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# Anti-Inflammatory Effects of PPAR-gamma in Surgical Brain Injury (SBI)

by

Amy Hyong

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Thesis submitted in partial satisfaction of
the requirements for the degree of
Master of Science in Physiology

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September 2008

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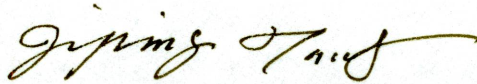
Each person whose signature appears below certifies that this thesis in his/her opinion is adequate, in scope and quality, and meets the requirements in fulfillment of the Masters of Science degree in Physiology.



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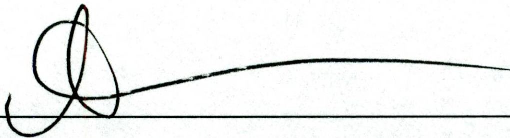

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ABBREVIATIONS

| | |
|---|---|
| ABC | Avidin-Biotin Complex |
| BBB | Blood Brain Barrier |
| COX | Cyclooxygenase |
| DBD | DNA Binding Domain |
| DMSO | Dimethyl Sulfoxide |
| GM-CSF | Granulocyte Macrophage Colony Stimulating Factor |
| HTAB | Hexadecyltrimethylammonium Bromide |
| ICAM | Intercellular Adhesion Molecule |
| IL | Interleukin |
| iNOS | Inductible Nitric Oxide Synthase |
| LBD | Ligand Binding Domain |
| MIP | Macrophage Inflammatory protein |
| MMP | Matrix Metalloproteinase |
| MPO | Myeloperoxidase |
| NFkB | Nuclear Factor kB |
| NO | Nitric Oxide |
| PPAR | Peroxisome Proliferator-Activated Receptor |
| PPRE | Peroxisome Proliferator Response Elements |
| ROS | Reactive Oxygen Species |
| RSG | Rosiglitazone |
| RXR | Retanoid X Receptor |
| SBI | Surgical Brain Injury |

| | |
|---|---|
| TBI | Traumatic Brain Injury |
| TNF | Tumor Necrosis Factor |
| TZD | Thiazolidinediones |

ABSTRACT OF THE THESIS

Anti-Inflammatory Effects of PPAR-gamma in Surgical Brain Injury (SBI)



by

Amy Hyong

Master of Science, Graduate Program in Physiology
Loma Linda University, September 2008
Dr. Jiping Tang, Chairperson



**Introduction:** Brain injury can be caused by neurosurgical procedures themselves, due to direct trauma, retractor stretch, intraoperative hemorrhage and electrocautery damage. As a result of this surgical brain injury (SBI), postoperative complications such as inflammation, brain edema, and cell death can occur in the susceptible brain areas. Cerebral inflammation is a known contributor to the pathophysiology of brain injury. Following brain injury, the release of inflammatory mediators facilitates the development of BBB breakdown, cerebral edema, oxidative stress and neuronal death, resulting in further tissue damage in the brain and poor neurological outcomes. This study evaluates whether the use of a PPAR- γ agonist RSG can reduce postoperative complications and provide neuroprotection in a rodent model of SBI.

**Methods**: SBI rat model incorporates partial resection of frontal lobe with reproducible blood brain barrier (BBB) disruption, brain edema, and neuronal death in the susceptible brain tissue. RSG was administered intraperitoneally in two treatment regimens: 1mg/kg/dose and 6mg/kg/dose. Animals were tested for neurological and sensorimotor deficits and euthanized at 24 hours postsurgery to

measure brain water content, neutrophil infiltration as an indication of inflammation (MPO), and BBB disruption through IgG staining.

**Results:** Brain edema was significantly higher in vehicle-treated rats when compared to sham rats. Treatment with both dosages of RSG did not attenuate brain edema. Increased IgG staining was qualitatively illustrated surrounding the site of surgical resection although no apparent reduction in IgG staining was observed in treated rats. RSG, however, significantly reversed the MPO activity, which was increased after surgery in vehicle-treated rats. There were no significant differences in neurological scores between groups.

**Conclusion:** Treatment with Rosiglitazone attenuated inflammation; however, did not reduce brain edema, improve BBB integrity or neurological outcomes after SBI. This may be attributed to a variety of causes including the fact that RSG may target different mechanisms in SBI as well as the early time course chosen for this study. Future studies will be directed towards understanding the mechanism and timing of inflammation in the SBI model that will enable potential neuroprotective agents to be administered at time points to produce the most beneficial effects.

# CHAPTER ONE

# INTRODUCTION

## Surgical brain injury

The invasive nature of neurosurgical procedures can result in unavoidable injuries irrespective of the surgeon's precision. These injuries can be attributed to the surgery itself due to inevitable circumstances such as brain retraction, intraoperative bleeding, and electrocautery damage (Jadhav et al., 2007b; Lee et al., 2008; Lo et al., 2007; Matchett, et al., 2007). The result of these injuries can produce postoperative complications including blood brain barrier (BBB) disruption, brain edema, and neuronal cell death leading to neurological deficits (Jadhav et al., 2007a; Jadhav et al., 2007b; Lee et al., 2008; Lo et al., 2007; Matchett, et al., 2007). The propensity to induce neurological complications in normal brain tissue can be due to the following etiologies: direct injury to the normal brain structures, brain edema, vascular injury or hematoma formation (Bernstein et al., 2000). In neurosurgical procedures such as malignant glial tumor resection, postoperative complications such as brain edema is one of the key factors related to neurological morbidity, which in severe cases can result in mortality of the patient (Bernstein et al., 2000).

Current treatment options for the management of postoperative complications such as cerebral edema include the use of diuretics, osmotic agents, corticosteroids, hypothermia and hyperventilation (Marmarou, 2007;

Raslan et al., 2007). However, various studies have questioned the efficacy of these agents in attenuating cerebral edema (Marmarou, 2007; Raslan et al., 2007). In addition, treatments using hypothermia has not been widely accepted into clinical practice although it has shown some beneficial effects (Baughman, 2002; Marmarou, 2007). The lack of consensus in effective treatments raises the need for further studies on current as well as potential neuroprotective agents.

Although technological advances have enabled neurosurgeons to better visualize and navigate through intracranial structures with more accuracy, thereby minimizing brain injury associated with dissection, risks are still associated with each procedure (Bernstein et al., 2000). In fact, due to these risks, medicolegal influences can render neurosurgeons to practice with extreme caution due to the fear of lawsuits (Jadhav et al., 2008). This may prevent neurosurgeons from adopting more aggressive approaches in their practice. This poses a need for a confirmed treatment strategy that can help prevent and treat complications experienced from surgical procedures. Through this application, it will allow neurosurgeons to attempt more neurosurgical procedures using more aggressive approaches in their practice.

Surgical Brain Injury (SBI) model is designed to simulate injuries experienced during neurosurgical procedures. The premise behind the SBI model is not aimed at mimicking a specified type of neurosurgical procedure; rather, it is to produce postoperative secondary injuries including blood brain barrier disruption, cerebral edema and neuronal cell death typically induced by this surgical brain injury (Bravo et al., 2008; Jadhav et al., 2007a; Jadhav et al.,

2007b; Jadhav et al., 2008; Lee et al., 2008; Lo et al., 2007; Matchett et al., 2007). Utilization of this experimental animal SBI model allows researchers to examine the affects of various treatment strategies that can be applicable in a clinical setting. In addition, this model allows the study of the mechanisms behind the pathophysiology of this surgical brain injury.

Due to the novelty of this model, which originated from our lab, the mechanisms behind the pathophysiology of the injury has not been fully elucidated. However, previous studies using the SBI model have looked at various underlying factors that may contribute to the pathogenesis of surgically induced injuries and can be potential targets in improving postoperative outcomes. These factors include oxidative stress, neuronal cell apoptosis, BBB disruption, and cerebral edema edema and neuronal cell death that are induced by this surgical brain injury (Bravo et al., 2008; Jadhav et al., 2007b; Lee et al., 2008; Lo et al., 2007; Matchett et al., 2007).

## Inflammation in brain trauma

Inflammation has been a target for neuroprotective intervention in various experimental models of brain injury such as focal ischemia, intracerebral hemorrhage, subarachnoid hemorrhage, surgical brain injury, and traumatic brain injury (Bright et al., 2008; Hyong et al., 2008; Luo et al., 2006; Mashaly et al., 2008; Zhao et al., 2007). The dual role of inflammation allows for the shift between beneficial and harmful effects of inflammation based on environmental factors (Kapadia et al., 2008; Schmidt et al., 2004; Yi et al., 2007). Inflammation following brain injury modulates tissue plasticity to promote mechanisms of repair

by acting as a scavenger for dead cells. However, these beneficial effects can be counteracted in times of excessive activation of inflammatory mediators triggering harmful outcomes that exacerbate secondary injuries, resulting in further tissue damage (Kapadia et al., 2008; Yi et al., 2007).

Following initial brain trauma, activated neurons, glial cells and astrocytes release elevated levels of pro-inflammatory cytokines into the brain (Schmidt et al., 2004; Scholz et al, 2007). Inflammation-induced cytokine stimulation activates adhesion molecules to compromise the BBB creating an excitotoxic environment. This release of cytokines mediates the expression of adhesion molecules and transmigration of neutrophils to the endothelium. In addition, the activation of matrix metalloproteinases (MMPs) propagates the breakdown of the extracellular matrix, facilitating leukocyte entry into the brain tissue (Figure 1). Increased release of these leukocytes into brain tissue can have detrimental effects on damaged brain tissue. (Holmin et al., 2000; Man et al., 2007; Morganti-Kossmann et al., 2007; Schmidt et al., 2004; Scholz et al., 2007; Yi et al., 2007).

Leukocytes, which include neutrophils, produce cytokines that release pro-inflammatory substances, cytotoxic proteases and free oxygen radicals. Increased leukocytes into the brain were observed within the brain parenchyma following brain trauma. Accumulation of these leukocytes has been indicated to be a main contributor in the progression of secondary damage to the brain (Scholz et al., 2007). Enhanced entry of leukocytes profused into the brain tissue plays a causative role in the pathophysiology of vasogenic brain edema,

sustained inflammation, and oxidative stress (Morganti-Kossmann et al., 2007; Scholz et al., 2007).

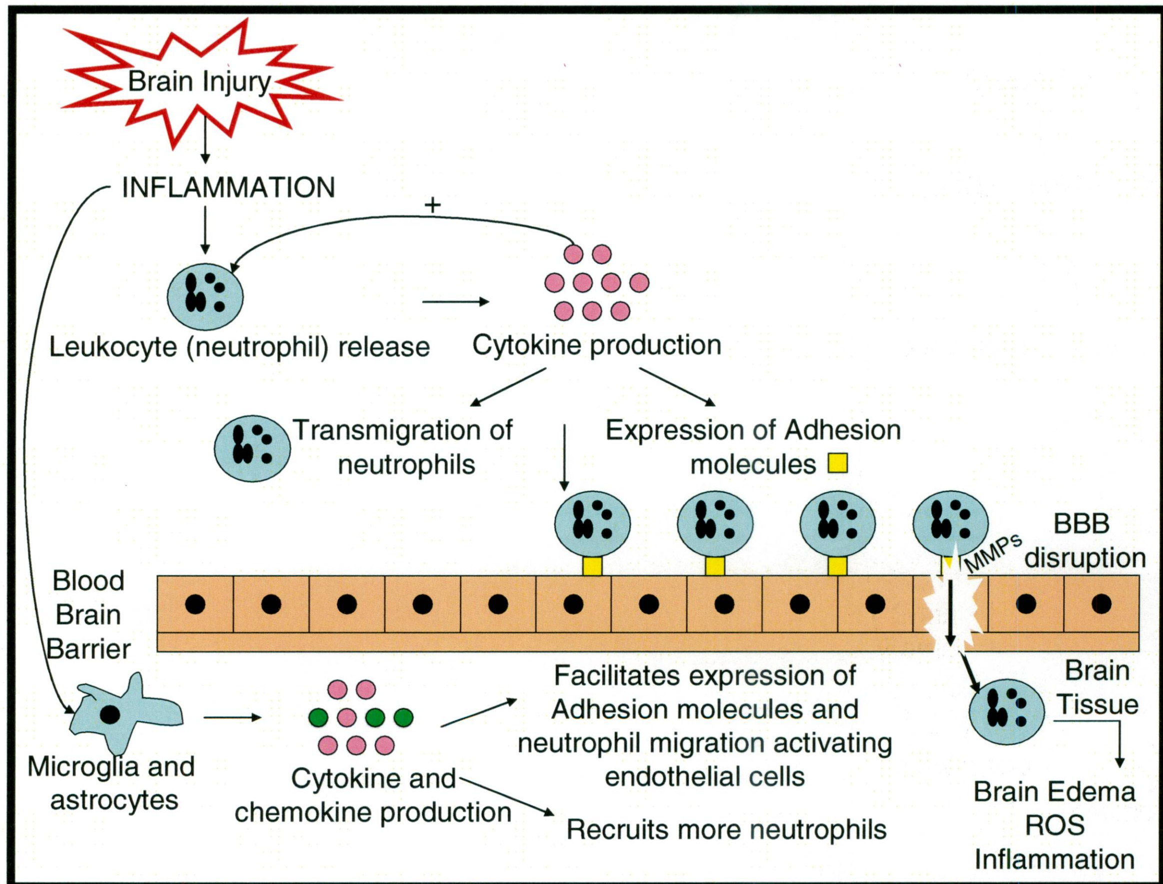


Figure 1. Inflammation following brain injury. Elevated levels of pro-inflammatory cytokines released into the brain stimulation activate adhesion molecules and transmigration of neutrophils to the endothelium. The activation of matrix metalloproteinases (MMPs) facilitates breakdown of the BBB allowing increased leukocyte entry into the brain resulting in brain edema, reactive oxygen species (ROS) and sustained inflammation.

### Mediators of Inflammation

Cytokines have been widely implicated for its known role in promoting secondary injuries following trauma to the brain in both experimental and clinical studies (Morganti-Kossmann et al., 2007; Scholz et al., 2007; Yi et al., 2007). The harmful effects of cytokines are expressed in various cell types including microglia, neurons, and astrocytes. Among the cytokines extensively studied, pro-inflammatory mediator interleukin (IL)-1 has been characterized for its neurotoxic role through the breakdown of the BBB following brain injury. IL-1 is present in two isoforms: IL-1α, the membrane bound form and IL-1β, the secreted form. Both isoforms are expressed following trauma; however, IL-1β is a main contributor to the IL-1 induced deleterious effects. Due to IL-1's pro-inflammatory role in exacerbating brain injury, many experimental studies have targeted IL-1 and its receptor (IL-1R) to attenuate inflammation. In a previous study, inhibition of cytokine IL-1 through IL-1 receptor antagonist (IL-1RA) demonstrated a reduction in neuronal damage (Schmidt et al., 2004; Scholz et al., 2007).

Tumor necrosis factor (TNF) is another cytokine that plays a critical mediating role in inflammation. It is well known for its ability to recruit leukocytes from the peripheral circulation and release proteolytic enzymes that result in the breakdown of the BBB following brain injury (Morganti-Kossmann et al., 2007). TNF is upregulated within a few hours after brain trauma and functions to express the adhesion molecule on astrocytes and regulate leukocyte movement

and chemokine expression in the injured brain (Schmidt et al., 2004; Wang et al., 2002).

Chemokines, a class of cytokines, are produced by resident cells of the brain including neurons, astrocytes and microglial cells. Through its chemotactic factor, chemokines trigger the intracranial infiltration of leukocytes into the injured brain tissue. Previous studies have highlighted the harmful role of chemokines IL-8 and macrophage inflammatory protein (MIP)-2 in mediating the degree of BBB dysfunction in TBI, resulting in the increased BBB permeability and elevated leukocyte extravasation into the brain tissue (Morganti-Kossmann et al., 2007; Schmidt et al., 2004).

Adhesion molecules such as intercellular adhesion molecule (ICAM)-1, and P-selectins have also been highlighted as important mediators in response to inflammation. Similar to other inflammatory mediators, adhesion molecules play a significant role in regulating leukocyte trafficking and extravasation into the brain following injury (Schmidt et al., 2004).

Previous studies indicate the concomitant expression of ICAM-1, TNF and MIP-2 exhibiting synergistic effects in modulating leukocyte accumulation. The combination of these inflammatory mediators suggest the critical role cytokines, chemokines and adhesion molecules in contributing to the pathogenesis of secondary injuries such as brain edema, inflammation, and cell death (Holmin et al., 2000; Schmidt et al., 2004).

## Peroxisome Proliferator-Activated Receptors (PPARs)

Peroxisome Proliferator-Activated Receptors (PPARs) belong to the superfamily of ligand-activated nuclear receptor transcription factors (Bright et al., 2008). They exert notable pleiotropic effects on glucose and lipid metabolism, cellular proliferation, differentiation and on the immune system (Straus et al., 2007 and Kapadia et al., 2008). Recently however, there have been increased efforts in PPAR research due to its anti-inflammatory effects, especially as a neuroprotective strategy following brain injury (Kapadia et al., 2008).

The protein structure of PPARs contains multiple domains with respective functions. The A/B domain at the N-terminal activates transcription (AF-1); C region includes the DNA-binding domain (DBD); D-domain is non-specific; and regions E and F at the C-terminal are the ligand-binding domain (LBD) (Bordet et al., 2006; Yi et al., 2007).

Three isoforms of PPARs are currently known: PPAR-α, -β/δ, and –γ. Each isoform exhibits a distinct function based on their encoded target gene (Bordet et al., 2006; Yi et al., 2007). PPAR-activation by both endogenous and synthetic ligands has been shown to govern lipid and lipoprotein metabolism. Widely expressed PPAR- β/δ is distinguished by its regulatory role in serum lipid profiles and fatty acid β-oxidation of muscle and adipose tissue during lipid metabolism. Endogenous ligands such as prostaglandins and synthetic ligands, which include anti-diabetic insulin-sensitizing thiazolidinediones (TZDs) activate PPAR- γ to regulate glucose metabolism (Bordet et al., 2006; Kapadia et al., 2008).

## PPAR- γ

PPAR- γ is expressed over a range of different cells including adipocytes, monocytes, B and T lymphocytes, dendritic cells, and epithelial cells. In these various cell types, the expression of PPAR- γ functions to promote cell differentiation, lipid metabolism, lipid storage, glucose metabolism, insulin sensitization, and inhibits inflammation, and angiogenesis (Kapadia et al., 2008). Within the brain, PPAR expression is mainly found in astrocytes and microglia, which play a predominant role in inflammation (Kapadia et al., 2008).

Prostaglandin J2 derivative, 15 –deoxy-delta 12, 14-PGJ2(15d-PGJ2), is an endogenous PPAR- γ agonist. It is present in low concentrations, often in insufficient amounts to activate PPAR- γ in mammalian cells (Yi et al., 2007). TZDs are a class of insulin sensitizing synthetic ligands that are powerful agonists due to their high affinity for PPAR- γ. Of these ligands, the most extensively studied among the synthetic PPAR- γ agonists are rosiglitazone and pioglitazone, which are currently approved by the FDA as anti-diabetic drugs (Yi et al., 2007).

## Mechanism of PPAR- γ

Upon ligand binding, PPAR- γ is activated and forms a heterodimer with retinoid X receptor (RXR). Transcription is then regulated through the binding to the peroxisome proliferator response elements (PPREs) at the target gene's enhancer sites. When a ligand is not present, PPAR- γ still forms the PPAR-γ:RXR heterodimer complex, however; this complex can activate co-repressors

that bind to PPRE resulting in the transcriptional repression of target genes (Kapadia et al., 2008; Yi et al., 2007).

### The role of PPAR- γ in inflammatory response

In addition to PPAR- γ's widely recognized effects on metabolism and cell differentiation, recent studies have indicated a critical role for PPAR- γ in attenuating inflammation and oxidative stress by governing immune function in macrophages, T and B lymphocytes, and dendritic and endothelial cells (Bright et al., 2008; Kapadia et al., 2008; Straus et al., 2007; Yi et al., 2007).

Activation of PPAR- γ enables the repression of transcription factors such as nuclear factor kB (NFkB), inhibiting the expression of downstream inflammatory response genes. NFkB is regulated through its inhibitor IkB, which can be described by its heterodimers IKK α and IKK β and its regulatory subunit IKK γ. When IKK subunits are phosphorylized, NFkB is activated and results in the translocation of subunits p65 and p50 into the nucleus promoting transcription of pro-inflammatory target genes. The transcription of genes produce pro-inflammatory cytokines IL-1β, TNF- α, granulocyte macrophage colony stimulating factor (GM-CSF) and chemokines IL-8, MIP-1 α and MCP-1. These mediators play a critical role within the pathophysiology of inflammation by influencing the inflammatory cells to migrate to the site of inflammation (Straus et al., 2007; Yi et al., 2007).

NF-kB also governs the expression of adhesion molecules ICAM-1, V-CAM1, and E and P-Selectins through cytokine production. Once activated, the effects of NFkB are usually sustained as a result of their downstream products,

especially cytokines IL-1β and TNF- α, which create a positive feedback loop to further stimulate the NFkB cascade, inducing a perpetuated inflammatory response (Yi et al., 2007). PPAR- γ agonists modulate the inflammatory response by inhibiting NFkB signaling cascade and stimulating IkB which prevents transcription of key downstream inflammatory genes (Chung et al., 2008). In addition, PPAR- γ agonists have been shown to down regulate NO, pro-inflammatory cytokines IL-1β, IL-6, TNF- α and chemokine MCP-1 production in microglia and astrocytes, resident cells of the brain. In macrophages, protein expression of iNOS, COX-2, MMP, and Stat-1 were also inhibited (Figure 2) (Kapadia et al, 2008; Yi et al, 2007).

## The role of PPAR- γ in oxidative stress

PPAR- γ directly regulates the production of cerebral oxidative stress and reactive oxidative species (ROS). In cerebral ischemia, the genes catalase and Cu/Zn-SoD which play a critical role in scavenging free oxygen radicals was found to be directly regulated by PPAR- γ. PPAR- γ agonists also induce inhibition of inflammatory markers including IL-1 β, COX-2 and iNOS. It has been shown that inhibition of COX-2 results from the inhibition of oxidative stress. This indicates that PPAR- γ plays a modulating role in linking oxidative stress and inflammation (Bordet et al., 2006; Kapadia et al., 2008).

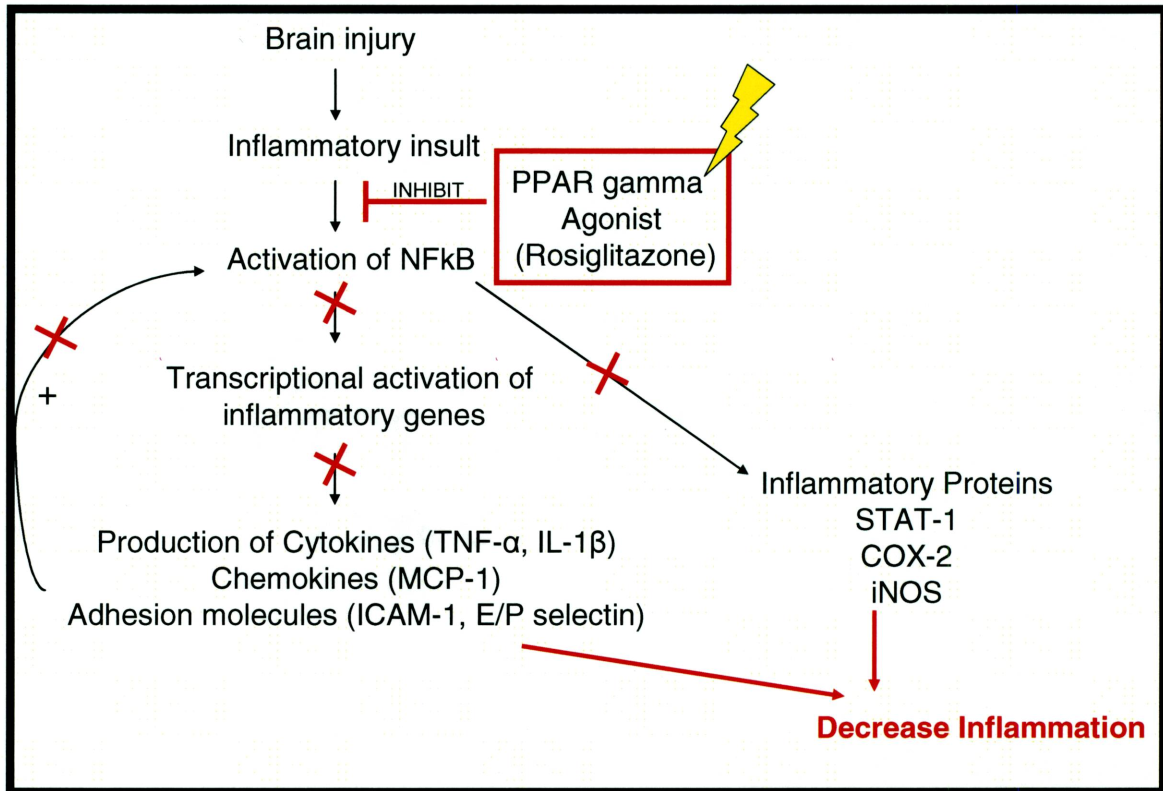


Figure 2: PPAR-γ agonist (RSG) mechanism of action. Following an insult to the brain, inflammation is activated by NF-kB. NF-kB regulates the transcription of inflammatory genes leading to the production of cytokines, chemokines and adhesion molecules, which can stimulate positive feedback mechanisms to further activate NF-kB. PPAR- γ agonists modulate the inflammatory response by inhibiting NFkB signaling cascade resulting in decreased inflammation.

**PPAR- γ: Target for inflammation in brain injury**

As the presence of inflammation can exacerbate the degree of cerebral injury, it is imperative that approaches are directed towards mitigating the harmful effects imposed by inflammation. Attenuation of inflammation by PPAR- γ agonists prevents the deleterious effects of inflammation that include oxidative stress and neuronal cell death (Bordet et al., 2006). The anti-inflammatory properties of PPAR- γ agonists have shown promising neuroprotective outcomes

in various experimental models of brain injury (Allahtavakoli et al., 2006; Chen et al., 2006; Luo et al., 2006; Pereira et al., 2006; Yi et.al, 2007;Zhao et al., 2007).

TZD's beneficial anti-inflammatory effect was demonstrated in multiple models of cerebral ischemia. Luo et al (2006) used Rosiglitazone in a mouse MCAO model and reported a decrease in infarct volume size in addition to improved neurological score. This study also showed decrease in adhesion molecules (ICAM-1), MPO activity, levels of cytokines TNF- α, IL-6, chemokine MCP-1 and the degree of activated microglia after Rosiglitazone treatment (Luo et.al, 2006; Yi et.al, 2007). Reductions in inflammatory mediators suggest a role of Rosiglitazone in attenuating inflammation following ischemia. In a focal ischemia study by Sundararajan et al., (2005), administration of TZDs troglitazone and pioglitazone decreased infarct volume and increased neurological score. Activated microglia and macrophages were reduced and inflammatory mediators COX-2, Stat-1 and iNOS were downregulated after treatment (Sundararajan et al. 2005; Yi et al., 2007).

Treatment with a PPAR- γ agonist displayed a decrease in infarct size, brain edema, activation of microglia and macrophages and inhibition of a variety of pro-inflammatory mediators. This beneficial anti-inflammatory role of PPAR- γ agonists in various models of brain injury suggests a potential role in producing favorable neurological outcomes in surgical brain injury.

## Rationale of study

The potent anti-inflammatory effects of PPAR- γ agonists following brain injury has great potential as a neuroprotective strategy that can be used in a

clinical setting. Both endogenous and synthetic PPAR- γ agonists showed reduction in inflammation, decreased levels of oxidative stress and neuronal cell death in experimental models of ischemia and intracerebral hemorrhage (Allahtavakoli et al., 2006; Chen et al., 2006; Luo et al., 2006; Pereira et al., 2006; Yi et.al, 2007; Zhao et al., 2007). Previous studies have used PPAR- α, another isoform within the PPAR family with anti-inflammatory properties, demonstrating its neuroprotective effects in traumatic brain injury (Besson et al., 2005). However, the use PPAR- γ agonists for its anti-inflammatory neuroprotective effects has not been tested in the SBI model.

Surgical brain injury (SBI) is an experimental animal model which aims to reproduce injuries incurred during neurosurgical procedures. Similar to other models of ischemia, hemorrhage and trauma, secondary injuries including inflammation, apoptosis, cerebral edema, and blood brain barrier disruption can also be observed in the SBI model following brain injury (Hyong et al., 2008. Jadhav et al., 2007b; Lo et al., 2007; Matchett et al., 2007; Morganti-Kossmann et al., 2007; Schmidt et al., 2004). However, an advantage to the SBI model, due to the predictability in preparation of scheduled neurosurgical procedures, is the ability to administer the drug prior to SBI. This pre-treatment strategy allows for potential increased tolerance to injury, which can result in more promising neurological outcomes.

My study aims to use PPAR- γ in an experimental model of surgical brain injury (SBI), to determine if the anti-inflammatory and neuroprotective effects

observed in previous studies can be duplicated in this model of brain injury to reduce postoperative complications and improve neurological outcomes.

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# CHAPTER TWO

## ROSIGLITAZONE, A PPAR GAMMA AGONIST, ATTENUATES INFLAMMATION AFTER SURGICAL BRAIN INJURY IN RODENTS

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## Abstract

Introduction: Surgical brain injury (SBI) is unavoidable during many neurosurgical procedures. This inevitable brain injury can result in postoperative complications including brain edema, blood-brain barrier disruption (BBB) and cell death in susceptible areas. Rosiglitazone (RSG), a PPAR-$\gamma$ agonist, has been shown to reduce inflammation and provide neuroprotection in experimental models of ischemia and intracerebral hemorrhage. This study was designed to evaluate the neuroprotective effects of RSG in a rodent model of SBI.

Methods: 65 adult male Sprague-Dawley rats were randomly divided into sham, vehicle and treatment groups. RSG was administered intraperitoneally in two dosages (1mg/kg/dose, 6mg/kg/dose) 30 minutes before surgery, and 30 minutes and 4 hours after surgery. Animals were euthanized 24 hrs following neurological evaluation to assess brain edema and BBB permeability by IgG staining. Inflammation was examined using myeloperoxidase (MPO) assay and double-labeling fluorescent immunohistochemical analysis of IL-1$\beta$ and TNF-$\alpha$.

Results: Localized brain edema was observed in tissue surrounding the surgical injury. This brain edema was significantly higher in rats subjected to SBI than sham animals. Increased IgG staining was present in affected brain tissue; however, RSG reduced neither IgG staining nor brain edema. RSG also did not improve neurological status observed after SBI. RSG, however, significantly attenuated MPO activity and qualitatively decreased IL-1$\beta$ and TNF-$\alpha$ expression compared to vehicle-treated group.

Conclusion: SBI causes increased brain edema, BBB disruption and inflammation localized along the periphery of the site of surgical resection. RSG attenuated inflammatory changes, however, did not improve brain edema, BBB disruption and neurological outcomes after SBI.

Keywords: Rosiglitazone, surgical brain injury, inflammation, brain edema, blood brain barrier, myeloperoxidase.

## Introduction

Neurosurgical operations can result in inevitable brain injury due to surgical trauma, retractor stretch, intraoperative hemorrhage and electrocautery damage (Andrews and Muto, 1992; Solaroglu et al., 2004, Jadhav et al., 2007a). This surgical brain injury (SBI) is unavoidable (Deletis and Sala, 2001) and results in brain edema, disruption of blood brain barrier (BBB), oxidative stress, and cell death in the vulnerable functional tissue along the periphery of surgical resection (Matchett et al., 2006; Jadhav et al, 2007a; Lo et al., 2007). SBI not only has medical significance but also has medicolegal implications due to the practice of 'defensive medicine' which results in excess expenditure to the tune of $ 70-126 billion a year ("Addressing the New Health Care Crisis," U.S. Department of Health and Human Services, March 2003).

Rosiglitazone (RSG), a peroxisome proliferating activating receptor-γ (PPAR-γ) agonist, is shown to be neuroprotective in focal cerebral ischemia and intracerebral hemorrhage (Allahtavakoli et al., 2006; Chen et al., 2006; Luo et al., 2006; Pereira et al., 2006; Zhao et al., 2007). RSG is a constituent of the thiazolidinediones nuclear hormone receptor superfamily and is known for its

anti-inflammatory actions via activation of PPAR- γ. The activation of PPAR- γ leads to the inhibition of the inflammatory NFkB pathway (Luo et al., 2006; Tureyen et al., 2007). Thus, RSG attenuates the expression of pro-inflammatory genes and cytokine production by regulating ligand activation of transcription factors (Luo et al, 2006; Chen et al, 2006).

Inflammation is a key component of brain injuries resulting from different etiologies such as trauma, ischemia, neurodegeneration, and excitotoxicity (Esiri M, 2007; Wang et al., 2007; Williams et al., 2007). Acute inflammation involves the activation of a range of cells including neutrophils and microglia, in addition to inflammatory mediators such as cytokines and chemokines (Wang et al, 2007), which can contribute to disruption of BBB leading to brain edema as well as neuronal damage (Stamatovic et al., 2006). Thus, inflammatory processes can exacerbate brain injury and worsen the neurological outcomes (Luo et al, 2006). In the present study we hypothesized that SBI causes inflammation in susceptible brain tissue and RSG provides neuroprotection via its anti-inflammatory actions.

## Results

### Brain Water Content Was Not Attenuated with Rosiglitazone Treatment

Brain water content in the frontal ipsilateral lobe (susceptible to SBI) was significantly higher in all animals subjected to SBI as compared with sham surgery group. However, there was no significant difference in brain water content between vehicle treated group and groups treated with either doses of RSG (1mg/kg, 6 mg/kg) (Figure 3). Other areas of the brain (frontal contralateral,

parietal ipsilateral/contralateral, cerebellum and brain stem) were also evaluated, however, there were no significant differences observed in brain water content between sham surgery, vehicle treated and RSG treated groups (contralateral frontal lobe data in Figure 3, other brain regions not shown) similar to previous reports (Jadhav et al., 2007a).

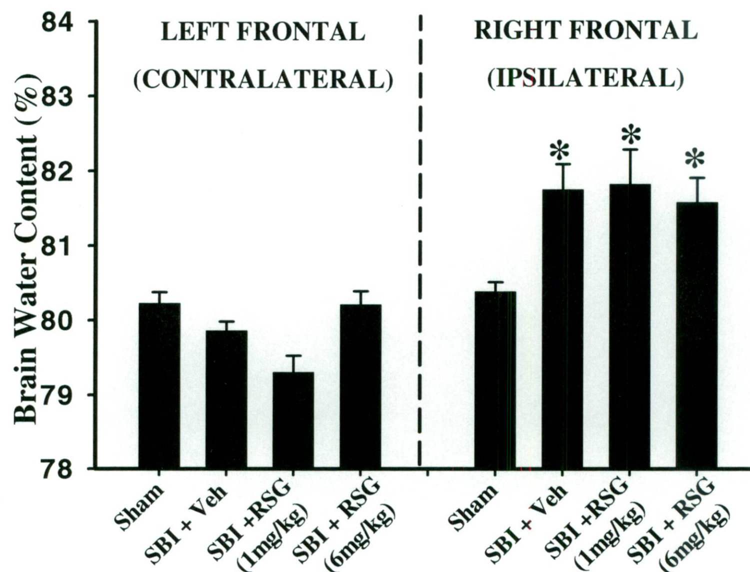


Figure 3. Effect of RSG on Brain Water Content. The figure shows quantified data representing brain water content (brain edema) in the contralateral and ipsilateral frontal lobes of the brain at 24 hrs after SBI. There is no significant difference in the brain water content between the sham surgery, vehicle treated and RSG treated groups (1mg/kg and 6mg/kg) in the contralateral frontal lobe. The ipsilateral frontal lobes show significantly higher brain water content in the vehicle and RSG treated groups (1mg/kg and 6mg/kg) as compared to the sham surgery group. However, there is no significant difference between the RSG treated groups and vehicle treated groups. $p<0.05$, (*) denotes significant difference compared to sham surgery group. 'n' number is as follows: sham surgery = 8, SBI + vehicle = 7, SBI + RSG 1mg/kg = 4, SBI + RSG 6mg/kg = 8.

Rosiglitazone Did Not Reduce Blood Brain Barrier Permeability.

The breakdown of the BBB was qualitatively determined by IgG staining similar to previous reports (Jadhav et al., 2007b, Yamaguchi et al., 2007). The frontal contralateral lobe which served as control did not show any IgG staining (brown staining). In contrast, in the frontal ipsilateral lobe, IgG serum proteins were increasingly present surrounding the site of resection (Figure 4).

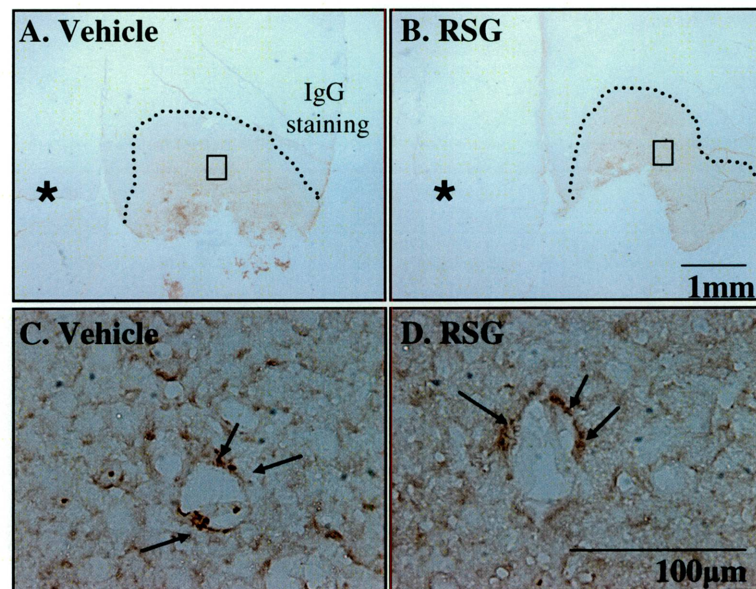


**Figure 4: BBB Permeability by IgG Staining.** The figure depicts IgG staining (brown color) in the vehicle treated (fig A and C) and RSG (6mg/kg, fig B and D) treated rats at 24 hrs after SBI. There is IgG extravasation in the affected brain tissue after SBI in the ipsilateral frontal lobe. The contralateral frontal lobe (marked by asterisk) does not show any staining. There appears to be no qualitative difference in the IgG staining between the vehicle treated and RSG treated groups (IgG staining demarcated posterior to dotted line). Similar findings were seen at higher magnification (figs C and D, ROI marked by boxes in figs A and B) which show extravasated IgG protein surrounding the microvasculature (depicted by arrows). The scale bars denote 1mm in panels A and B and 100μm in panels C and D. The figure is representative of data from 3 animals per group.

Higher magnification indicated that that the extravasation of IgG was more around the micro vasculature (Figure 4). However, RSG treatment (6mg/kg) did not show any qualitative difference in IgG staining thus, indicating that it did not decrease BBB permeability after SBI.

Inflammation Was Reduced with Rosiglitazone Treatment.

Myeloperoxidase (MPO), a marker for the infiltration of neutrophils, was assayed for quantitative indication of the presence of inflammation in the ipsilateral frontal lobes from different groups.

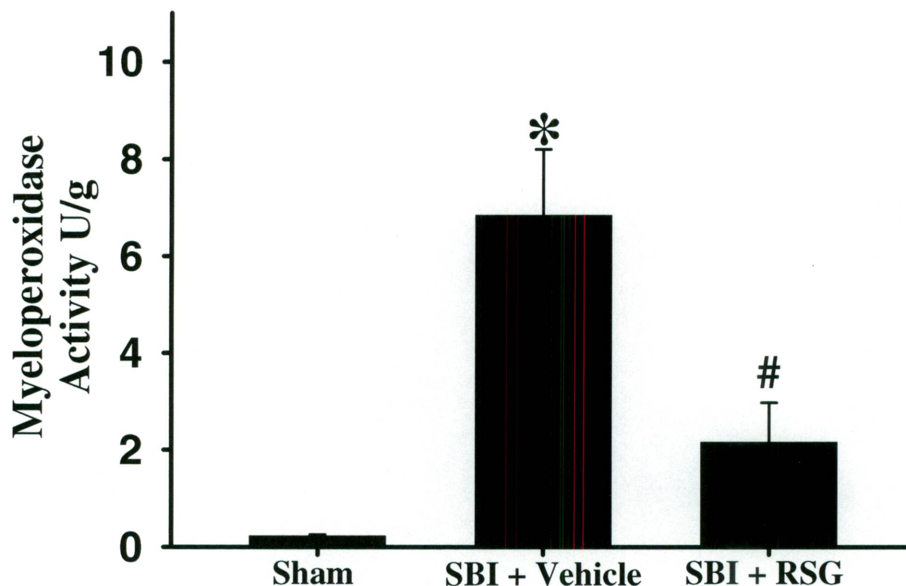


Figure 5. Effect of RSG on Inflammation.
Figure 5A. Myeloperoxidase Activity. Shows the effect of RSG (1mg/kg) on myeloperoxidase (MPO) activity at 24 hrs after SBI in the ipsilateral frontal lobe. There is increased MPO activity after SBI in the vehicle treated group as compared to the sham surgery. This increased MPO activity indicative of inflammation was attenuated by RSG treatment (1mg/kg). $p<0.05$, (*) denotes significant difference compared to sham surgery group and (#) denotes significant difference compared to vehicle treated group. Number of animals is as follows: sham surgery = 4, SBI + vehicle = 6 and SBI + RSG = 4.

MPO observed at high optical density (460nm) signifies an increased presence of inflammation. Vehicle treated groups had a significantly higher MPO (6.85 ± 1.35 U/g) compared to sham surgery groups (0.22 ± 0.03 U/g). In RSG (1 mg/kg) treated groups, there was a significant decrease in MPO (2.15 ± 0.82 U/g) compared with vehicle treated groups demonstrating its anti-inflammatory properties (Figure 5A).

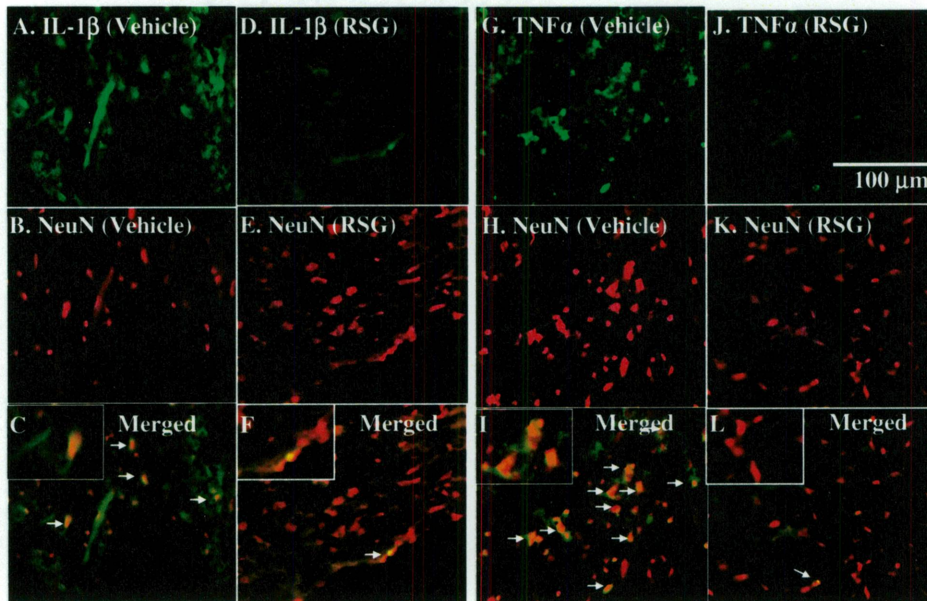


Figure 5B: Inflammatory Markers. Shows double fluorescent immunohistochemical representative pictographs depicting inflammatory markers IL-1β and TNFα (green color, FITC) co-stained with NeuN, a marker for neuronal cells (red color, Texas Red) in ipsilateral frontal lobe at 24 hrs after SBI. The region of interest (ROI) from the sections used for fluorescent immunostaining was obtained from the ipsilateral frontal lobe, more precisely from the edge of the resection as depicted in figures 4A and 4B for vehicle and RSG treated groups respectively. The distribution of immunoreactivities in the vehicle treated groups (panels A-C and G-I) suggests that neurons express inflammatory mediators IL-1β and TNFα after SBI. Qualitatively, these markers are attenuated in the RSG treated (6mg/kg) group (panels D-F and J-L). The merged images (panels C, F, I and L) show magnified images of the cells in the insets. Arrows depict the merged immunoreactivities of the inflammatory markers and Neun. The scale bar denotes 100μm. The figures are representative of data from 3 animals per group.

Immunohistochemical analysis of well known inflammatory markers TNF-α and IL-1β indicated that these markers were increased in the neurons (NeuN, neuronal marker) in the affected brain tissue in the vehicle treated rats. However, in animals treated with RSG (6mg/kg), there appeared to be decreased levels of both inflammatory markers in the affected brain tissue, further illustrating that treatment with RSG decreased inflammation (Figure 5B).

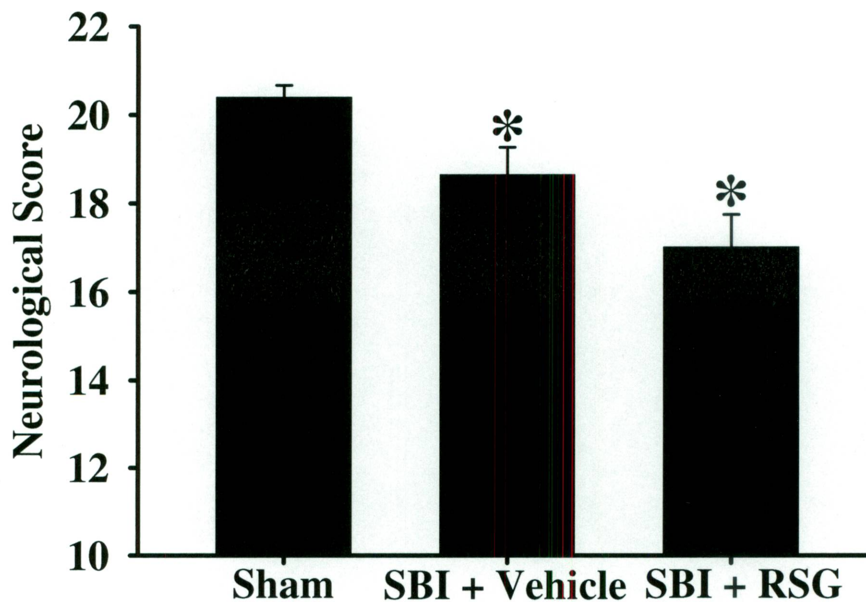


Figure 6: Neurological Outcomes The figure shows quantified data of sensorimotor neurological evaluation at 24 hrs after SBI in different groups. The graph shows that both vehicle treated and RSG treated animals showed significantly greater neurological deficits as compared to sham surgery animals. However, there was no significant difference between the vehicle treated and RSG treated animals. $p<0.05$, (*) denotes significant difference compared to sham surgery group. The number of animals is as follows: sham surgery = 9, SBI + Vehicle = 11 and SBI + RSG 6mg/kg = 4.

### Neurological Evaluation

A 21 point sensorimotor scoring was performed by a blinded observer to evaluate the neurological outcomes similar to previous reports (Lo et al., 2007). As expected, SBI decreased the neurological score in the vehicle treated animals as compared to the sham surgery group. However, RSG group did not show any significant difference compared to the vehicle treated group.

## Discussion

The present study shows for the first time that inflammatory changes occur in the affected brain tissue after SBI. We demonstrated that RSG, a PPAR-$\gamma$ agonist reduces inflammation however does not provide neuroprotection after SBI in rodents. Previous studies have elucidated that oxidative stress, disruption of BBB and brain edema are critical in the pathophysiology of SBI (Jadhav et al., 2007, Matchett et al., 2006, Lo et al., 2007). BBB disruption and brain edema are common complications after SBI which can lead to deteriorating neurological outcomes (Fasano and Penna 1992; Manninen et al. 1999; Tommasino 1992). Inflammation has been reported to play a role in the development of vasogenic brain edema (Zhang et al, 2006) which occurs as a result of BBB disruption. Furthermore, our previous study also showed that matrix metalloproteinases which play an important role in inflammation are increasingly activated after SBI (Yamaguchi et al., 2007). Our present results indicated that inflammation was mitigated with RSG treatment, which is consistent with numerous experimental studies; validating anti-inflammatory effects of RSG and its neuroprotective potential in cerebral ischemia and intracerebral hemorrhage (Allahtavakoli et al.,

2006; Chen et al., 2006; Luo et al., 2006; Park et al., 2007, Pereira et al., 2006; Zhao et al., 2007). The neuronal cells were co-localized with inflammatory markers and we also observed significant neutrophil infiltration after SBI (MPO assay). These changes were reversed after RSG treatment. The involvement of other inflammatory cells such as microglia (Kelly et al., 2007; Byrnes and Faden, 2007; Bye et al., 2007) needs to be further elucidated in SBI.

A parallel effect was not shown in this study because we observed that there was no improvement in brain edema at the lower (1mg/kg) as well as higher (6mg/kg) doses of Rosiglitazone. Based on these findings, we evaluated whether the higher dose of Rosiglitazone (6mg/kg) had any effect on blood brain barrier (BBB) permeability which can precede the development of brain edema. However, the higher dose did not affect the BBB disruption. Thus, a parallel dose effect was not established for the rest of the analyses and one dose was adopted for quantification of inflammation (a) using myeloperoxidase assay (lower dose - 1mg/kg) and (b) immunohistochemical analyses (higher dose - 6mg/kg) to provide evidence of anti-inflammatory action of RSG. Using additional doses for the latter experiments would not have provided further scientific information to this study.

The anti-inflammatory actions of RSG, however, did not have any beneficial effects on brain edema after SBI. One explanation may be that RSG is not specific in targeting edema, particularly vasogenic edema. The formation of vasogenic edema is characterized by an increased permeability of the BBB (Xi et al, 2002). Yet treatment with Rosiglitazone did not appear to preserve the BBB,

as shown with the IgG staining. The anti-inflammatory effects of RSG were not sufficient to reduce brain edema and preserve BBB integrity possibly also due to the severity of the injury. SBI showed an increase of approximately 2% in brain water content in the affected brain tissue resulting in localized brain edema.

Another possibility for ineffectiveness of RSG may be the early time course for evaluation of brain edema in this study. The time course for this study was based on our previous study, which indicates that brain edema peaks at 24 hours after SBI which also correlates with the clinical scenario (Jadhav et al, 2007). However, inflammation and its deleterious outcomes may be a delayed effect after SBI. Future studies can be designed to address whether anti-inflammatory treatment with RSG or other pharmacological agents will reduce brain edema and BBB disruption at later time points after SBI. However, in our opinion, a delayed therapeutic potential may not be clinically viable in SBI wherein the common and major complications such as BBB disruption and brain edema are likely to result in first 24 hrs after neurosurgical procedures.

SBI caused inflammatory changes in susceptible brain tissue along with localized brain edema and disruption of BBB and deterioration in neurological outcome. RSG, a PPAR-$\gamma$ agonist did not reduce brain edema and BBB disruption or improve the neurological status; however it attenuated inflammation in the affected brain tissue.

## Experimental Procedures

All experimental procedures were evaluated and approved by Loma Linda University Institutional Committee for Animal Care and Handling (IACUC).

### Surgical Brain Injury

65 adult male Sprague Dawley rats (350-485g) were used for SBI modeling as previously described (Jadhav et al., 2007b, Yamaguchi et al., 2007). Briefly, animals were anesthetized with 3% isoflurane (in mixture of 70% medical air, 30% oxygen) and positioned in a stereotactic frame (Benchmark) under a surgical operating microscope. A midline incision was made through the skin and connective tissue layer to expose the frontal region of the cranial bone. A microdrill was used to fashion a square cranial window (5mm each side) 2mm lateral and 1mm posterior to the sagittal and coronal planes through the bregma respectively. The dura was reflected to expose the brain. Two incisions were made using a flat blade (width = 1.5mm and length = 6mm) along the sagittal and coronal planes of the 5 mm x 5 mm window plane leading away from the bregma and the excised brain was then lifted and removed as previously described (Jadhav et al., 2007b, Matchett et al., 2006, Yamaguchi et al., 2007). Bleeding was controlled through intraoperative packing and saline irrigation. Once hemostasis was achieved, the dura and bone were restored to their original positions and skin was sutured using a 3-0 silk on reverse cutting needle (Ethicon). All vital signs were monitored throughout the surgery. Animals' body temperature was controlled and maintained between 35-37$^0$ celsius indicated by rectal probe. Sham surgery included only a craniotomy without dural incisions.

Postoperative monitoring included maintenance of body temperature and post-operative fluids (saline). All animals were sacrificed 24 hours after surgery.

## Animal Groups

Four groups, namely sham, vehicle treated and RSG treated (1mg/kg and 6mg/kg) were studied. RSG (Avandia™, GlaxoSmithKline, USA) was used in two concentrations, 1mg/kg and 6mg/kg, based on previous studies (Luo et al, 2006, Pereira et al, 2006).

The drug was dissolved in vehicle (10% DMSO for 1mg/kg, 25% DMSO for higher dosage of 6mg/kg) while vehicle treated animals received 25% DMSO. The higher concentration of DMSO (25%) was required to dissolve higher concentration of RSG (6mg/kg) whereas 10% DMSO was sufficient for 1mg/kg dose of RSG based on previous studies (Luo et al; Peirera et al., and Allahtavakoli et al). We used the higher concentration of DMSO (25%) in the vehicle group due to preliminary studies indicating that DMSO (10%) and DMSO (25%) did not display significant differences in brain water content (unpublished data). We used a 1:4 ratio which in comparison to the animal's body weight, the volume of DMSO was very small.

Three applications of RSG treatments were administered intraperitoneally 30 minutes prior to surgery, and 30 minutes and 4 hours post-surgery.

## Brain Water Content

Animals were placed under deep anesthesia and sacrificed 24 hours post-surgery. Brains were removed under ice and sectioned into the following six

regions: frontal ipsilateral, frontal contralateral, parietal ipsilateral, parietal contralateral, cerebellum and brain stem. Sectioned brains were weighed immediately (wet weight) and placed in an oven set at $104^0$ celsius for 48 hours to obtain the dry weight. Brain water content was calculated using the following equation: Percent brain water content= ((Wet weight)-(Dry weight)/ (Wet weight)) x 100 (Xi et al., 2002).

Blood Brain Barrier Permeability by Immunoglobulin G (IgG) Staining

According to methods previously described (Richmon et al, 1998) perfused brains were sectioned and incubated with goat anti-rat IgG (1:200) biotin-conjugated antibody (Santa Cruz Biotechnology, CA) overnight. Protocol using the Avidin-biotin complex (ABC) kit was followed accordingly for staining.

Immunohistochemical Staining

Following euthanization, brains were perfused with 200 ml of PBS (Sigma-Aldrich Corp, MO) followed by 100 ml of 4% phosphate buffered formalin (Fisher Scientific Inc). Brain tissue was then fixed with sucrose and formalin and stored at $4^0$ celsius. Frozen brains were sectioned and immunofluorescence studies followed standard protocols. (Shimamura et al, 2006). Antibodies used for immunofluorescence were mouse anti NeuN (Chemicon, MAB377), goat anti TNF- α (1:100) (Santa Cruz Biotechnology, CA), rabbit anti IL-1β (1:100) (Santa Cruz Biotechnology, CA). Photomicrographs of sections were viewed and analyzed using an Olympus BX51 fluorescent microscope and MagnaFire SP Version 2.1B software.

Myeloperoxidase (MPO) Assay

MPO assay was used to determine inflammatory changes in the affected brain tissue. All animals were deeply anesthetized and sacrificed at 24 hours. As previously described perfused brains were sectioned into frontal ipsilateral, frontal contralateral, parietal ipsilateral, and parietal contralateral and then frozen at $-80^0$ celsius (Lo et al., 2007). However, solely the frontal ipsilateral section was used to measure MPO activity. Frontal ipsilateral brain tissue was thawed and weighed. Thereafter, brain tissue was homogenized with 1ml 50mmol/L Tris-HCl. Tissue was then homogenized with 3ml sodium buffer, divided into three 1.5 ml (Eppendorf) tubes, centrifuged at 4C, max G, for 30 minutes. The supernatant was discarded; pellet was re-suspended in 0.3ml of 0.5% hexadecyltrimethylammonium bromide (HTAB) in potassium phosphate buffer for 2 minutes. The brain tissue was then frozen in liquid nitrogen and then thawed at 25C. Sonication of brain tissue for 10 seconds at $25^0$ celsius, freezing of brain tissue and thawing at $25^0$ celsius were performed three times. Thereafter, samples were incubated at $4^0$ celsius for 15 min and centrifuged at 12500 g for 15 minutes. Three tubes of supernatant (0.9ml) were collected and mixed with the same volume of 0.005% o-dianisidine dihydrochloride and hydrogen peroxide solution. Absorbance was measured spectrophotometrically at 460 nm at three time points, every 4 minutes. 50 mmol/L sodium phosphate was used as the blank solution. MPO was calculated by taking the average of the three timepoints using the following equation: Absorbance (time point) / (weight of brain tissue sample(g)).

Neurological Evaluation

Neurological outcomes were assessed by a blinded observer just before euthanization at 24 hrs using a 21 point sensorimotor scoring system as per previous reports (Lo et al., 2007). Briefly, the scoring system consisted of seven tests with scores of 1–3 for each test. These seven tests included: (i) spontaneous activity, (ii) symmetry in the movement of four limbs, (iii) forepaw outstretching, (iv) lateral turning, (v) climbing, (vi) body proprioception, and (vii) response to vibrissae touch. The score given to each rat at the completion of the evaluation was the summation of all seven individual test scores. The minimum neurological score was 3 and the maximum was 21.

Statistical Analysis

All data was analyzed using ANOVA with post hoc Holm-Sidak tests for differences between groups and expressed as mean ± standard error using Sigma Stat software, version 3.0.1. Data was found to be significant at $p<0.05$.

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# CHAPTER THREE

# CONCLUSIONS

## Discussion

Cerebral inflammation is a known contributor to the pathophysiology of brain injury in various models of brain trauma, ischemia and hemorrhage injury (Allahtavakoli et al., 2006; Chen et al., 2006; Luo et al., 2006; Pereira et al., 2006; Yi et.al, 2007;Zhao et al., 2007). The presence of uncontrolled inflammation involves multiple mechanisms including the activation and release of neutrophils, pro-inflammatory cytokines, adhesion molecules and chemokines. These inflammatory mediators facilitate the development of BBB breakdown, cerebral edema, oxidative stress and neuronal death. The combination of these factors leads to further tissue damage in the brain and poor neurological outcomes (Holmin et al., 2000; Man et al., 2007; Morganti-Kossmann et al., 2007; Schmidt et al., 2004; Scholz et al., 2007; Yi et al., 2007).

TZDs are widely characterized for their role in glucose and lipid metabolism, and cell differentiation. Furthermore, they are now extensively studied for their anti-inflammatory properties (Straus et al., 2007; Kapadia et al., 2008). In this study, we have shown that inflammation is activated following SBI. Using Rosiglitazone, a PPAR- γ agonist, this inflammation was reversed which was in accordance with previous studies. However following SBI, there were no significant changes in brain edema, BBB integrity, and neurobehavior observed.

This discord between the results obtained from my study and those of previous published reports may be due to a variety of reasons. The most apparent explanation for this disparity can be attributed to the type and severity of brain injury. Among the brain injury experimental models, whether hemorrhagic, ischemic or traumatic, each present a different etiology for every pathological outcome. Therefore, the inflammatory mechanism shown following brain trauma may not present the same characteristics as those seen in surgical brain injury.

The fact that no significant changes in brain edema or BBB disruption were observed despite reduction in inflammation may be due to Rosiglitazone's effects on various pathways other than on vasogenic brain edema. According to Chu et al. (2006), Rosiglitazone promotes angiogenesis following focal ischemia, in addition to reducing inflammation. This suggests the possibility that Rosiglitazone may target angiogenesis-promoting or anti-apoptotic pathways following surgical brain injury increasing cell differentiation. Further studies would be necessary to determine whether treatment with Rosiglitazone is targeting cell survival through its anti-inflammatory effects.

A limitation of this study is the lack of various time points observed post surgery. A 24 hour time point was used due to previous studies indicating the peak edema formation was at 24 hours after surgical brain injury (Jadhav et al., 2007). In addition, peak neutrophil infiltration is noted to be within 24 hours after injury in other experimental models including traumatic brain injury (TBI) (Lenzlinger et al., 2001; Morganti-Kossmann et al., 2007). However, increased

expression levels of inflammation markers have been detected days following brain injury (Stoll et al., 2002; Williams et al., 2007). Therefore, it would be beneficial to observe the effects of inflammation at multiple time points following SBI. This may determine whether the effects of inflammation on brain edema, BBB disruption, and cell death is being exerted at a later time point.

An additional factor that should be considered is the dual role of inflammation which may affect the cascade of inflammatory responses. Determinants such as timing, type of inflammatory mediators released, and the amount of inflammatory activation establishes whether the inflammation will produce beneficial or detrimental effects in the brain (Schmidt et al., 2004). Therefore, it is difficult to assess the extent of injury to which will produce a beneficial or harmful effect of inflammation. Additional studies are necessary to fully comprehend the complex role of inflammation and its dichotomous effects.

This study showed that inflammation was reduced with treatment of Rosiglitazone, a PPAR- γ agonist, indicating that cerebral inflammation following surgical brain injury may activate multiple pathways that still need to be identified. With this critical knowledge, it may reveal additional targets that can reduce the degree of secondary injuries incurred following SBI and lead to improved neurological outcomes. Due to the complex nature of inflammation, a greater understanding of the time course, cellular mechanisms, and the immune response is imperative to develop an effective therapeutic pharmacological target that can promote the beneficial effects of inflammation as well as inhibit the detrimental effects of inflammation following surgical brain injury.

## Future Directions

Future studies will need to be performed to characterize the mechanism of inflammation in SBI. This should include the study of which pathways are activated by SBI due to inflammation as well as the pro-inflammatory mediators that are expressed. In addition, a time course study is essential in understanding at which time point inflammation casts its beneficial and detrimental effects. This will allow pharmacological agents to be selected to target critical mediators in inflammation and to be delivered at time points which would produce the most beneficial effects.

The use of combination therapy in targeting brain injury may have potential for future studies. Due to multiple pathways are activated following brain injury, targeting only one pathway may not be suffice in reducing complications experienced after surgery.  Therefore, future studies utilizing a combination of pharmacological agents, may present beneficial synergistic outcomes that can reduce secondary injuries and improve neurological outcomes following SBI.

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