100 g/min, protein synthesis is inhibited, adenosine triphosphate (ATP) is depleted and alterations in intracellular calcium and extracellular potassium occur. Additionally, at this reduced level of flow, excitatory neurotransmitters, such as glutamate and aspartate are released into the extracellular space and may bind freely to cell membrane receptors, specifically the N-
methyl D-aspartate (NMDA) receptor (Donnarumma et al., 1997). This activity results in an opening of transmembrane channels, allowing an influx of water, sodium and calcium ions. Severe intracellular acidosis ensues and rapid, irreversible damage to individual cell membranes and destruction of the surrounding tissue can occur (Ames, Wright, Kowada, Thurston, & Majno, 1968). Intracellular acidosis as a result of the ischemic event is toxic to brain cells, causing cellular edema and eventually cell death (Donnarumma et al., 1997).

Ischemia-Reperfusion, Inflammation and Leukocyte Activation

Early restoration of blood flow to the ischemic brain, known as reperfusion, is the treatment of choice to limit cell death (NINDS rt-PA Stroke Study Group, 1995). However, reperfusion initiates a complex inflammatory response characterized, in part, by early activation and accumulation of leukocytes in the blood vessels, and local expression of proinflammatory cytokines. Activation, or the inducement of a pathophysiologic response in the leukocyte, is mediated by a number of chemotactic factors following ischemia-reperfusion. In turn, as a result of this inflammatory process, activated leukocyte accumulation can contribute to additional injury to brain cells and blood vessels and has been termed a secondary neuronal injury (Jean et al., 1998). Initiation of this inflammatory response occurs very rapidly after cerebral reperfusion leading to rapid expression of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (DeGraba, 1998). These key cytokines, followed by the release of interleukin-6 (IL-6) and interleukin-8 (IL-8), and coupled with prostaglandin and oxygen free radicals all contribute to leukocyte activation and early migration to the area of cellular injury (Hallenbeck, 1996).
Early Leukocyte Accumulation

Leukocyte accumulation in the blood vessels is an initial event in the inflammatory response initiated by ischemia-reperfusion (Ritter, Wilson, Williams, Copeland & McDonagh, 1995). Ames et al. (1968) first described recruitment of leukocytes to the ischemic zone of the brain as potentially leading to reocclusion of microvessels and contributing to a "no-reflow" phenomenon, a process in which recirculation remains sluggish after clot lysis. In addition, activated leukocytes become stiff, less deformable and may become trapped in the capillaries, adhering to the endothelium and causing plugging of the microvasculature (Ritter & McDonagh, 1997; Schmid-Schönbein, 1993). This process can result in no-reflow as well. Activated leukocytes also produce proteolytic enzymes and oxygen free radicals, which may injure endothelial tissue in addition to their direct neuronal effects (Matsuo et al., 1995). Leukocyte accumulation may also play a role in thrombus formation (Issekutz, Ripley, & Jackson, 1983). The initiation of thrombosis is evidenced by leukocyte-platelet aggregates found on histopathologic examination of cerebral thrombosis (Kochanek & Hallenbeck, 1992). However, these processes remain unclear; it also has been argued that leukocytes exhibit antithrombotic properties in cerebral ischemia (Grau, Graf, & Hacke, 1994).

Adhesion Molecules and Leukocyte-Endothelial Interactions

The adhesion of leukocytes to surface endothelium and subsequent transmigration from the microvessels into the brain parenchyma is mediated by a variety of molecules located on the surface of both leukocytes and endothelium (Pantoni et al., 1997). Typically, under normal conditions, there is very little cell-surface expression of adhesion
molecules (Osborn, 1990). However, in the face of cerebral ischemia, adhesion molecule expression is upregulated, induced by various inflammatory processes and mediated by cytokines (Pantoni et al., 1997). There are four main families of adhesion molecules: integrins, the immunoglobulin (Ig) superfamily, cadherins and selectins. It is the selectin family of adhesion molecules that mediate the initial, rolling attachment of leukocytes on ischemic endothelium. L-selectin is expressed on unactivated, circulating leukocytes, while P-selectin and E-selectin are expressed on stimulated endothelial cells. The endothelial-based selectins interact with carbohydrate ligands, sialyl-Lewis ligands, on leukocytes, forming a tether-like attachment, enabling the leukocyte to roll along the surface of the endothelium. Upon activation of the leukocyte, L-selectin is shed; a conformational change occurs and leukocyte-endothelial interaction is intensified (Jean et al., 1998). Subsequently, a firm cell-to-cell adhesion ensues, which is mediated predominately by leukocyte integrins (CD11b/CD18), platelet activating factor (PAF) and endothelial intercellular adhesion molecule-1 (ICAM-1), a member of the Ig superfamily (Perry & Granger, 1991). In the reperfused, proinflammatory environment, circulating cytokines, such as IL-1 and others, further support leukocyte-endothelial adhesion (Naka, Stern, & Pinsky, 1996). As a result of this accumulation and adhesion of leukocytes to endothelium, mediated by early expression of selectins, it has been postulated that through a variety of mechanisms, leukocytes can further damage the brain by extending the ischemic area, expanding neuronal tissue necrosis.

*Leukocytes and Additional Injury*

The complicated process described in the preceding paragraphs, initiated by
ischemia-reperfusion and resulting in leukocyte accumulation, can contribute to several destructive mechanisms in vascular and neural cells. The injury-inducing mechanisms include leukocyte occlusion of the microvessels, potential “no-reflow” phenomena, thrombosis initiation, vasoconstrictive mediator release, direct endothelial cell damage via oxygen radical and cytotoxic enzyme release, potential migration into brain tissue with further damage to parenchymal cells (Härtl, Schürer, Schmid-Schönbein, & del Zoppo, 1996). Final injury outcomes as a result of these occurrences include increased vascular permeability and edema (Matsuo et al., 1994), extension of infarction area (Härtl et al., 1996), decreased electroencephalogram (EEG) function (Vasthare, Heinel, Rosenwasser, & Tuma, 1990), and a change in neurocognitive function (Clark, Madden, Rothlein, & Zivin, 1991).

**Summary**

Acute stroke is one of our country’s most serious health problems (AHA Heart and Stroke Update, 1999). Early intervention after stroke is essential to avoid brain cell death. However, reperfusion itself may cause additional injury (Jean et al., 1998). The antagonizing effects of reperfusion following acute ischemic stroke have been well documented in the literature. The pathophysiology surrounding the cascade of deleterious biochemical processes, inducing the expression of proinflammatory mediators and characterized by leukocyte activation and accumulation via adhesion molecules, provided the framework to guide this study.
CHAPTER 2

REVIEW OF THE LITURATURE

It was once thought that, in response to injury, the primary role of the leukocytes was to act as scavengers of tissue debris. It is now known that leukocyte activation and accumulation contributes to secondary cell injury after a primary injury, for example, organ ischemia. In the brain, the most compelling evidence for secondary, postischemic leukocyte-mediated injury comes from research performed in animal models of ischemia-reperfusion. Valuable insight regarding the importance of leukocyte-mediated tissue injury in the ischemic brain has been gained from studies employing anti-leukocyte interventions (Härtl et al., 1996). Most of these studies target polymorphonuclear leukocytes, hereafter referred to as simply leukocytes. Therefore, the focus of this review is to discuss studies that utilized animal models of cerebral ischemia and reperfusion to examine the role of leukocytes in cerebral I-R injury.

Methods and Models for Studying Leukocyte-Mediated Cerebral I-R Injury

The study of cerebral ischemia in animals began with models of global cerebral ischemia, first reported in the late 1970s (Tamura, Kawai, & Takagi, 1997). Rodent models of global ischemia have contributed to an “accumulation of vast amounts of knowledge on the underlying mechanisms of and therapeutic approaches against ischemic injury” (Tamura et al., 1997, p. 273). Methods of global ischemia include a four-vessel occlusion model (Pulsinelli & Brierley, 1979) and more commonly, bilateral common carotid artery occlusion with hypotension (Smith, Auer, & Siesjö, 1984, as cited in Tamura et al., 1997). Although still used today, models of global ischemia lack clinical
relevance. Many investigators are now employing models of focal ischemia, such as middle cerebral artery occlusion (MCAO), as they resemble stroke in humans, making the model more clinically applicable (Tamura et al., 1997). In addition to the MCAO technique, a number of studies describe the use of neurological assessments for functional analysis of stroke and tissue staining and imaging techniques for analysis of the size of infarction after stroke and reperfusion. Using these methodologies, investigators have examined the effects of leukocyte depletion, leukocyte activation and adhesion molecule inhibition after cerebral ischemia and reperfusion.

**Middle Cerebral Artery Occlusion**

Ischemic injury caused by cerebral thrombosis is the most common type of stroke in humans (AHA Heart and Stroke Facts, 1994). Occlusion of a single trunk artery, particularly the internal carotid or middle cerebral artery (MCA), is the most frequent mechanism of stroke (NSA Stroke/Brain Attack Briefing, 1996). To mimic the human focal ischemic state observed in acute stroke, rat MCAO models are now the most widely used (Tamura et al., 1997). The duration of ischemia in MCAO can be permanent, or transient with a resumption of blood flow.

Early restoration of blood flow with t-PA is considered the treatment of choice for acute ischemic stroke. However, careful inspection of the possible negative influence of reperfusion must be taken into consideration (Aronowski, Strong, & Grotta, 1997). Yang & Betz (1994) demonstrated that three hours of focal ischemia followed by three hours of reperfusion in the rat produced more damage that six hours of continuous ischemia without reperfusion, indicating that reperfusion itself contributes to additional damage.
Examination of mechanisms of damage after focal cerebral ischemia and reperfusion is difficult in humans. Therefore, a reliable animal model of focal cerebral ischemia and reperfusion is required.

The model developed by Zea Longa, Weinstein, Carlson and Cummins (1989) mimics focal stroke in humans. It is relatively noninvasive and is a reversible method of MCAO. Advantages of this rodent model include occlusion and reperfusion of the MCA without invasive craniectomy and its relative simplicity for the experienced surgeon (Zea Longa et al., 1989). Disadvantages of this model are the high mortality rate and the sacrifice of the external carotid artery, which may result in a reduction in blood flow to the scalp on the ischemic side and possibly a change in brain temperature (Tamura et al., 1997).

Varying time periods of ischemia can be employed using the MCAO model. One to 6 hour ischemic time periods are commonly reported (Aronowski et al., 1997; Soriano et al., 1996; Yanaka et al., 1996; Zhang et al., 1996). Garcia et al. (1993) found profound histological abnormalities in neurons subjected to more than 6 hours of ischemia, characteristic of progression from an ischemic lesion to an infarct. These investigators also reported that qualitatively minimal histological irregularities were evident in animals subjected to less than 2 hours of ischemia. However, acute microscopic changes were detectable as early as 30 minutes after MCAO (Garcia et al., 1993). Current recommendations regarding therapeutic intervention with t-PA for humans with acute stroke is within 3 hours of onset of ischemic stroke (Adams et al., 1996). Thus, an ischemic period of 1 to 6 hours in an animal model has clinical application as well.
Coupled with an effective method for evaluation of therapeutic strategies on stroke, MCAO is a model that has achieved wide application in basic research.

Reperfusion after focal stroke using the MCAO model in the rat is an effective method for examination of leukocyte contribution to ischemia-reperfusion (I-R) injury, and has been used in a number of studies. For example, Shiga et al. (1991), using unilateral MCAO and varying time periods of reperfusion found a reduction in edema and infarction size in rats treated with antileukocyte monoclonal antibodies. Using similar methodology, Matsuo et al. (1994) reported comparable results.

**Analysis of Cerebral Damage**

It is essential to establish effective methods for determining the degree of ischemic damage following stroke. A combination of functional and pathological evaluations can assist in the investigation of the pathogenesis of stroke and evaluation of anti-leukocyte treatment regimens. Bederson et al. (1986) reported that staining using 2, 3, 5-triphenyltetrazolium chloride (TTC) is a rapid, convenient, inexpensive and reliable method for the detection and quantification of cerebral infarction in rats 24 hours after the MCAO. For these reasons, TTC staining is often reported (Bendar et al., 1991; Heinel et al., 1994; Matsuo et al., 1994). A water-soluble salt, TTC has been used as a stain to detect ischemic infarction in mammalian tissue since 1958 (Sandritter & Jestadt, 1958, as cited in Bederson et al., 1986). It acts as a proton acceptor for nucleotide-linked enzyme systems within the mitochondrial membranes of living cells. Therefore, TTC staining results in a deep red color in healthy tissue. Non-viable, infarcted tissue does not react with the TTC and remains white. Using this method, a clearly defined border between
normal and infarcted tissue can be observed. Although TTC staining is a valuable method for infarction analysis, it does have limitations. Its use should be limited to samples obtained 24 hours after the onset of ischemia. At 24 hours, the size of the area of infarction can be unequivocally delineated (Bederson et al., 1986). Staining tissue before 24 hours after the injury can result in poorly defined lesions that are stained pink (Bederson et al., 1986). In addition, staining tissue after 36 hours of injury can obscure the margin of ischemia, because infiltration of macrophages with intact mitochondria can occur (Tamura et al., 1997).

**Analysis of Neurological Function**

Neurological tests to measure functional changes have proved beneficial to evaluate outcomes of experimental stroke. It is possible to detect functional changes in a rat with even a very small cortical infarction (Robinson, 1979). A variety of methods for the assessment of motor and behavioral function and various scoring systems have been reported. Bederson et al. (1986) developed a neurological exam using a 0-3 grading scale based on three tests. First, the rat was suspended by the tail to observe forelimb flexion. Animals with infarction consistently flexed the forelimb contralateral to the injured hemisphere; those animals with larger infarctions also exhibited adduction with internal rotation of the shoulder. The investigators then placed the rats on soft paper and applied lateral pressure behind the shoulders until forelimbs slid and attempted to grip the paper. Animals with neurological deficits exhibited reduced resistance to the lateral push toward the paretic side. Last, the animals were allowed to roam freely and were observed for a circling pattern to their gait. Animals with infarction spontaneously circle toward their
paretic side and those with severe neurological deficits circle continuously or do not move at all (Bederson et al., 1986). Menzies, Hoff and Betz (1992) reported a similar method of clinical evaluation of ischemia, utilizing the same tests and grading animals 0-4. Yanaka et al. (1996) and Zea Longa et al. (1989) also used the above tests and added a level of consciousness assessment, observing the animal’s level of curiosity and spontaneous movement. Several investigators have reported neurological testing as an adjunct to pathological data to examine effects of a variety of treatments including anti-leukocyte therapy (Connolly et al., 1996, 1997; Matsuo et al., 1994; Soriano et al., 1996).

Leukocyte Depletion

Animals can be rendered leukopenic using genetic manipulation, antileukocyte serum, antineoplastic agents or monoclonal antibodies (MoAb). Antileukocyte serum is a preparation used to deplete leukocytes in one animal by immunizing them with leukocytes from another species. Using this method, Grögaard, Schürer, Gerdin and Arfors (1989) achieved up to a 95% reduction in circulating leukocytes. Employing the global ischemia model of bilateral middle cerebral artery occlusion (MCAO) and arterial hypotension, these investigators found that rats treated with antileukocyte serum had improved postischemic local cerebral blood flow (CBF) within one hour after the ischemic insult. Using the same model, Schürer et al. (1990) reported similar results. They also reported an improvement in early CBF and decreased brain edema in animals treated with antileukocyte serum compared to sham operated controls. Using a model of thromboembolic stroke, Bendar, Raymond, McAuliffe, Lodge and Gross (1991) reported improved CBF, reduced intracranial pressure (ICP) and smaller ischemic defect size in
rabbits pretreated with antileukocyte serum when compared to controls. These improvements did not occur in a group treated with antiplatelet antiserum, emphasizing the importance of leukocytes to I-R injury.

Antineoplastic treatments are also effective in depleting white blood cells in animal models, achieving an average of 70-90% WBC reduction (Härtl et al., 1996). Vasthare et al. (1990) found that rats rendered leukopenic with vinblastine and subjected to global forebrain ischemia with bilateral common carotid occlusion and hypotension had preserved EEG activity and somatosensory evoked response (SSER) than control animals. Heinel et al. (1994) reported similar results using the same method. In addition, these investigators observed a greater percentage of viable tissue after TTC staining in the leukopenic group. Shiga, Onodera, Matsuo and Kogure (1992) found that leukopenia induced by cyclosporin pretreatment led to a reduction in brain edema and smaller infarct size in rats subjected to MCAO and reperfusion. Pretreating animals 3 to 4 days prior to the ischemic insult is required when using this method of myelosuppression. Complications such as nausea and vomiting leading to dehydration, metabolic alkalosis and cardiovascular collapse can occur making this method of study difficult (Härtl et al., 1996). In spite of this, cyclosporin treatment results in cerebral protection after stroke, similar to other methods of leukocyte depletion.

Investigators have reported significant reductions in infarction size after using another, more specific method of PMN depletion. Treatment with the antileukocyte MoAb, RP-3 results in 85-90% PMN reduction without affecting other WBC lines (Härtl et al., 1996). Using this method of leukocyte manipulation, Shiga et al. (1991) and
Matsuo et al. (1994) found that rats pretreated with RP-3 and subjected to one hour of focal cerebral ischemia and reperfusion had a reduction in brain edema and infarct size when compared to controls. Although successful in showing the effects of leukocyte depletion in reducing cerebral damage following an ischemic event, the above methods are not without weaknesses. The toxic side effects of antineoplastic agents previously mentioned often limit the observation periods to a few hours. Additionally, Engler and Covell (1987) suggest that even very small numbers of circulating neutrophils may produce postischemic microvascular dysfunction. Therefore, because 100% depletion of leukocytes using these methods is not possible, attributing the observations to leukocyte depletion alone may be in question. In addition, leukocyte depletion is not a clinically applicable model of leukocyte manipulation in stroke research. Recently, more studies have examined the use of agents that could possibly be used in humans.

Inhibition of Mediators of Leukocyte Activation

Cytokines are considered to be among the principal mediators of immunologic and inflammatory responses (Pantoni et al., 1998). Therefore, they have been defined as potent activators of leukocytes (Yamasaki et al., 1995). In addition, cytokines not only activate leukocytes, but also activate endothelial cells and platelets, thereby exacerbating the inflammatory response in cerebral tissue (Pantoni et al., 1998). Interleukin-1 (IL-1) appears to be a key component in the induction of endothelial cell adhesion molecule expression and promotion of infiltration of leukocytes into cerebral tissue (Jean et al., 1998). Additionally, an overexpression of IL-1 has been documented during brain ischemia. Liu et al. (1993) reported a substantial increase in IL-β mRNA synthesis was
observed after MCA occlusion. Yamasaki et al. (1992) treated animals with an intraventricular injection of exogenous recombinant human IL-1β (rh IL-1β) during reperfusion following MCAO. A neurological assessment was performed to assess for complete ischemic insult. They found that, after rh IL-1β treatment, animals exhibited an increase in neutrophil infiltration and increased brain edema.

Other investigations report a reduction in cerebral infarct size after administration of IL-1 antagonists. For example, in a follow up study, Yamasaki et al. (1995) found that animals injected with anti-IL-1β into the cerebral ventricle during reperfusion following MCAO, had a decreased number of neutrophils and a significantly reduced area of infarction. Using a TTC staining method for analysis of infarction size, Relton, Martin, Thompson and Russell (1996) reported similar results following peripheral administration of rh IL-1 receptor agonist. These studies indicate a leukocyte-mediated proinflammatory role of IL-1 in cerebral I-R.

Like IL-1, TNF-α is one of the proinflammatory cytokines and is expressed in ischemic brain (Liu et al., 1994). One important inflammatory effect of TNF-α is up-regulation of adhesion molecules on endothelial cells. This action facilitates migration of activated leukocytes into the ischemic brain and might result in secondary damage following brain ischemia (Kochanek & Hallenbeck, 1992; Hallenbeck, 1996). Using TTC staining for infarction analysis, Barone et al. (1997) found that direct administration of TNF-α exacerbated ischemic injury, thereby increasing infarction size, following MCAO in rats. They also found that anti-TNF-α antibodies had a neuroprotective effect, reducing infarct size in the treated group. In a similar study, inhibition of TNF-α, in mice with
permanent MCAO lead to a smaller infarct volume, compared to controls (Nawashiro, Martin & Hallenbeck, 1997). However, conflicting data does exist on this cytokine. In a separate study performed by Nawashiro, Tasaki, Ruetszler and Hallenbeck (1997), TNF-α administered to mice 48 hours before ischemic insult resulted in a neuroprotective effect, with a significant reduction in infarction size as observed with TTC staining techniques. Further experimentation of this cytokine is needed to determine its relevance to leukocyte-endothelial interactions in I-R injury.

Adhesion Molecule Inhibition

Recent studies reported that the expression of endothelial adhesion molecules P-selectin and ICAM-1 is increased following I-R, and that these molecules participate in the pathogenesis of leukocyte recruitment and cerebral tissue damage during reperfusion after stroke (Connolly et al., 1996). Therapies targeting these molecules have been investigated with promising results.

ICAM-1 mediates firm leukocyte adhesion to activated endothelial cells by binding to the β2-integrin subunit CD18 expressed on the cell surface of the leukocyte. Using mice deficient in CD18, Prestigiacomo et al. (1999) found that animals subjected to 45 minutes of MCAO and reperfusion, demonstrated an 82% decrease in leukocytes in the ischemic zone. In addition, significantly smaller infarcts were found in CD18 deficient animals compared to controls. When permanent occlusion of the MCA was performed and cerebral tissue was not reperfused, no difference in infarct volume was seen. Using monoclonal antibodies against CD11a, CD18 and ICAM-1, Matsuo et al. (1994) found a reduction in post-ischemic edema formation and infarction extension in the brain of rats.
subjected to MCAO and reperfusion. In addition, by blocking these adhesion molecules, a reduction in leukocyte infiltration was seen in the treatment group. Soriano et al. (1996) reported that ICAM-1 deficient mice were less susceptible to cerebral injury following MCAO and reperfusion. These investigators reported a significant reduction in infarction volume in ICAM-1 deficient animals when compared to their wild-type littermates.

The role of another family of adhesion molecules, the selectins, and their relationship to cerebral I-R injury is not well understood, making this an important area for stroke research. From studies done on cat mesenteric venules, it appears that the selectins mediate the initial rolling attachment of leukocytes to endothelium before firm adhesion is accomplished by the integrins and ICAM-1 (Perry & Granger, 1991). In another study in mesentery venules, Ley et al. (1991) observed, in-vivo, a rapid expression of P-selectin on ischemic endothelium and L-selectin on leukocytes. In the primate model, using immunocytochemical techniques, Okada et al. (1994) found that focal cerebral I-R stimulated upregulation of endothelial P-selectin, beginning during ischemia and persisting through 4 hours of reperfusion. Additionally, Okada et al. reported ICAM-1 was transiently expressed at 1 and 4 hours of reperfusion in this study. These data provide evidence for selectin involvement in the early accumulation of leukocytes to ischemic tissue following early reperfusion. A limited number of studies targeting selectins in the cerebral vasculature have been reported. Morikawa, Zhang, Seko, Toyoda and Kirino (1996) found that rats, treated with a selectin oligopeptide that selectively blocks selectin-mediated cell adhesion, had a decrease in the size of ischemic injury following MCAO-R. They found no difference in infarction size in another treated group subjected to
permanent ischemia when compared to controls. Using mice genetically deficient in P-selectin and subjected to MCAO-R, Connolly et al. (1997) reported a reduction in cerebral blood flow after reperfusion, a decrease in leukocyte accumulation and smaller infarction volumes when compared to normal mice expressing the P-selectin gene. In the same study, these investigators reported similar results in mice treated with a monoclonal antibody directed against P-selectin. The unique feature of this thesis study is the examination of the effects of fucoidin (FCN), an inhibitor of both P and L-selectin in early reperfusion after MCAO.

Fucoidin, a sulphated, fucosylated polysaccharide from seaweed, inhibits both P- and L-selectin from binding to their respective ligands. Thus, FCN is postulated to be a potent inhibitor of selectin-mediated adhesion of leukocytes to vascular endothelium during ischemia-reperfusion (Patankar, Oehninger, Barnett, Williams & Clark, 1993). Ritter, Copeland and McDonagh (1998) observed a significant decrease in leukocyte accumulation in the rat coronary microcirculation during postischemic reperfusion after FCN treatment. In addition, Miura et al. (1996) found that selectin blockade with FCN in the neonatal lamb heart model resulted in better recovery of left ventricular function and coronary blood flow following ischemia, despite a higher circulating white blood cell count. Using intravital microscopy, Kubes, Jutila and Payne (1995) observed a profound increase in leukocyte rolling and adhesion following I-R in cat mesentary venules. When high dose FCN (25 mg/kg) was administered, leukocyte rolling was reduced by >90%, and normal reperfusion-induced leukocyte adhesion was attenuated by 50%. To date, the use of a P and L-selectin inhibitor, such as FCN, has not been tested during early cerebral I-R,
making this a unique area of study.

Attenuation of leukocyte accumulation following I-R has been evidenced by adhesion molecule blockade (Matsuo et al., 1994; Prestigiacomo et al., 1999). Fucoidin (FCN) inhibits both P and L-selectin and studies have demonstrated a decrease in leukocyte accumulation following administration of this agent (Kubes et al., 1995; Ritter et al., 1998). In addition, information gained from the use of FCN in early I-R can be used to correlate to microcirculation findings recently reported by Ritter, Orozco, Coull and McDonagh (in press). They observed a significant accumulation of leukocytes in cerebral venules after 2 hours of MCAO and 1 hour of reperfusion. Thorough evaluation of the therapeutic impact of attenuating leukocyte rolling and subsequent accumulation with interventions such as FCN has not yet been reported.

Leukocytes and Cerebral Edema

Formation of vasogenic brain edema following a stroke can be detrimental to patient outcome. Edema formation following focal ischemia can lead to increased intercranial pressure, further ischemia, and secondary cell death (Härtl et al., 1996). The edema is of particular concern in the brain, as opposed to peripheral organs, where edema formation does not have such harmful consequences (Härtl et al., 1996). Because of the clinical consequences of cerebral edema following an ischemic event and it’s impact on experimental studies, identification of the contributors to brain edema formation has been an important area of study. The inflammatory response and subsequent leukocyte accumulation has been identified as one of the contributing mechanisms of this pathological phenomena (Kochanek & Hallenbeck, 1992).
Leukocytes play a significant role in ischemic edema formation in the brain, predominantly by increasing vascular permeability (Shiga et al., 1991). Using the filament method of MCAO-R, Shiga et al. (1991) demonstrated that depletion of circulating neutrophils in rats using anti-neutrophil monoclonal antibody (RP3), suppressed postischemic brain edema. In a similar study with RP3, Matsuo et al. (1994) reported that depletion of circulating leukocytes throughout focal cerebral I-R suppressed postischemic edema formation as observed by TTC at 24 hours of reperfusion. The time course of maximum leukocyte invasion into brain parenchyma in rats subjected to MCAO appears to be 24 hours and correlates well with brain edema in non-treated control animals (Matsuo et al., 1994; Garcia et al., 1994). The exact contribution of activated leukocytes to increased vascular permeability and edema formation following stroke is controversial (Kochanek & Hallenbeck, 1992). Attenuating specific mechanisms of the inflammatory response following an ischemic event, such as leukocyte-endothelial interactions, may assist in further clarification of the issue of cerebral edema formation.

Swelling of ischemic tissue may result in enlargement of the infarcted zone, leading to overestimation of infarct volume (Lin, He, Wu, Khan, & Hsu, 1993). This potential distortion of infarct volume by brain edema creates a problem, particularly when interpreting the effects of therapeutic interventions (Lin et al., 1993). Recognizing that cerebral edema is an outcome of MCAO has led to the development of more precise calculations of cerebral infarction volume. Currently, most investigators report cerebral infarction volumes that have been corrected for edema (Aronowski et al., 1997; Lin et al., 1993; Soriano et al., 1996, 1999; Swanson et al., 1990; Zhang et al., 1996). Using these
calculations also yields more information regarding the effects of therapies on edema formation.

Summary

Recent FDA-approved thrombolytic therapy (t-PA) for stroke and the ascent of educational programs emphasizing the early signs and symptoms of stroke have made reperfusion strategies for acute stroke viable and successful treatment options for stroke victims. There is strong evidence supporting the deleterious effect of leukocyte accumulation in the cerebral microvasculature during reperfusion after stroke. Recent research has focused on attenuating leukocyte accumulation and leukocyte-endothelial interaction via a number of methods targeting leukocyte modulation. However, little is known about selectin blockade in the cerebral microvasculature following I-R. The information that was gained from this investigation can add to the body of knowledge regarding the leukocyte contribution to cerebral I-R injury. Subsequently, this information may be useful in developing early treatments for acute ischemic stroke.
CHAPTER 3
METHODOLOGY

This study used an experimental design using a control \( (n = 9) \) and a treatment group \( (n = 9) \), to test the effect of fucoidin (FCN) on infarct size and neurological function. This chapter presents the methodology used in the study. Detailed descriptions of the methods, experimental protocol, data collection processes and data analyses are presented.

Methods

Animal Preparation

All animal experiments in this study were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health [NIH] Publication No. 80-23, revised 1985). A study protocol including the work to be done in this study was submitted to and approved by the University of Arizona Institutional Animal Care and Use Committee (Appendix A).

Male Sprague-Dawley rats weighing 250 to 350 grams were used. The animals were fasted 12 hours prior to surgery and allowed free access to water. The rats were initially anesthetized with 3.5% halothane with air and 1 L/min oxygen in a plexiglass chamber. Maintenance of anesthesia via a face mask was achieved with 1.5-2.5% halothane with air and 1 L/min oxygen. Body temperature was maintained at 36.0-37.5°C with a heating pad and recorded with a rectal probe. Respirations were monitored throughout the procedure and recorded with temperature readings at three time points:
(a) after induction of anesthesia, (b) after induction of ischemia, and (c) after reperfusion. The anterior neck and the chest, from chin to forelimbs, were cleansed with saline and clipped prior to surgical procedure. Appendix B presents examples of the surgical set-up used in these experiments.

**Middle Cerebral Artery Occlusion**

Middle cerebral artery occlusion (MCAO) was induced by the intraluminal filament method described by Zea Longa et al. (1989). Under an operating microscope, a midline incision was made and retractors placed on opposite sides of the surgical site. The right common carotid artery was exposed following separation of the omohyoid muscle. The external carotid artery (ECA) was then carefully dissected from surrounding nerves and fascia. The occipital artery and superior thyroid artery branches of the ECA were then cauterized. Next, the ECA was tied with 4.0 silk suture and cauterized. The internal carotid artery (ICA) was isolated and the pterygopalatine branch of the ICA was then dissected free in order to visualize the correct placement of the filament into the artery. A microvascular clamp was applied to the external carotid stub and a small hole was cut above the clip with microdissecting scissors. Prior to the surgical procedure, a 30mm segment of 4.0 nylon monofilament (Harvard Apparatus) was prepared by rounding the tip to a diameter of 0.25mm with a cautery tool. The prepared filament was then introduced into the vessel through the small hole and a 5.0 silk suture was tied around the vessel and the filament to prevent bleeding. The microvascular clamp was then removed, and the filament was advanced 18mm into the ICA, or until a subtle bending of the filament was visualized, indicating that the blunted filament tip had reached ostia of the MCA, thus
occluding distal flow into the artery. After the filament was placed, the neck incision was sutured and the animals were allowed to recover. All animals underwent a 4-hour period of ischemia.

After 4 hours of ischemia, animals were re-anesthetized in the manner described above. The surgical incision was then re-opened and preparations were made for filament removal. For reperfusion, the filament was carefully withdrawn until the tip became visible at the origin of the lumen of the ECA, thus, restoring blood flow through the MCA.

The animals were again allowed to recover. In the treatment group, animals were injected with FCN (Sigma # F-5631, Saint Louis, MO), 25 mg/kg, 10 minutes prior to reperfusion via a jugular catheter. Fucoidin was injected slowly, over 10 minutes. Treatment animals received a second dose of FCN 1 hour post reperfusion. In the control group, 1 ml of phosphate buffered saline (PBS) was injected at the same time points.

**Neurological Evaluation**

Neurological function of the animals was assessed 1-2 hours after ischemia was induced and 24 hours after reperfusion just prior to sacrifice for infarct size analysis. The neurological evaluation used in this study was adapted from examinations described by others (Bederson et al., 1986; Menzies et al., 1992; Yanaka et al., 1996; Zea Longa et al., 1989) (see Table 1). Animals were scored in four categories of behavior: (a) level of consciousness (LOC), (b) spontaneous circling, (c) front limb symmetry, and (d) front limb paresis. In the LOC assessment, the alertness and curiosity of the animal was assessed. In the spontaneous circling category, the animal was allowed to move freely and
Table 1.
Neurological Evaluation Scoring after MCAO and Reperfusion

<table>
<thead>
<tr>
<th>Neurological Function</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LOC</strong></td>
<td>Alert, energetic, curious, normal behavior</td>
<td>Moves slowly, somewhat curious</td>
<td>Does not move unless stimulated, no curiosity</td>
<td>Semi-comatose or comatose, difficult to arouse, not sitting upright</td>
</tr>
<tr>
<td><strong>Spontaneous Circling</strong></td>
<td>No circling behavior demonstrated, advances equally in all directions</td>
<td>Circles, occasionally, favors left side, usually heads straight</td>
<td>Circles most of the time, almost to left side, occasionally heads straight</td>
<td>Circles all of the time, always to left side, or does not move at all</td>
</tr>
<tr>
<td><strong>Front Limb Symmetry</strong></td>
<td>Both front limbs symmetrically extended to the ground</td>
<td>Slight, subtle asymmetry, occasional retraction of left limb toward body</td>
<td>Moderate, obvious asymmetry, frequent retraction of left limb, occasional body retraction to left</td>
<td>Prominent, severe asymmetry, constant retraction of left limb, body continuously retracted to left</td>
</tr>
<tr>
<td><strong>Tail Suspension</strong></td>
<td>No evidence of left sided weakness, normal gait</td>
<td>Slight, subtle weakness noted in left front limb, leans/favors left occasionally</td>
<td>Moderate, obvious weakness noted in left front and back limbs, lifts limbs slowly, leans/favors left usually, may drag limb</td>
<td>Prominent, severe left sided weakness, left front and back limbs curl toward body, leans/favors left always, drags left limbs</td>
</tr>
</tbody>
</table>

assessed for nondirectional circling toward the paretic side. The symmetry of the animal’s front limb was assessed by briefly suspending the rat by its tail. Retraction of the limb toward the body on the affected side was evaluated and compared with the non-affected limb in this test. Observing the movement of the animal assessed the paralysis of the affected limb. The degree of leaning or dragging of the limb was evaluated and scored in the last category. Appendix C contains examples of each of the categories scored.

Neurological function was graded on a scale of 0 to 3 for each category, based on the level of neurological impairment. An animal with no neurological deficits, exhibiting
normal behavior, received a score of 0; severe impairment received a score of 3 for the category. The scores in each category were added together to yield a total neurological score. To be included in the study, animals had to receive an initial total neurologic score between 6 and 10.

Staining for Infarction Volume

Prior to retrieval of the brain for infarction size analysis, the animals were deeply anesthetized with 3.5% halothane and 1 L/min oxygen. After deep anesthesia was achieved, the brain was quickly removed, placed in iced phosphate buffered saline, and put in a −10°C freezer for 5 minutes. The specimen was then placed in a brain slicer (Braintree Scientific Inc., BS-4000C) and sectioned into seven, 2 mm coronal slices. Slices were identified as sections 1 through 7 in the frontal to occipital direction. For delineation of area of infarction, the brain sections were placed in individual wells in a 12-well specimen plate and then immediately incubated at 37°C in 2% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma # T-8877, St Louis, MO). After 30 minutes, TTC was removed from the wells and replaced with 10% formalin for tissue fixing. The plate was then protected from light with aluminum foil and refrigerated until the sections were photographed.

Infarction Volume Analysis

Photographed images of the brain sections were obtained 5-7 days after fixation in formalin. Labels identifying the type of experiment, date of procedure, animal number and section number were made for each specimen. Each section was removed from the well plate, placed briefly on blotting paper and placed under the operating microscope on a
plain colored background. Care was taken to minimize handling of the delicate, infarcted tissue. The appropriate label and a millimeter ruler were placed in the field. Using a dissecting microscope (Zeiss, Stemi-2000C) at 6.5x magnification, the section was visualized and captured on 35mm color film (Kodak GOLD, 200 speed). After film development, the images were scanned (Mustek 600 III EP Plus scanner) and converted into a digital format (Micrografx Picture Publisher, Version 6.0). The digital images were stored using JPEG File Interchange format at a resolution of 200 dots per inch (dpi).

The digitized images of the brain sections were analyzed for area of infarction using image analysis software (Zeiss KS 300 Imaging System, Version 3.0). Infarcted brain was visualized as an area of unstained white tissue, in contrast to viable tissue, which stained red. The area of infarct and the ipsilateral (hemisphere with the infarction) and contralateral (hemisphere without the infarction) hemispheric areas (mm$^2$) were measured by tracing the desired area on the computer screen. For each coronal section, the following direct measurements were performed: (a) area of the contralateral total hemisphere, (b) area of the ipsilateral total hemisphere, (c) non-infarcted area of the ipsilateral hemisphere, and (d) infarcted area of the ipsilateral hemisphere. (Connolly et al., 1996; Soriano et al., 1996). The investigator performed all measurements.

When fragility of the specimen resulted in small fragments of missing tissue, two procedures for determining areas were used. Morikawa et al. (1996) reported that, in the MCAO model of stroke, the ipsilateral hemisphere had 4% greater hemispheric area than the contralateral hemisphere. Therefore, in the present study, if part of the ipsilateral hemisphere was missing, the contralateral hemisphere area plus an additional 4% was used.
as the ipsilateral hemisphere area for that section. When a part of the contralateral hemisphere was missing, 4% of the ipsilateral hemisphere area was subtracted and this value substituted for the contralateral hemisphere area. In the second procedure, the area of missing tissue was traced by visually comparing the area to the intact, opposite hemisphere. The areas obtained from each procedure were remarkably similar (Appendix D). However, to maintain the most consistency between samples, calculations using the 4% correction for edema were used if tissue was missing. If tissue was missing from both the contralateral and ipsilateral hemispheres in the same section, the specimen was not included in the study.

**Fucoidin Treatment**

The P and L-selectin blocker, FCN, was used in this study to examine leukocyte contribution to cerebral injury following I-R. Previous studies have shown that treatment with FCN significantly decreases leukocyte rolling on vascular endothelium (Kubes et al., 1995) and leukocyte accumulation in the microcirculation after ischemia and reperfusion (Ritter et al., 1998). In this study, FCN was given to the treatment group at a dosage of 25 mg/kg 10 minutes prior to reperfusion and 1 hour post reperfusion as described by Kubes et al. (1995).

**Experimental Protocol**

To examine the leukocyte contribution to ischemia-reperfusion (I-R) injury following focal stroke, the research design of this study used middle cerebral artery occlusion and reperfusion (MCAO-R) in the rat. Using FCN, the effects of both P and L-
selectin blockade on the size of cerebral infarction was analyzed. The effect of selectin blockade on neurological function was also examined. Standard operating procedures (SOPs), developed by the investigator and others, were followed during the experiments (see Appendix E for samples). The data collected during the experiments was recorded on a data collection form (Appendix F) and the forms were placed in a laboratory manual.

Two groups were studied: (a) operated controls (N = 9), and (b) operated animals treated with FCN (25 mg/kg) (N = 9). Prior to the day’s experiments, animals were randomly assigned to the experimental group or the control group by a coin toss. The operated control group underwent the same surgical procedures as the treated group. The animals were anesthetized and a jugular catheter was inserted. An experienced surgeon then performed the MCAO procedure. Reperfusion was initiated by removing the filament after 4 hours of ischemia. In order to obtain maximal effects of selectin adhesion molecule blockade during the first minutes of reperfusion, FCN was administered 10 minutes prior to reperfusion and 1 hour post reperfusion in the experimental group. The control group received 1 ml of phosphate buffered saline (PBS) 10 minutes prior to reperfusion and 1 hour post reperfusion. The animals were assessed for neurological function at 1 hour after MCAO and at 24 hours post reperfusion. After reperfusion, the animals were allowed to recover with access to water and a diet of softened food pellets. At 24 hours of reperfusion, animals were sacrificed and the brains retrieved for analysis of infarction size. After retrievals, brains were sliced and incubated in TTC, fixed in formalin and refrigerated. Approximately 5-7 days later, the specimens were appropriately labeled and photographed. Images were scanned into a digital format and computer analysis.
performed to determine infarction size in both groups. A comparison of neurologic assessment scores for both groups was also performed.

Data Analysis

All data collected in these experiments were tabulated on Microsoft Exel 97 spreadsheets. The physiologic parameters of weight, temperature, respiratory rate are reported.

Digitalized images of the brain slices were analyzed for area of infarction using image analysis software. To reduce experimenter bias during analysis, the images did not reflect group assignment and specimens were labeled with animal number and date only. Information regarding group assignment was kept in a laboratory manual until data collection was finished. Initially, a tracing was performed three times and the average area was recorded for each section. After tracing several sections, the variability between tracings of the same section became negligible (Appendix G). Therefore, for the remainder of the analysis, the areas of a section were traced once.

In order to compare different analysis methodologies reported in the literature, two methods of measurement were employed in analysis of the infarct size data. In the direct measurement method, the infarcted area of the ipsilateral hemisphere was traced in sections 1-7 for each brain. If the section did not have any area of injury, the infarct value was 0. The area of each section is presented as infarct area per section. The total volume (mm$^3$) of infarcted tissue for each brain was then determined by integrating the appropriate area with the section thickness (2mm). Data are expressed as a volume of infarction and
as a percentage of the total ipsilateral hemispheric volume. The direct method of measuring infarction size has been reported by a number of investigators (Connolly et al., 1996; Garcia et al., 1993; Prestigiacomo et al., 1999; Zhang et al., 1994).

To minimize the effect of brain edema, a second method, the calculated measurement method of determining infarction size, was performed. Using this method, calculation of the infarcted area was accomplished indirectly by subtracting the area of noninfarcted tissue in the ipsilateral hemisphere from the total area of the contralateral hemisphere. Infarction areas determined this way are expressed as they are in the direct measurement method, that is, as infarct area per section. Total infarct volume for each brain was determined by multiplying the calculated infarct area by the section thickness (2mm). In contrast to the direct method, the calculated method expresses infarct volume as a percentage of the contralateral hemisphere. The calculated method corrects for cerebral edema, which increases the size of the ipsilateral hemisphere. Recent studies report the use of this method of determining infarction size because this measurement more accurately reflects the true, uninflated value of tissue infarction (Lin et al., 1993; Soriano et al., 1996, 1999; Zhang et al., 1996).

The extensive data collected using the above methods for determination of infarction size revealed an interesting finding in terms of cerebral edema. Therefore, in order to explore the effect of FCN on cerebral swelling, calculations were performed on the specimens in the control and FCN treated groups. According to the methods of Kaplan (1991), neocortical edema volume was estimated by subtracting the volume of the contralateral hemisphere from that of the ipsilateral hemisphere. Additionally, percent
edema was determined by the following formula:  
\[
\text{Edema (\%)} = \frac{\text{ipsilateral hemispheric volume} - \text{contralateral hemispheric volume}}{\text{contralateral hemispheric volume}} \times 100 
\]
(Morikawa et al., 1996, p.953).

Infarct volumes, determined by both direct and corrected measurement methods, cerebral edema, physiological variables and neurological examination scores were compared between control and treatment groups (GB-Stat\textsuperscript{TM} for Windows\textsuperscript{TM}, Version 6.5). A Student’s \textit{t}-test for unpaired variables was used to determine whether a significant difference in infarct size existed between the control and treatment groups. Comparisons between groups in terms of physiologic parameters, and edema volume were also made using a Student’s \textit{t}-test. Neurological scores did not exhibit normal distribution and required analysis with a non-parametric test. Therefore, the non-paired Wilcoxon-Rank Sum test was used to compare the neurological scores between the control and FCN groups. A paired Wilcoxon-Rank Sum test was used to identify significant differences in ischemia and reperfusion neurological scores within the FCN group. Physiological parameters, infarction size and edema volume data are presented as means ± standard error of the means (SEM). Neurological scores are presented as medians and range. In all analyses a value of \( p < 0.05 \) was considered statistically significant.

Using an effect size of 0.5, (\( \alpha = 0.05 \), \( \beta = 0.2 \), power = 0.8), a sample size of 17 animals per group was estimated (Cohen, 1977). However, to ensure prudent use of animals, data analysis was performed periodically to determine statistical significance.
Summary

To study the effects of dual selectin inhibition with FCN on infarct size and neurological function following MCAO-R, two groups were studied. In these experiments, control (n = 9) and treatment (n = 9) groups underwent focal stroke via the filament method of MCAO. After 4 hours of ischemia, the filament was removed, inducing reperfusion of the brain. In the treatment group, FCN (25mg/kg) was administered 10 minutes prior to reperfusion and a second dose was administered one hour after reperfusion. Neurological function was assessed following ischemia and 24 hours after reperfusion just prior to retrieval of the brains for analysis of infarction size. A time line of the experimental protocol is presented in Appendix H. Multiple measurements were obtained using image analysis software and different analysis methodologies were employed for infarct size analysis. Cerebral edema was also investigated. The results obtained following analysis of these data are presented in the next chapter.
CHAPTER 4
PRESENTATION AND ANALYSIS OF DATA

This chapter presents the characteristics of the sample and the statistical analysis of the physiologic parameters, infarction size, cerebral swelling and neurological scores.

Characteristics of the Sample

Surgery was performed on study animals over a 12-week period. Periodic analysis to determine statistical significance was performed and a total sample size of 18 animals demonstrated statistically significant results. All 18 animals met the requirements for study inclusion (Control = 9; FCN treated = 9).

Statistical Analysis of Physiologic Parameters

The physiologic parameters of weight, temperature and respiratory rate were obtained in these experiments. Weights (gms) were obtained prior to the initial surgical procedure. Temperature (°C) and respirations (rate/min) were monitored throughout the surgical procedures and recorded at three time points as described previously. Table 2 summarizes physiologic parameter data from all time points obtained in each group. A significant difference in the temperature readings was found between the two groups. The mean temperature of the FCN treated group was 0.62 °C higher than the control group ($p = 0.024$). The two groups did not differ with respect to body weight and respiratory rate. Raw data collected at each time point for each of the animals included in the study is presented in Appendix I.
Table 2.
Physiologic Parameters in the Two Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Weight (Gm)</th>
<th>Temp (°C)</th>
<th>Resp Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>9</td>
<td>289.2 ± 7.9</td>
<td>36.5 ± 0.2</td>
<td>72.0 ± 4.8</td>
</tr>
<tr>
<td>FCN</td>
<td>9</td>
<td>287.2 ± 6.7</td>
<td>37.1 ± 0.2*</td>
<td>80.4 ± 2.2</td>
</tr>
</tbody>
</table>

Note. Values are mean ± SEM; Temp and Resp Rate recorded at three time points: (a) after induction of anesthesia, (b) after induction of ischemia, and (c) after reperfusion. * Significantly different than controls ($p = 0.024$)

Statistical Analysis of Infarction Size

To account for missing tissue that was lost in the process of sample retrieval, sectioning, and staining, calculations were performed for data analysis as described in the Methods section. In the control group, 12 of the total 62 brain sections had small pieces of tissue missing that resulted in calculation adjustments. In the FCN treated animals, 7 of the total 63 sections had missing tissue, which resulted in calculation adjustments. In the control group, the area of section 1 was calculated in only 8 animals due to an error in slicing technique. Appendix D summarizes the specific samples involved and the adjustments made.

Fucoidin Effect on Pattern of Infarction following MCAO-R

Infarction area patterns in the two groups was calculated using two different methods. Figures 2 and 3 represent the mean infarction areas for each coronal section using the direct and corrected methods, respectively.

Using either method for infarct calculation, coronal sections 3 and 4 demonstrated
the largest infarction areas in both control and FCN treated groups. Figure 2 depicts mean infarction areas (mm$^2$) using the direct method of measurement. Fucoidin treatment resulted in a significant reduction in mean infarct area in section 3 (Control = 39.17 mm$^2$; FCN = 23.87 mm$^2$; $p = 0.0218$), section 4 (Control = 40.09 mm$^2$; FCN = 12.35 mm$^2$; $p < 0.0001$), and section 5 (Control = 14.64 mm$^2$; FCN = 2.09 mm$^2$; $p = 0.0196$).

The corrected method of measuring infarct volume corrects for edema of the injured tissue in the ipsilateral hemisphere. Using this method, a significant reduction in infarct area was also observed in section 3 (Control = 39.16 mm$^2$; FCN = 22.57 mm$^2$; $p = 0.0215$), section 4 (Control = 37.38 mm$^2$; FCN = 14.68 mm$^2$; $p < 0.0004$), and section 5 (Control = 16.30 mm$^2$; FCN = 2.81 mm$^2$; $p = 0.0102$) (Figure 3).

Figure 4 illustrates representative images from the control (left) and FCN treated (right) groups. The coronal sections 3, 4, and 5 demonstrate significant differences in infarction size following FCN treatment.

**Fucoidin Effect on Infarction Volume following MCAO-R**

The previous graphs depicted the areas of ischemic injury in each coronal section for the two groups. Integration of the areas with the interval thickness of each section (2 mm) yielded the total volume measurements obtained. Table 3 summarizes the volume measurements obtained in the control and FCN treated groups. The contralateral and ipsilateral hemispheric volumes were similar in each group, although the volume of the ipsilateral hemispheres tended to be greater than the contralateral hemispheres. Infarction volume was calculated using the direct method and the method that corrects for edema.
Figure 2. Using the direct measurement method of infarct calculation, the pattern of infarction for each 2 mm coronal section (1-7) from anterior to posterior in Control (n = 9) and FCN treated (n = 9) groups is depicted. Points represent the mean infarct area per section; error bars depict standard errors of the means.

* Statistically different from controls
Figure 3. Using the corrected measurement method of infarct calculation, the pattern of infarction for each 2 mm coronal section (1-7) from anterior to posterior in Control (n = 9) and FCN treated (n = 9) groups is depicted. Points represent the mean infarct area per section; error bars depict standard errors of the means.

* Statistically different from controls
Figure 4. A representative specimen from both the control (left) and FCN treated (right) groups. The statistically significant sections (3-5) are shown.
Table 3.

Hemispheric Volume and Infarction Size in Control and FCN treated groups using the direct and corrected methods of measurement

<table>
<thead>
<tr>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CON (n = 9)</td>
<td>904.1 ± 14.0</td>
<td>920.8 ± 14.0</td>
<td>243.5 ± 20.5</td>
<td>26.6 ± 2.5%</td>
<td>233.7 ± 20.3</td>
<td>26.0 ± 2.5%</td>
</tr>
<tr>
<td>FCN (n = 9)</td>
<td>878.9 ± 15.6</td>
<td>880.4 ± 14.9</td>
<td>105.8 ± 23.4*</td>
<td>12.0 ± 2.7%*</td>
<td>110.1 ± 21.0*</td>
<td>12.6 ± 2.5%*</td>
</tr>
</tbody>
</table>

Note. Volumes are in mm$^3$; Values are mean ± SEM
* Statistically different from controls ($p = 0.001$)
There was a significant decrease in infarction volume in the FCN treated group compared to controls, as measured by both methods ($p = 0.001$) (Table 3). The size of infarction was also expressed as a percent of the ipsilateral side using the direct method (Control = $26.6 \pm 2.5\%$; FCN = $12.0 \pm 2.7\%$) and as a percent of the contralateral side using the corrected method (Control = $26.0 \pm 2.5\%$; FCN = $12.6 \pm 2.5\%$). There were no significant intergroup differences between the direct versus the corrected calculation methods. Therefore, the correction for edema did not affect the significance of the results.

Using the corrected method, Figure 5 represents the integration of the areas of injury to yield a total infarct volume (mm$^3$). The total mean infarct volume for the control and FCN groups was $233.67 \pm 20.3$ and $110.12 \pm 21.0$, respectively. Therefore, fucoidin treatment reduced infarction volume by approximately 50%.

**Statistical Analysis of Cerebral Swelling**

*Fucoidin Effect on Edema*

The effect of FCN on cerebral edema was determined in two ways. Figure 6 demonstrates the effect of FCN on mean edema volume (mm$^3$). Although a decrease in edema volume was observed following FCN treatment, this change was not statistically significant ($p = 0.083$). The decrease in percent edema with FCN treatment, calculated using the formula described in the Methods section, is illustrated in Figure 7. A significant decrease was not demonstrated ($p = 0.085$).
Figure 5. Fucoidin effect on mean total infarct size, corrected for edema; Error bars depict standard error of the means
* Statistically different from controls ($p = 0.001$)
Figure 6. Fucoidin effect on neocortical edema volume (mm$^3$) estimated by subtracting the volume of the contralateral (nonischemic) cortex from that of the ipsilateral (ischemic) cortex; Vertical lines depict standard error of the means.
Figure 7. Fucoidin effect on Edema (%) calculated by the following formula:

\[
\text{Edema (\%)} = \frac{\text{ipsilateral hemispheric volume} - \text{contralateral hemispheric volume}}{\text{contralateral hemispheric volume}} \times 100;
\]

Vertical lines depict standard error of the means.
Statistical Analysis of Neurological Scores

*Fucoidin Effect on Neurological Function*

Functional neurologic data was obtained during ischemia (before FCN treatment) and again during reperfusion (after treatment with FCN). There were no significant differences in individual or total neurologic scores between the control and FCN treated groups. However, within the FCN group, there was a significant decrease in limb symmetry ($p = 0.0431$) and a significant decrease in the total neurologic score ($p = 0.03$) after reperfusion compared to ischemic scores. Table 4 summarizes the results obtained from both groups in each scoring category.

**Summary**

The results of this study confirmed the first hypothesis, which stated that animals treated with the dual selectin blocker, FCN, would have smaller cerebral infarcts versus non-treated control animals following MCAO-R. The second hypothesis stated that animals treated with FCN would exhibit improved neurological functioning following MCAO-R, the results in this study confirmed this hypothesis as well. Additionally, a trend toward decreased cerebral edema was observed.
Table 4.

Neurological Scores following Ischemia and Reperfusion

<table>
<thead>
<tr>
<th>Category</th>
<th>Level of Consciousness</th>
<th>Spontaneous Circling</th>
<th>Front Limb Symmetry</th>
<th>Limb Paresis</th>
<th>Total Neurological Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>R</td>
<td>I</td>
<td>R</td>
<td>I</td>
</tr>
<tr>
<td>CON (n=9)</td>
<td>2 (1-2)</td>
<td>2 (1-2)</td>
<td>1 (1-3)</td>
<td>1 (1-3)</td>
<td>2 (2-3)</td>
</tr>
<tr>
<td>FCN (n=9)</td>
<td>2 (2)</td>
<td>2 (2)</td>
<td>1 (1-2)</td>
<td>1 (0-2)</td>
<td>2 (1-3)</td>
</tr>
</tbody>
</table>

Note. I = Ischemic period, score obtained 1-2 hours after ischemia; R = Reperfusion period, score obtained 24 hours after reperfusion
Data expressed as Medians and (Range)
† Statistically different from fucoidin ischemia value using paired Wilcoxon-Rank Sum
The advent of thrombolytic therapy for stroke has made restoration of blood flow, or reperfusion, the desired treatment to limit brain cell death following acute ischemic stroke. However, there is increasing evidence to suggest that reperfusion following an ischemic episode initiates a complex inflammatory response in the brain that may cause additional injury to cerebral blood vessels and surrounding neuronal tissue. Leukocyte accumulation in the blood vessels is an initial event in the inflammatory response initiated by ischemia-reperfusion, or I-R (Ritter et al., 1995). Activation of leukocytes and endothelial cells occurs very rapidly after cerebral reperfusion, resulting in an upregulation of leukocyte and endothelial surface adhesion molecules. Adhesion molecules promote adherence of leukocytes to endothelium (Pantoni et al., 1997). It is the selectin family of adhesion molecules that mediate the initial, rolling attachment of leukocytes on endothelium. As a result of this early accumulation and adhesion of leukocytes to endothelium, mediated in part, by early expression of selectins, leukocytes may further damage cerebral tissue. Through a variety of mechanisms, (a) microvascular plugging, (b) toxic mediator release, (c) thrombosis initiation, and (d) leukocyte migration into the parenchyma, leukocytes may extend the ischemic area, expanding neuronal tissue necrosis.

To examine the early leukocyte contribution to I-R injury, the purpose of this study was to determine the effects of selectin adhesion molecule blockade on cerebral infarction size and neurological functioning following middle cerebral artery occlusion and
reperfusion. A discussion of the study results, clinical implications, and recommendations for further study are presented in this chapter.

Discussion of Results

The results of this study demonstrated that administration of fucoidin, an inhibitor of P and L-selectin, significantly reduces infarction size in the rat following 4 hours of focal ischemia and 24 hours of reperfusion. Additionally, selectin blockade significantly improved neurologic function within the treated group after 24 hours of reperfusion compared to pre-treatment (ischemic) values. Control animals showed no improvement in neurological functioning after 24 hours of reperfusion. These data support the hypothesis that early leukocyte activation and accumulation is a contributing factor in ischemic neural cell damage, and that attenuation of this process is an effective therapeutic intervention following transient focal ischemia in an animal model of stroke.

Physiologic Parameters

There were no significant differences in the control and FCN treated groups with respect to body weight or respiratory rate. The normal body temperature range for the rat is 36.0 - 39.5°C (Karibe, Chen, Zarow, Graham, & Weinstein, 1994). In these experiments normal temperature range was maintained in both groups. However, body temperature in the control group (36.5°C) during surgical procedures was significantly lower than in the FCN group (37.1°C). Animals in both groups were exposed to room temperature following MCAO. It is possible that hypothermia during the surgical procedure protected the control animals. It is known that postischemic hypothermia has a
neuroprotective effect and temperature management of experimental animals during and after surgery may also affect infarct volume (Karibe et al., 1994). Therefore, the size of infarction in the control group may be an underestimation. However, even with this possible underestimation in the control group, there was a statistically significant difference in infarct size between groups.

*Anti-selectin Effect on Infarct Pattern*

In this study, the areas of injury on coronal brain sections (1-7) after focal cerebral ischemia and reperfusion exhibited a pattern of infarction similar to what has been reported by others. Sections 3 and 4 exhibited the largest area of injury in the control and FCN treated groups. Using similar MCA occlusion and section preparation techniques, Chopp et al. (1994), Morikawa et al. (1996), and Zhang et al. (1996) reported the greatest area of infarct in sections 3 and 4 as well. This pattern of infarction appears independent of the time of ischemia, the time of reperfusion or the method used to induce stroke. For example, using a method of focal ischemia involving tandem occlusion of the MCA and common carotid artery (CCA), Morikawa et al. (1996) subjected animals to either 2 hours of permanent occlusion or 2 hours of occlusion and 30 minutes of reperfusion. The greatest area of infarction was in sections 3 and 4. Chopp et al. (1994) and Zhang et al. (1996) utilized the filament method of MCAO-R used in this study, with 2-hour periods of ischemia and 48 hours of reperfusion. These investigators reported sections 3 and 4 exhibiting the greatest areas of injury.

In the present study, a significantly smaller area of infarction was exhibited in coronal sections 3, 4, and 5 following treatment with FCN. Similar results have been
reported in other experiments targeting adhesion molecules to attenuate leukocyte-endothelial interactions. Zhang et al. (1996) demonstrated significant decreases in infarction size in coronal sections 4, 5, and 6 when targeting E-selectin with administration of CY-1503, an analog of sialyl Lewis$^x$ (Sle$^x$), the ligand for E-selectin. In addition, Chopp et al. (1994) reported significant differences observed in area of infarct in sections 2, 3, and 4 following administration of a monoclonal antibody targeting the CD11b/CD18 (Mac-1) integrin. These data indicate that anti-selectin therapy is effective in reducing cell death in the portions of the brain that are most likely to be affected with MCAO.

**Anti-selectin Effect on Total Infarct Volume**

The acute inflammatory reaction of leukocyte recruitment, accumulation and adhesion to endothelium following reperfusion of ischemic cerebral tissue has been shown to exacerbate brain damage following reperfused stroke through a variety of injury-inducing mechanisms (Härtl et al., 1996). Through depletion of circulating leukocytes, and the use of agents that interfere with leukocyte infiltration or manipulation of leukocyte-endothelial interactions, investigators demonstrated significant reductions in the volume of ischemic injury in the brain (Barone et al., 1997; Connolly et al., 1996, 1997; Matsuo et al., 1994; Morikawa et al., 1996; Prestig et al. 1999; Shiga et al., 1991, 1992; Soriano et al., 1996; Yamasaki et al., 1992, 1995; Zhang et al., 1996).

The focus of this study was the inhibition of the selectin family of adhesion molecules, which are responsible for the initial attraction, or rolling, of leukocytes on endothelium (Pantoni et al., 1997). The molecular mechanisms underlying leukocyte rolling has received a great deal of attention in recent years. This interest is due, in large
part, to the fact that the initial process of leukocyte rolling is a prerequisite for subsequent firm leukocyte adhesion and diapedesis, and therefore may be an early target for therapeutic intervention in the ischemia-reperfusion injury cascade (Kubes et al., 1995). During inflammation following I-R in the brain, local endothelium rapidly translocates P-selectin from Weible-Palade secretory granules and E-selectin on the cell’s surface. In addition, L-selectin is expressed on the leukocyte cell membrane (DeGraba, 1998).

Fucoidin (FCN), a sulfated, fucosylated, polysaccharide derived from marine algae, shares structural similarities with the selectin ligands (Angstwurm et al., 1995), which are sialylated carbohydrates. Therefore, FCN readily binds to P- and L-selectin (Kubes et al., 1995). Thus, by inhibiting both P- and L-selectin from binding to their respective ligands, FCN acts as a competitive inhibitor of selectin-mediated leukocyte adhesion to vascular endothelium during I-R (Patankar, 1993). Fucoidin does not inhibit E-selectin (Bevilacqua & Nelson, 1993). Because both the blood (Shiga et al., 1991) and the endothelium (Schmid-Schönbein, 1995) may be activated in I-R (DeGraba, 1998), it is an advantage to utilize a compound such as FCN which targets the blood (L-selectin) and endothelial (P-selectin) components (Kubes et al., 1995).

Ritter et al. (1998) reported a significant decrease in leukocyte accumulation in the coronary venules and capillaries of the rat during postischemic reperfusion following treatment with FCN. Using ultravital microscopy, Kubes et al. (1995) observed a >90% reduction in leukocyte rolling in postischemic mesentary venules in the cat with FCN (25 mg/kg). This finding translated into a 50% attenuation of the normal reperfusion-induced leukocyte adhesion to the endothelium. The results of the present study demonstrated that
administering the same concentration of FCN (25 mg/kg) prior to reperfusion and one hour after reperfusion reduced cerebral infarction size by approximately half. This observation suggests that a reduction of leukocyte adhesion, via P- and L-selectin inhibition, significantly reduces the area of injury in cerebral tissue after stroke and reperfusion.

Other investigators reported similar decreases in cerebral infarction size using different anti-selectin treatments. Morikawa et al. (1996) observed a significant reduction in infarct volumes using a treatment aimed at inhibiting selectin-mediated cell adhesion. The investigators reported a 42% decrease in infarction volume following administration of a selectin oligopeptide in rats subjected to 2 hours of focal cerebral ischemia followed by 24 hours of partial reperfusion. However, treatment with the oligopeptide did not reduce infarct volume in the group subjected to permanent ischemia (Morikawa et al., 1996). Interestingly, these data indicate that the injury mechanisms associated with reperfusion, but not permanent occlusion, may be responsive to anti-leukocyte and specifically, anti-selectin, adhesion therapy.

Using the MCAO model of 45 minutes of focal cerebral ischemia followed by 1 hour of reperfusion, Connolly et al. (1997) reported a 77% reduction in infarct volume in mice that were homozygous null for the P-selectin gene (PS -/-), as compared to wild-type mice (PS +/+). In the same study, Connolly et al. (1997) reported significantly reduced cerebral infarction volumes in the PS +/+ mice subjected to I-R and treated with monoclonal rat anti-mouse P-selectin antibody (clone RB 40.34).
The role of E-selectin in the pathogenesis of focal cerebral I-R injury has also been examined. After 2 hours of ischemia and 46 hours of reperfusion using the MCAO-R method, Zhang et al. (1996) reported a 42% reduction in cerebral infarct volume in rats treated with an analog of sialyl Lewis$^x$ (SLe$^x$), the carbohydrate ligand for E-selectin.

Collectively, these data and the results of this study suggest that selectin adhesion molecules are important contributors to leukocyte accumulation and the subsequent damage they incur in reperfused stroke. Therefore, attenuating these responses with anti-adhesion molecule therapies may benefit the recipients by reducing the size of brain injury and improving neurological function.

**Direct vs. Corrected Method of Infarction Size Analysis**

Correcting for edema using the corrected method of measuring infarct volume did not affect the statistical comparison of infarct size between groups. Morikawa et al. (1996) also reported that a correction for brain edema did not affect comparisons of the volumes of injury among groups with permanent or transient focal ischemia. However, correcting for edema not only avoids overestimation of actual volume of injured tissue, but yields information regarding the extent of cerebral edema after a given treatment. The majority of investigators report cerebral infarct volumes that have been corrected for edema (Kaplan et al., 1991; Lin et al., 1993; Morikawa et al., 1996; Soriano et al., 1999; Swanson et al., 1990).

**Anti-selectin Effect on Cerebral Swelling**

Little information exists regarding the effect of anti-selectin therapies on cerebral edema. However, several studies demonstrated an association between leukocyte
accumulation and cerebral edema following focal stroke and reperfusion (Kochanek & Hallenbeck, 1992; Lin et al., 1993; Matsuo et al., 1994; Shiga et al., 1991). Using the filament method of MCAO-R, Shiga et al. (1991) reported a suppression of postischemic brain edema in rats following a depletion of circulating neutrophils with anti-neutrophil monoclonal antibody (RP3). To evaluate the role of intercellular adhesion molecule-1 (ICAM-1) and integrins CD11a and CD18 on postischemic brain edema formation in rats subjected to MACO-R, Matsuo et al. (1994) used monoclonal antibodies targeting these molecules. They demonstrated that pretreatment with antibodies against CD11a, CD18, or ICAM-1 significantly reduced brain edema in the area of the cerebral cortex following 24 hours of reperfusion. However, edema was not significantly reduced with antibody treatment in the caudate putamen (considered the ischemic core in MCAO) in their study.

Although quantifying the edema in separate portions of the brain was not done in the present study, after careful examination of the images obtained, it did appear that infarct reduction occurred mainly in the cortex. Therefore, we can postulate that the majority of edema reduction after FCN treatment occurred in the cortex as well. Although the reduction in cerebral edema was not statistically significant \( p = 0.0847 \), less edema was observed in the FCN treated group (0.20 %) versus the control group (1.85 %). This finding suggests that an increase in postischemic vascular permeability and subsequent cerebral edema may be mediated, in part, by P- and L-selectin.

*Anti-selectin Effects on Neurological Function*

In this study, neurologic evaluation was performed for study inclusion and for evaluation of treatment. Neurologic function was assessed by scoring the level of
consciousness, spontaneous circling, limb symmetry, and limb paresis during ischemia and after reperfusion. A higher score indicates a more severe stroke. A significant improvement was observed in neurological function within the FCN treated group, when ischemia and reperfusion values were compared. A statistically significant difference between control and FCN treated groups was not demonstrated during ischemia or reperfusion.

Yanaka et al. (1996) reported significant improvements in neurological functioning in rats subjected to MCAO-R and treated with fibronectin peptides to leukocyte adhesion molecules when compared to controls. They also reported a significant decrease in infarction volume in treated animals compared to controls. There have been no previous reports of an improvement in neurologic function with the use of anti-selectin therapy, after cerebral I-R in the rat. Although post-reperfusion neurologic function in the FCN treated group (total score 7; range 6-10) did not statistically differ from the control group (total score 7; range 5-9), there was a trend toward better neurological function in the FCN treated group. The significant reduction in infarction size and the modest decrease in cerebral edema likely accounted for the observed improvement in neurological function in treated animals.

Clinical Implications

Recent clinical trials have demonstrated that early reperfusion with t-PA following acute ischemic stroke can reduce neurological damage (NINDS rt-PA Stroke Study Group, 1999; STARS Study Group, 2000). Reperfusion of ischemic tissue is essential for
cellular metabolism. However, there is overwhelming research that demonstrates the damaging effects of inflammation following reperfusion of ischemic cerebral tissue, termed I-R injury. Leukocyte infiltration of reperfused tissue is the hallmark of I-R injury. Leukocyte accumulation in cerebral infarcts of patients has been associated with poor clinical outcomes (Akopov, Simonian, and Grigorian, 1996). Mediated by selectin adhesion molecules, leukocyte rolling on the vascular endothelium is the initial step in the inflammatory process. Therefore, there is some intuitive appeal to interrupting the inflammatory process at its earliest stages (Miura et al., 1996).

As previously discussed, anti-leukocyte treatment was not effective in reducing infarction size after permanent occlusion in animal studies. Prestigiacomo et al. (1999) reported diminished leukocyte recruitment and cerebral protection in CD18 deficient mice in the setting of reperfused stroke. In permanent focal cerebral ischemia, the lack of CD18 was not protective. Bowes, Rothlein, Fagan, and Zivin (1995) demonstrated similar results with CD18 antibody and ICAM-1 antibody in a rabbit model of embolic stroke. However, when antibodies were administered following reperfusion with t-PA, improved neurological outcome was observed. These studies suggest that leukocyte anti-adhesive strategies may be most effective during the reperfusion period. In light of the current practice to administer the thrombolytic, t-PA, for reperfusion in acute ischemic stroke, it is reasonable to hypothesize that the addition of an early anti-inflammatory agent, such as anti-selectin treatment, to the pharmacological regime may improve outcomes after stroke.

Clinical studies support a role for activated leukocytes in the injury process following stroke (Kochanek & Hallenbeck, 1992). Pozzilli et al. (1985) reported that an
elevated peripheral leukocyte count 72 hours following stroke was associated with significantly larger infarct size, impaired LOC, and poor clinical outcome. Other clinical studies support the consequence of vascular obstruction (Mercuri, Ciuffetti, Robinson & Toole, 1989), and vasoconstictor effects (Mugge et al., 1991) by activated leukocytes in cerebral circulation. It is studies such as these that may assist in explaining the leukocyte contributions to additional cerebral damage following stroke.

Adhesion molecule expression following ischemic events may further characterize the role of leukocyte activation in human stroke. Fassbender et al., (1995) characterized the pattern of release of adhesion molecules in human patients with acute ischemic stroke. When compared to healthy controls, Fassbender et al. reported those patients with acute stroke had significantly higher levels of a selectin-type adhesion molecule (sELAM-1) located primarily on surfaces of activated endothelial cells 8 hours, 10 hours and day 1 following stroke. Also observed in this study were significantly increased levels of immunoglobulin-type adhesion molecule (ICAM-1) in patients with acute stroke when compared to controls. This increase in ICAM-1 was observed until day 5 following ischemic stroke. It was also reported in this study that patients with risk factors for atherosclerosis (e.g. smoking, hypertension, diabetes mellitus, or hypercholesterolemia) had increased levels of immunoglobulin-type adhesion molecule (ICAM-1) in the absence of stroke. A decrease in circulating, leukocyte-derived L-selectin acute stroke was found in patients with risk factors which and may be explained by L-selectin binding to upregulated endothelial counterreceptors. Elevated levels of circulating adhesion molecules have also been reported in diabetic patients (Roep, Heidenthal, deVries, Kolb &
Martin, 1994). These data suggest that anti-inflammatory treatments, such as anti-selectin therapy may improve outcome following acute ischemic stroke. Additionally, the elevated levels of circulating adhesion molecules found in patients with risk factors for vascular disease suggests chronic endothelial inflammation in these patients. This inflammation may be amenable to anti-inflammatory treatments, thus decreasing risk for acute events such as stroke.

Today's patient often presents to their healthcare provider armed with information and questions regarding the latest research study, new medications, new diagnostic tests, or the latest technological advances. The Internet has recently exposed individuals to a wealth of healthcare facts (and fiction) that, previously, was not easily accessed. Because stroke is the third leading cause of death in the United States, it is one of the priority health problems reported in the electronic media, the news, and medical literature. Health care providers, and nurses in particular, can play a key role in educating patients regarding risk factors for cerebrovascular disease, warning signs of stroke, signs and symptoms of acute stroke, and new therapies for stroke intervention and treatment. To successfully fulfill this role, one must keep abreast of the latest data available in their field of interest. Although anti-inflammatory therapies are not a part of the current pharmacologic regime for acute stroke, the balance of research findings suggest that they may be beneficial if used in combination with thrombolytic therapy and/or other neuroprotective agents (De Keyser, Sulter & Luiten, 1999).
Recommendations for Further Study

The events surrounding I-R injury in the brain are complex and multifaceted. There are many aspects of the acute inflammatory response following I-R that merit further investigation. Of particular interest are blood cell to blood cell interactions in acute inflammation in the brain. Little is known about the mechanisms and functional significance of leukocyte-platelet interactions after stroke. It has been suggested that when activated leukocytes and platelets interact with each other, their inflammatory and thrombotic cell responses may be exaggerated, potentially increasing cell damage in cerebral tissue following stroke. Inhibition of leukocyte-platelet interactions with anti-selectin therapies (FCN) and anti-platelet therapies (GPIIb/IIIa inhibitors) and the effects on leukocyte accumulation in the cerebral microvasculature, on infarct size, and on neurological function are in need of further study.

Summary

Early intervention after acute ischemic stroke is essential to avoid brain cell injury and death. Reperfusion of the ischemic brain with thrombolytic therapy such at t-PA has achieved excellent outcomes and has a sustained benefit (NINDS rt-PA Stroke Study Group, 1999). Paradoxically, reperfusion itself may cause additional injury. It is known that early leukocyte accumulation and interaction with endothelium contributes to this additional injury. The selectin family of adhesion molecules mediates the initial, rolling attachment of leukocytes to endothelium. P-selectin is rapidly expressed on ischemic endothelium in the brain and L-selectin is expressed on leukocytes. Fucoidin inhibits
P- and L-selectin binding to their natural carbohydrate ligands and has been reported to decrease leukocyte accumulation during postischemic reperfusion (Ritter et al., 1998). Little was known about the effects of both leukocyte and endothelial selectin inhibition after cerebral ischemia and reperfusion. The purpose of this study was to determine the effects of selectin adhesion molecule blockade on cerebral infarction size and neurological function following middle cerebral artery occlusion and reperfusion.

The results of this study demonstrate that attenuating the leukocyte-endothelial response early in reperfusion via selectin blockade is protective. The extent of ischemic injury is decreased and neurologic function is improved with fucoidin treatment.

The knowledge gained from this study more clearly characterizes leukocyte-endothelial interactions that occur initially following reperfused stroke. In addition, information from this and similar studies may aid in development of optimal anti-inflammatory therapies for stroke that will limit brain injury and reduce disability and mortality from stroke.
APPENDIX A

Study Protocol Approval
Verification of Review
By The Institutional Animal Care and Use Committee (IACUC)

Final Approval Granted
PHS Assurance No. A-3248-01 -- USDA No. 86-3

TITLE: PROTOCOL CONTROL #97-042

“Cerebral Vascular Leukocyte Accumulation After Stroke”

PRINCIPAL INVESTIGATOR/DEPARTMENT:
Leslie S. Ritter, PhD - Neurology

SUBMISSION DATE: March 19, 1997           APPROVAL DATE: May 22, 1997

GRANTING AGENCY:
NIH NRSA

The University of Arizona Institutional Animal Care and Use Committee reviews all sections of proposals relating to animal care and use. The above named proposal has been granted Final Approval according to the review policies of the IACUC.

NOTES:

*** Full approval of this control number is valid through May 21, 2000

* When projects or grant periods exceed past the above noted expiration date, the Principal Investigator will submit a new protocol proposal for full review. Following IACUC review, a new Protocol Control Number and Expiration Date will be assigned.

*** Continued approval for this project was confirmed May 23, 1997

*** Revisions (if any), are listed below:

Michael A. Cusanovich, Ph.D.
Vice President for Research

DATE: May 23, 1997
APPENDIX B

Surgical Set-Up
Anesthesia Set-Up

Surgical Prep

Surgical Procedure
APPENDIX C

Categories of Neurological Scoring
Limb Symmetry

Level of Consciousness

Spontaneous Circling

Limb Paresis
APPENDIX C

Samples with Tissue Missing
Associated Calculations

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<th>Estimated Measurement</th>
<th>Adjustment Made**</th>
<th>Corrected Measurement††</th>
<th>Difference (mm²)</th>
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<td>40.126</td>
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<td>Ipsil Hem</td>
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<td>+ 4% Contra</td>
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<td>+ 4% Contra</td>
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</tr>
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Note. Ipsil Hem = Ipsilateral Hemisphere  Contra Hem = Contralateral Hemisphere
** To account for missing tissue and correct for edema, 4% was added to the contralateral hemisphere measurement of the involved section if ipsilateral tissue was missing, and 4% was subtracted from the total ipsilateral hemisphere measurement of the involved section if contralateral tissue was missing (Morikawa, 1996). The % variability is the % difference between the estimate and the corrected measurement, (+) indicates the investigators overestimation and (-) indicates an underestimation. †† These measurement were used for data analysis.
APPENDIX E

Selected Standard Operating Procedures
Preparation of Fucoidin (FCN) for 25mg/kg dosage
10 ml of solution is adequate for treatment of 4 animals

Equipment Needed:

- Fucoidin 1G (Sigma # F-5631)
- PBS
- 15 ml centrifuge tube
- Balance (Mettler AE200)
- 4 x 4 weighing paper
- spatula
- 0.2um Acrodisc filters
- 20 ml syringe
- 20 g needle
- 1ml syringes

Example for 300 g rat:

300 g rat = 0.300 kg
25 mg x 0.300 = 7.5 mg/ml
7.5 mg x 10 ml = 75 mg FCN
(adjust dose mixed based on average weight in animals being used in experiment)

Procedure:

1. Determine average animal weight for the day’s experiment and mix drug based upon findings. (Example is for 300 g animal)
2. Pipette 10 ml of PBS into a 15 ml centrifuge tube.
3. Weigh 75.0 mg (0.075 g) Fucoidin and add to the PBS.
4. Cap the tube and vortex for 15 sec.
5. Using a 20 ml syringe and a 20g needle, withdraw the Fucoidin from the tube.
6. Remove the needle from the syringe, replacing it with the 0.2um Acrodisc filter.
7. Filter the fucoidin into a sterile 15 ml centrifuge tube.
8. Determine exact amount needed per animal and draw up in 1 ml syringe.
   Example: 275 g animal
   25 mg x 0.275 = 6.875 mg
   6.875 mg/7.5 mg/ml = 0.92 ml of prepared FCN solution
9. Discard any remaining fucoidin after completion of experiment.
Procedure for TTC Staining of Brain Slices

Equipment needed:

- 37 °C water bath
- 12 well plate
- 10 ml pipette
- TTC (Sigma # T-8877 )
- PBS
- 10% formalin
- Several microtome blades
- 2 one-sided razor blade
- 1mm section brain slicer
- Ice bucket and ice
- 2-100 ml glass beaker

Procedure:

1. At least one hour before brain slices are to be stained, prepare 2% TTC solution using 100 ml PBS in a glass beaker and put in the 37 °C water bath.
2. At least one hour before brain slices are to be stained, fill ice bucket and put 100 ml of PBS in a beaker and the brain slicer on the ice.
3. Immediately after the animal is euthanized, remove the brain and put in the iced PBS for 10 min.
4. After sitting in the iced PBS, place the beaker with the brain in the -20 °C freezer for 5-7 min just until the PBS begins to crystallize.
5. Pipette 4 ml of TTC into 9 wells of the 12 well plate immediately before the brain is sliced.
6. Remove brain from the freezer, and place in the 1mm section brain slicer.
7. Place a one-sided razor into the slicer against the cerebellar portion of the brain.
8. Place another one-sided razor into the slicer against the frontal lobe portion of the brain.
9. Starting at the frontal lobe, gently place one microtome blade every 2mm along the brain’s surface, take care not to exert pressure against the tissue.
10. After all the blades are in place, carefully place your fingers on top of all blades at the sides of the slicer and push down in one smooth motion making all 2mm slices at one time.
11. Very gently, place each section into a separate well in the plate.
12. Place the plate in the 37 °C water bath for 30 min.
13. After 30 min, remove the TTC with a pipette and discard in the sink.
14. With a new pipette, add 4 ml of formalin to each well.
15. Seal the plate with parafilm, cover with foil and label with experiment date, type, and signature.
16. Place in the 4 °C refrigerator for later analysis.
APPENDIX F

Data Collection Form
Date: _______ Experiment: Anti-Leukocyte Therapies/MCAO: FCN 4 hour Ischemia

Anesthesia: ___________ Induction: ___________ Maintenance: ___________

Animal #: ___________ Tail Band #: ___________ Weight (gms): ___________ Treated or Control

Start time: ___________ Resp Rate (after induction): ___________ Temp: ___________

Filament in: ___________ Resp Rate (after filament placed): ___________ Temp: ___________

End time: ___________ Total procedure time: ___________

Neuro Assessment: (See page 5 of lab manual N2 for scoring criteria)

Time post-op: _______ (Approx 1-2 hours post-op)

SCORE: LOC: ___________
Spon Circ: ___________
Limb Sym: ___________
Limb Paresis: ___________ TOTAL: ___________

Treatment:
Name: ___________ Concentration: ___________ Dose: ___________
Route: ___________ Dosing criteria/time: Dose 1 ___________ Dose 2 ___________

Special Instructions: _______________________________________________________

Filament out: ___________ Resp Rate (after filament out): ___________ Temp: ___________

Date: ___________
Neuro Assessment: (See page 5 of lab manual N2 for scoring criteria)

Time post-op: _______ (Approx 24 hours post reperfusion)

SCORE: LOC: ___________
Spon Circ: ___________
Limb Sym: ___________
Limb Paresis: ___________ TOTAL: ___________

Retrivial Time: ___________ TTC Stained: ___________

Comments:
APPENDIX G

Differences in Tracing Areas of Selected Sections
Raw Data

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<th>Animal # / Section #</th>
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Note. Tracing 1-3 are three sequential measurements of the specimen indicated. The % difference was calculated as the % difference between the largest measurement and the smallest measurement for that section: smallest measurement \( \div \) largest measurement \times 100 and subtracted from 100%.

#16FS4h and #18FS4h: Contralateral hemisphere measurements
#26FS4h: Ipsilateral hemisphere measurements
APPENDIX H

Time Line of Experimental Protocol
MCAO \rightarrow \text{Reperfusion}

\begin{array}{c}
4 \text{ hr Ischemia} \\
\text{FCN} \\
\text{FCN} \\
\text{Neuro exam} \\
\text{Image analysis}
\end{array}

\begin{array}{c}
24 \text{ hr Reperfusion} \\
\text{Neuro exam} \\
\text{Sectione} \\
\text{TTC stained} \\
\text{Infarction volume}
\end{array}
APPENDIX I

Physiologic Parameters
Raw Data

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<th>Temp-b</th>
<th>Temp-c</th>
<th>RR-a</th>
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<th>RR-c</th>
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Note. Temp = Temperature °C RR = Respiratory Rate
Weight was obtained preoperatively. Temperature and respiratory rate was
obtained at three time points: (a) after induction of anesthesia, (b) after induction
of ischemia, and (c) after reperfusion.
REFERENCES


Soriano, S. G., Coxon, A., Wang, Y. F., Frosch, M. P., Lipton, S. A., Hickey, P. R., & Mayadas, T. N. (1999). Mice deficient in Mac-1 (CD11b/CD18) are less susceptible to cerebral ischemia/reperfusion injury. Stroke, 30, 134-139.


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