ACETAMINOPHEN CONFERS NEUROPROTECTION DURING EARLY CEREBRAL ISCHEMIA-REPERFUSION

by

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A Dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

and The Graduate School of Biomedical Sciences

University of Medicine and Dentistry of New Jersey

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Graduate program in Physiology and Integrative Biology

written under the direction of

Gary F. Merrill, Ph.D.

and approved by

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New Brunswick, New Jersey

May, 2009

ABSTRACT OF THE DISSERTATION

Acetaminophen confers neuroprotection during early cerebral ischemia-reperfusion

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Stroke is the leading cause of disability in the United States. It usually occurs from a clot blocking a cerebral artery, resulting in cerebral ischemia and infarction. Few stroke therapeutics are available that definitively improve patient outcome. Acetaminophen is a readily available, inexpensive drug with a good safety profile - properties desirable of a neuroprotectant. We have previously shown acetaminophen to be effective following myocardial infarction. Since similar cell-destructive events occur during cerebral ischemia as in myocardial infarction, we sought to determine whether acetaminophen would protect neuronal cells in a similar fashion. Biochemical and molecular assays were employed to assess cell death and mitochondrial function. Acetaminophen significantly reduced cerebral infarct size, mitochondrial swelling and rupture as well as apoptosis. Acetaminophen also preserved mitochondrial membrane potential. Furthermore, acetaminophen prevented cerebral edema, as seen histopathologically. Taken together, these data suggest that acetaminophen is effective in preventing neuronal stroke damage.

ACKNOWLEDGEMENTS

I would like to thank my family, especially my parents and fiance, for supporting me emotionally (and at times financially) throughout my graduate career. Thank you for encouraging me and being my soundboard these past five years.

To Drs. Denhardt, Firestein, Ma and Weiss: Thank you for taking the time to serve as members of my committee. I would like to thank Dr. David Denhardt, for letting me work in his laboratory. I greatly appreciated your kindness, support, and advice, all of which contributed to my growth as a scientist. Additionally, I would like to thank Dr. Bonnie Firestein for graciously donating rats to my study as well as the use of her laboratory equipment. I would like to thank former and present members of the lab – Katie Jaques-Robinson, and Drs. Norell Hadzimichalis and Roseli Golfetti - for all of their help and support. Lastly, I would like to thank my PI, Dr. Gary Merrill, for his support and advice during this time.

My friends – from my past and the present – thank you for making the last five years easier to bear. To those that listened, to those that provided advice/help, and especially to those who provided laughter – thank you. Also, I would like to thank Carole Lewandowski for all the help she provided with the Systems lab, but also for her advice and help with my thesis.

DEDICATION

To my grandparents and parents,

For instilling the importance of education in me

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INTRODUCTION

1. Background

1.1 Stroke

Stroke results from a decrease in blood flow in a major cerebral vessel (usually the middle cerebral artery), which if not resolved within a short period of time, leads to severely ischemic brain tissue, ultimately resulting in cell death. In humans, there are two common types of stroke: that induced by a total loss of blood flow to the brain, such as during a cardiac arrest, or cerebral ischemia arising from a focal loss of blood flow to the brain due to an artery blockage (Figure 1) (Crack and Taylor, 2005). It is a disease process that can strike abruptly, with severe consequences.

More than half a million people experience a new or recurrent stroke each year in the United States. About 610,000 of these are first attacks, and 200,000 are recurrent attacks. The estimated number of non-hospitalized stroke survivors increased from 1.5 million to 2.4 million from the 1970s to the 1990s. However, mortality due to stroke is also significant. Stroke accounted for about 1 of every 15 deaths in the United States in 2003 (Thom et al., 2006), making it the third among all causes of death, behind heart disease and cancer (when considered separately from other cardiovascular diseases). On average, someone is affected by a stroke every 45 seconds in the United States, with individuals dying from stroke every 3 minutes. Interestingly, about 50% of these deaths occur out of hospital. A majority (88%) of all strokes are ischemic in nature (Lloyd-Jones et al., 2008). The prevalence of transient ischemic attacks (TIA), defined as stroke-like

symptoms that last less than 24 hours, is 2.7% for men aged 65–69. Stroke incidence rates in men are 1.25 times greater than in women at younger ages (Rosamond et al., 2007).

Risk Factors for Stroke

The prototypical victim of stroke will have the following characteristics: he/she is of more than 60 years of age, has diabetes mellitus, has focal symptoms of weakness or speech impairment, and has had a TIA lasting longer than 10 minutes. As with many disease states, smoking puts an individual at twice the risk of a non-smoker in the same demographic. In older adults (ages 55 and above), the lifetime risk for stroke is greater than 1 in 6. Blood pressure (BP) can determine the risk of stroke occurrence. The risk was cut in half when subjects presented with BP less than 120/80 mm Hg, compared to subjects with hypertension (Lloyd-Jones et al., 2008).

In 1999, more than a million American adults who suffered from stroke reported difficulty with activities related to daily living. According to the National Heart, Lung and Blood Institute's (NHLBI), Framingham Heart Study (FHS), 14% of persons who survive a first stroke or TIA will have another one within 1 year. With males, 22% who suffer from an initial stroke die within a year. This percentage is higher among people above the age of 65. The severity of a stroke can determine the length of time to recovery. Approximately 50–70% of stroke survivors regain functional independence, but

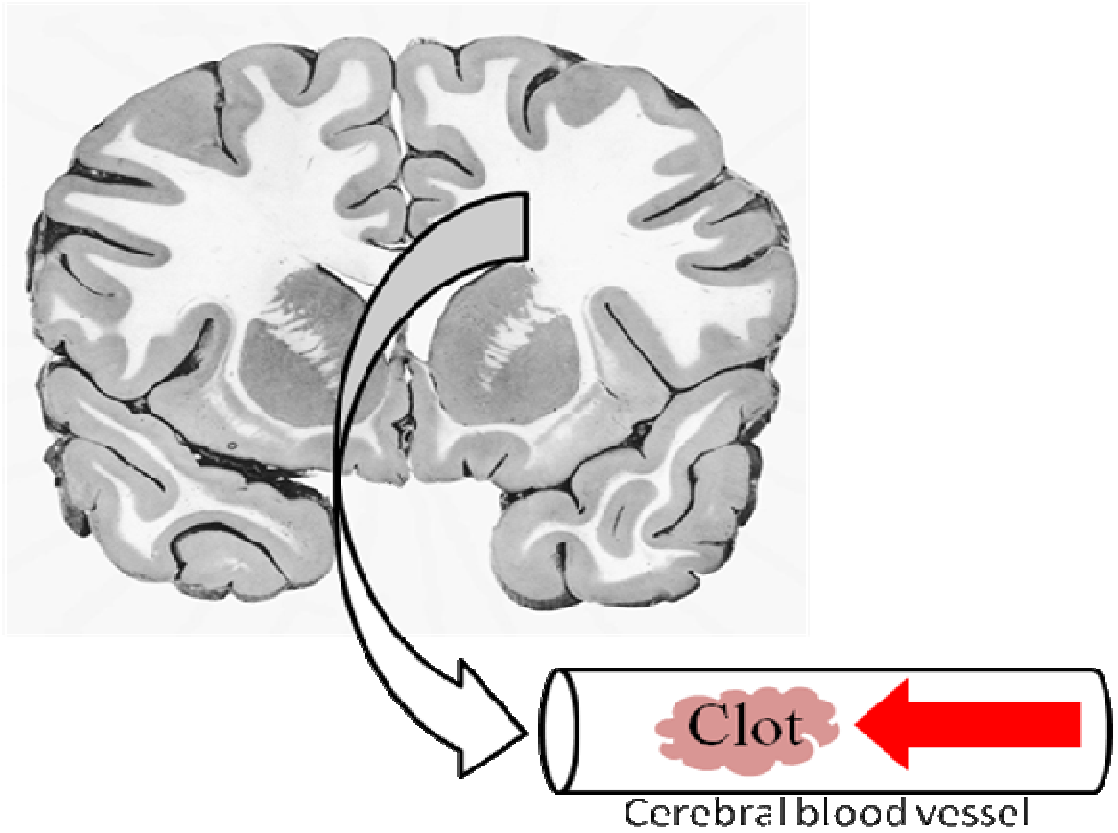


Figure 1. Ischemic stroke. An obstruction (clot) blocks blood flow (red arrow) to regions of the brain downstream from the clot.

15–30% are permanently disabled, and 20% require institutional care 3 months after stroke onset. In the NHLBI's FHS, ischemic stroke survivors 65 years and above

exhibited the following disabilities 6 months post-stroke: 50% had some hemiparesis, 30% required assistance while walking, 26% were dependent in activities of daily living, 19% had aphasia, 35% had symptoms of depression, and 26% were institutionalized in a nursing home. Data from the Paul Coverdell National Acute Stroke Registry showed the majority of stroke admissions were ischemic strokes (52–70%) with TIA and intracerebral hemorrhage comprising the bulk of the remainder (Rosamond et al., 2007).

Of patients with ischemic stroke in the California Acute Stroke Pilot Registry, 23.5% arrived at the ER within 3 hours of symptom onset. Data from the Behavioral Risk Factor Surveillance System (BRFSS) study of the CDC – the world's largest, on-going telephone health survey system, tracked health conditions and risk behaviors in 17 states and the US Virgin Islands. This study showed that public awareness of major stroke warning signs was high, where 94.1% of individuals questioned correctly identified sudden numbness or weakness of the face, arm or leg as a symptom of stroke, 87.9% identified sudden confusion, trouble speaking or understanding, 87.9% identified sudden trouble walking, dizziness or loss of balance or coordination, 85% identified sudden trouble seeing in 1 or both eyes, and 68.1% identified sudden severe headache with no known cause. Only 37.8% incorrectly reported sudden chest pain as a sign of stroke. Another study was conducted of patients admitted to an emergency department presenting with possible stroke, to determine the extent of knowledge of stroke. Overall,

only 40% of patients admitted with a possible stroke did not know the signs, symptoms and risk factors of stroke. In the past decade, awareness of stroke has increased as a telephone survey in a study from 2000 with 2100 respondents showed that 70% of respondents correctly named at least 1 established stroke warning sign versus 57% in 1995, and 72% correctly named at least 1 established risk factor versus 68% in 1995. Fifty-seven percent of queried individuals correctly listed at least 1 of the established warning signs and 68% correctly listed 1 of the established risk factors. Patients with a history of TIA were most likely to be aware of their risk. These data prove that many patients who have strokes could potentially benefit from neuroprotective drugs, early in the course of their cerebrovascular event (Thom et al., 2006).

Cost

The estimated direct and indirect cost of stroke for 2006 was $57.9 billion. In 2006, $3.9 billion (approximately $7449 per patient) was paid to Medicare beneficiaries discharged for stroke. On average, the lifetime cost of ischemic stroke in the United States is estimated at $140,048. This includes inpatient care, rehabilitation and follow-up care necessary for lasting deficits. In a population study of stroke costs within 30 days of an acute event, the average cost was $13,000 for mild ischemic strokes and $20,300 for severe ischemic strokes. Inpatient hospital costs for an acute stroke event account for 70% of the post-stroke costs during the first year. Severe strokes cost twice as much as mild strokes, despite similar diagnostic testing (Fisher and Bogousslavsky, 1998; Lloyd-

Jones et al., 2008; Pancioli et al., 1998; Rosamond et al., 2007; Stephenson, 1998; Thom et al., 2006).

1.2 Types of cell death during stroke

Blockage of a cerebral artery results in the interruption of the blood flow and supply of nutrients, glucose and oxygen to the brain. Blood flow levels are important in determining the size of the infarct by providing the conditions essential for maintenance of cellular energy hemostasis. Decreased blood flow leads to a reduction in phosphocreatinine and ATP. In prolonged cases of ischemia, the energy depletion will be sufficient to lead to severe impairment of cellular function by disruption of ATP-dependent processes. Since neurons do not have the ability to regenerate on a mass scale, neuronal cell death can result in deleterious consequences for stroke victims with crucial motor and speech abilities unable to be fully recovered (Barone and Feuerstein, 1999). The energy needs of the brain are supplied by metabolism of glucose and oxygen for the phosphorylation of ADP to ATP. Most of the ATP generated in the brain is utilized to maintain intracellular homeostasis and transmembrane ion gradients of sodium, potassium, and calcium. Energy failure results in collapse of ion gradients, and excessive release of neurotransmitters such as dopamine and glutamate (Adibhatla and Hatcher, 2008). Certain features of cell death, such as loss of energy stores, mitochondrial failure, macromolecular breakdown, and free radical production, are shared regardless of the pathway employed. Overall, the differences between the types of cell death are primarily

attributed to the timing and the subcellular location of the death pathway initiated by the cell (Choi, 1996).

Role of apoptosis

A quarter century ago, Kerr, Wyllie, and Currie (Kerr et al., 1972) coined the term 'apoptosis' to characterize cells dying under certain circumstances, such as during normal development or after exposure to ionizing radiation. These cells were initially considered anomalies as they died with a distinctive morphology, characterized by coarse chromatin aggregation and progressive loss of cell volume with minimal damage to surrounding cells. In contrast, necrotic cell death occurring as a result of various environmental insults, was characterized by early cell swelling, dilation of mitochondria, and the eventual rupture of organelle and plasma membranes. Recent breakthroughs in determining the molecular basis of apoptosis has produced a surge of interest as it is now considered the final execution of highly regulated genetic programs (Choi, 1996).

The number of cells in a multicellular organism is tightly regulated from conception up until death. If cells are no longer needed, they are programmed to commit suicide by activating an intracellular death program. This process is called apoptosis. For example, apoptosis occurs during various stages of embryonic development. Mouse paws and human hands are sculpted by cell death during this stage. Also, cells die when the structure they form is no longer needed, for example the tail of a tadpole after it has changed into a frog. Cell death also helps regulate cell numbers. In the case of neuronal

development, cell death adjusts the number of nerve cells to match the number of target cells that require innervation (Campbell et al., 1999).

Cells that undergo apoptosis appear to die neatly, without damaging their neighbors. Intially, the cell shrinks and condenses. Then, the cytoskeleton collapses, the nuclear envelope disassembles, and the nuclear DNA breaks up into fragments. Most importantly, alterations in the cell surface induce the expression of properties that cause the dying cell to be rapidly phagocytosed, either by a neighboring cell or by a macrophage before any leakage of its contents occur. Not only does this preventthe damaging consequences of cell necrosis but also allows the organic components of the dead cell to be recycled by the cell that ingests it.

The intracellular machinery responsible for setting the process of apoptosis into motion appears to be conserved between species. A family of proteases that cleave their target proteins are called caspases. Caspases are synthesized in the cell as inactive precursors, or procaspases, that are activated by cleavage at aspartic acids by other caspases. Once activated, caspases cleave in turn activating other procaspases, thus amplifying the proteolytic cascade. Some of the activated caspases then cleave other key proteins in the cell. Activation of the intracellular cell death pathway is usually triggered in a complete, all-or-none fashion. The protease cascade is not only destructive and self-amplifying but also irreversible, so that once a cell reaches a critical point along the path to destruction, it cannot turn back (Campbell et al., 1999).

Procaspase activation can also be triggered by adaptor proteins that induce aggregation of multiple copies of initiator procaspases. Some initiator procaspases have a small amount

of protease activity that is amplified when brought into proximity with other procaspases, thus triggering activation via cleavage. Procaspase activation can also be triggered from outside the cell by the activation of death receptors on the cell surface. If the Fas ligand is expression on surrounding cells, it binds to the death receptor protein Fas on the surface of the target cell. The subsequent aggregate of Fas proteins attracts procaspase-8, which upon aggregating themselves, cleave and activate one another. Activated caspase-8 molecules then activate downstream procaspases and continue the apoptotic cascade. Stressed or damaged cells initiate apoptosis by producing both the Fas ligand and the Fas protein, thereby triggering an intracellular caspase cascade. Alternatively, stressed/damaged cells can initiate apoptosis by triggering procaspase aggregation and activation within the cell (Campbell et al., 1999).

Apoptosis can also occur when certain organelles within the cell release pro-apoptotic compounds. Specifically, the mitochondria have been highlighted as sensitive organelles that respond to cellular insults by releasing a pro-apoptotic protein known as cytochrome *c*. Once present in the cytosol, it binds and activates an adaptor protein called Apaf-1 that recruits procaspase-9 molecules and activates them in a process similar to the activation cascade of procaspase-8 molecules. This mitochondrial pathway of procaspase activation is recruited in most forms of apoptosis to initiate, accelerate and amplify the caspase cascade, shown in Figure 2.

Some proteins can serve dual (and conflicting) roles within the cell when apoptosis is initiated. They may protect or mediate the apoptotic cascade by participating in more than one pathway. One of the major proteins involved in this dual-signaling is the Bcl-2

family of intracellular proteins. These proteins not only regulate procaspase activation and block the release of cytochrome *c* from the mitochondria; they can also promote procaspase activation and cell death. Proteins within this family can inhibit apoptotic activity (Bcl-2), promote the pro-apoptotic activities of other proteins within the family (Bid), or work solely in mediating apoptosis (Bax, Bak and Bid) depending on cellular signals (Campbell et al., 1999).

Another important aspect to consider is the work of intracellular anti-apoptotic regulators. Specifically, the IAP (inhibitor of apoptosis) family works to prevent apoptosis. IAP proteins were originally discovered in the virus as a means of preventing cell death prior to viral replication.These proteins inhibit apoptosis in two ways: they prevent procaspase activation, and they inhibit the activity of caspases.

Neuronal apoptosis due to ionic imbalance

The mitochondria are not the only means of triggering apoptosis within a cell. Ionic imbalance occurring as a result of a neuronal insult can also occur. The disruption of ionic gradients across excitable (neuronal) and nonexcitable (glial) membranes owing to loss of ATP is characterized by efflux of K^+ from the cells, cellular depolarization, and influx of Na^+, Cl^-, and Ca^{2+} into the cells (Siesjo, 1992a; Siesjo, 1992b). Of these events, the increase in extracellular K^+, along with a decrease in pH, precedes the other ionic changes. In this phase, rapid depletion of ATP stores occurs and ultimately results in marked changes in ion conductance. Supposedly, the increase in K^+ can reach levels sufficient to release neurotransmitters such as glutamate, which in turn will stimulate

Na^+/Ca^{2+} channels coupled to the NMDA receptor; these events will further lead to Na^+, Cl^-, and H_2O accumulation, cell swelling, and cytotoxic edema. In parallel, extracellular Ca^{2+} enters into cells through both voltage-operated and receptor-operated calcium channels, leading to elevated free cytosolic Ca^{2+}, which causes mitochondrial calcium overload and further compromise of ATP production. Transient extracellular K^+-induced depolarizations can also contribute to the expansion of the neuronal lesion. These K^+-induced depolarizations produces disruption of ionic gradients and transmitter release, with the associated accumulation of free Ca^{2+} in the cells leading to rapid and extensive breakdown of phospholipids, proteins, and nucleic acids by activation of calcium-dependent phospholipases, proteases, and endonucleases. Depolarization causes an increase in intracellular calcium and an increase in extracellular glutamate. Glutamate is an excitatory neurotransmitter implicated in causing ischemic neuronal damage. Neuronal damage results from glutamate excitotoxicity where excessive extracellular glutamate kills neurons through an increase in intracellular calcium. Glutamate-mediated excitotoxicity is thought to occur because of excessive activation of AMPA and NMDA synaptic glutamate receptors. The NMDA receptor channel conducts Na^+ and calcium as do some AMPA receptor channels. AMPA receptors also control the initial membrane depolarization caused by glutamate, and affect the opening of the NMDA receptors. (Barone and Feuerstein, 1999)

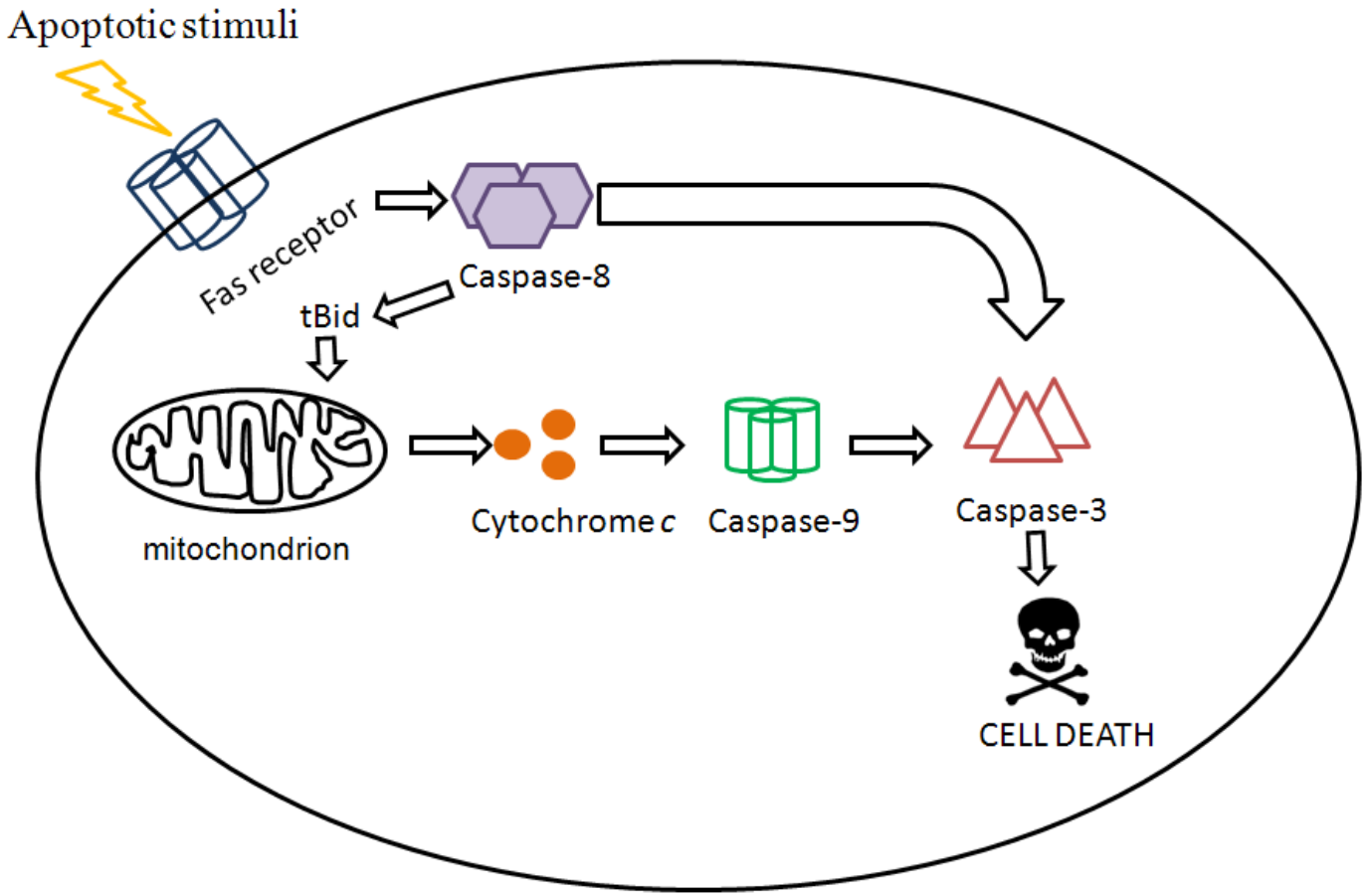


Figure 2. Apoptotic pathways. Agents within the cell can stimulate release of cytochrome *c*, that activates caspase-9, leading to cell death. Apoptotic stimuli can activate Fas receptors that activate procaspase-8, leading to activation of Bid, a protein that disrupts mitochondrial membranes, leading to the release of cytochrome *c*.

1.3 Events leading to cell death

Oxidative stress

Reactive oxygen species (ROS) have recently been hypothesized to play a role in the coordinated mechanism of cellular signaling. They have been found to stimulate a number of signal transduction pathways that are important in maintaining cellular homeostasis in the neuron. In the normal physiological state, the coordination and regulation of ROS is controlled by the cell's endogenous antioxidants. It is when this regulation is impaired or is unable to cope with the level of ROS present that oxidative stress ensues. High levels of oxidative stress can have a negative impact on specific signal transduction systems leading to a predisposition to disease of both an acute and a chronic nature. The contribution of oxidative stress after ischemia-reperfusion injury can lead to a vicious cycle as it impinges upon mitochondrial dysfunction, excitotoxicity, lipid peroxidation, and inflammation (Crack and Taylor, 2005). Accumulation of products such as free fatty acids (especially the unsaturated) that are metabolized to toxic lipid peroxides (via lipid peroxidation) further contribute to structural and functional perturbations of the membrane and cell function. Free radicals (highly reactive oxygen species characterized by a free electron) are generated during ischemia and can cause considerable damage to lipids, DNA, and proteins, all the while contributing to the process of neuronal death. Free radicals also contribute to the breakdown of the blood-brain barrier and brain edema. Levels of free radical scavenging enzymes (e.g., superoxide dismutase) decrease during ischemia and nitric oxide levels are elevated.

Nitric oxide generated primarily by neuronal and inducible nitric oxide synthases promote neuronal damage after ischemia (Barone and Feuerstein, 1999).

Several experimental studies suggest that xanthine oxidase (XO) might not be a major source of free radicals. This enzyme catalyzes the oxidation of hypoxanthine to xanthine and also xanthine to uric acid, generating superoxide anions. Moreover, xanthine deshydrogenase (XDH), another form of this enzyme, catalyzes a similar reaction but uses NAD, instead of oxygen, as an electron acceptor, and consequently does not produce free radicals. In healthy tissue, most of the enzyme exists as XDH, whereas XDH is converted to XO in many ischemic tissues. Nitric oxide synthases (NOS) constitute another source of free radicals. These enzymes use arginine and oxygen as substrates and produce nitric oxide (NO), which is then able to combine with superoxide anions to generate the strong oxidant peroxynitrite anions. Thus, in models of transient cerebral ischemia, NOS 1 or NOS 2 selective inhibitors and antisense against NOS 2 mRNA decrease the production of nitrotyrosine, a marker of peroxynitrite anion; knockout mice for the same NOS isoforms also exhibited reduced nitrotyrosine formation (Margaill et al., 2005).

Inhibition of protein synthesis

Much focus has been directed to the effect of oxidants produced during ischemia and reperfusion. However, other significant changes in brain metabolism occur during the ischemia that precedes damage experienced during reperfusion. Immediately upon the

onset of reperfusion after global ischemia, protein synthesis is inhibited in neurons throughout the entire brain. Dependent on the duration of ischemia, protein synthesis will gradually recover in brain regions more resistant to ischemic damage, but remains suppressed in selectively vulnerable neurons (DeGracia et al., 2002). In the most vulnerable brain region, the CA1 of the hippocampus, protein synthesis never recovers fully during reperfusion even after relatively brief ischemia (Araki et al., 1990). Participants in the process of protein translation known as eukaryotic initiation factors (eIF) are the rate limiting step. eIF subunits are regulated by phosphorylation, an event that occurs rapidly in response to diverse cellular stresses, such as nutrient deprivation, and is particularly dramatic during postischemic brain reperfusion. In the focal ischemia model of Althausen *et al.* (2001), immunohistochemical staining also showed low expression of phosphorylated eIF in normal brain sections but intense staining of perinuclear cytoplasm and nuclei in cortical neurons after 1 hour of reperfusion.

Mitochondrial dysfunction

Mitochondria are organelles with two well-defined compartments: the matrix, surrounded by the inner membrane (IM), and the intermembrane space, surrounded by the outer membrane (OM). The IM is folded into numerous cristae, which greatly increases its surface area. It contains the protein complexes from the electron transport chain, the ATP synthase and the adenine nucleotide translocator (ANT). To function properly, the IM is almost impermeable in physiological conditions (although periodic transient increases in permeability may occur to selected ions and metabolites of less than 1,500 Da, as a

consequence of various ion channels and uniporters), thereby allowing the respiratory chain to create an electrochemical gradient. The electrochemical gradient results from the respiration-driven, electron-transport-chain-mediated pumping of protons out of the IM and is required for driving the ATP synthase which phosphorylates ADP to form ATP. ATP generated on the matrix side of the IM is then exported in exchange for ADP by the ANT. The most abundant protein of the OM is the voltage-dependent anion channel (VDAC), which in normal conditions is permeable to solutes up to about 5,000 Da. Apoptotic OM permeabilization involves the release of proteins which are normally confined to the intermembrane space of these organelles, including cytochrome *c*, certain 'pro-caspases', adenylate kinase 2 and apoptosis-inducing factor. Mitochondria manifest signs of OM and/or IM permeabilization when exposed to a variety of pro-apoptotic second messengers. Pro-apoptotic members of the Bcl-2 family (Bad), which bear resemblance to channel-forming bacterial toxins, induce mitochondrial membrane permeabilization when added to purified mitochondria as recombinant proteins. This has been shown for Bax, Bak and Bid, and probably reflects the principal mechanism by which these proteins trigger cell death (Kroemer and Reed, 2000). The mitochondria have been identified as targets of cytoprotection for several disease states, mainly due to the high susceptibility of the organelle to cellular insults. In the brain, the mitochondria are especially vulnerable due to the high metabolic activity of the brain that is key in producing significant levels of cell-damaging oxidants (Szeto, 2006a; Szeto, 2006b). Mitochondria isolated from a variety of sources can show a sudden increase in the permeability of the inner mitochondrial membrane to solutes with a molecular mass of less than 1,500 Da, which results in the loss of $\Delta\psi$ (mitochondrial membrane potential),

mitochondrial swelling, and rupture of the outer mitochondrial membrane (Halestrap et al., 2002; Zoratti and Szabo, 1995). This process is called the mitochondrial membrane permeability transition (MPT). The MPT can be induced under various conditions, such as exposure of mitochondria to Ca^{2+} together with inorganic phosphate. Although the molecular mechanisms of the MPT are largely unknown, it is widely believed that it occurs after the opening of a channel complex that has been termed the permeability transition pore (PTP), which is thought to consist of the voltage-dependent anion channel (VDAC: outer membrane channel), the adenine nucleotide translocator (ANT: inner membrane channel), cyclophilin D (Cyp D), and possibly other molecules. For a long time, it has been unclear whether the CsA-sensitive MPT plays an important role in the apoptotic increase of mitochondrial membrane permeability. However, studies of Cyp D-deficient mice have finally provided some evidence that further clarifies this issue. Various cells isolated from Cyp D-deficient mice, such as thymocytes, embryonic fibroblasts (MEFs), and hepatocytes, undergo apoptosis normally in response to various stimuli, including etoposide, stautosporine, and tumor necrosis factor-α (Basso et al., 2005; Nakagawa et al., 2005; Schinzel et al., 2005). Small intestinal cells from Cyp D-deficient mice are also as sensitive to X ray-induced apoptosis as cells from control mice (Nakagawa et al., 2005). These results provide compelling evidence that the CsA-sensitive MPT is not essential for apoptosis. Of course, it remains possible that some forms of apoptosis might be mediated by the CsA-sensitive MPT, and thus inhibited by CsA (Tsujimoto and Shimizu, 2007).

1.4 Current treatments for stroke

Stroke is a leading cause of serious, long-term disability in the United States. The median time from stroke onset to arrival in an emergency room is between 3 and 6 hours, according to a study of at least 48 unique reports of hospital-related delay time for stroke patients, TIA or stroke-like symptoms. Improved clinical outcome at 3 months was seen for patients with acute ischemic stroke when intravenous thrombolytic treatment was started within 3 hours of the onset of symptoms. In 2003, an estimated 117,000 inpatient endarterectomy procedures were performed in the United States. Carotid endarterectomy is the most frequently performed surgical procedure to prevent stroke (Lloyd-Jones et al., 2008; Rosamond et al., 2007; Thom et al., 2006). This is a surgically invasive procedure that removes carotid plaque in the hopes of preventing future occurrence of a stroke.

In plasma, the primary function of plasmin is digestion of fibrin, and thus tPA is used as a thrombolytic agent for treatment of ischemic stroke. Thrombolysis is the breakdown of blood clots by pharmacological means. Clearing the cross-linked fibrin mesh (the backbone of a clot) makes the clot soluble and subject to further proteolysis by other enzymes, thereby restoring blood flow in the occluded blood vessel. More than 12 years after FDA approval, tPA remains the only approved therapy for stroke, but because of its severe inclusion criteria (narrow 3 hr time-to-treatment window and no apparent hemorrhagic complications), <5% of stroke patients get benefit from this clot dissolving agent. Clinical studies have shown that patients receive little or no benefit if tPA therapy is initiated more than 3 hrs after the onset of stroke, which excludes most stroke patients

from tPA treatment due to the time required for transportation to medical facilities and proper diagnosis. Another side of tPA in stroke is that it is more than just a thrombolytic agent. In addition to dissolving the blood clot, tPA may damage the basal lamina of the blood vessels, resulting in edema, disruption of the blood-brain barrier (BBB), or hemorrhage. Exogenous tPA exacerbated ischemic injury in both wild-type and tPA-null mice. These studies were conducted in a suture model of stroke where no clot formation is involved (Wang et al., 1998b), thus the effects of tPA on ischemic injury could be evaluated in the absence of its beneficial effects as a clot-dissolving agent. The damaging effects of tPA in stroke may include the amplification of damaging calcium currents during ischemic excitotoxicity. The tPA/PAI-1 complex is cleared from the circulation by the liver *via* a scavenging receptor, the low-density lipoprotein receptor-related protein-1 (LRP). Because of this, tPA has a half-life in the bloodstream in humans of 5 to 10 min (Gravanis and Tsirka, 2008). This short half-life prevents tPA from acting on subsequent and continued vessel occlusions that occur (Adibhatla and Hatcher, 2008).

Dietary supplements have also been found to exert neuroprotection following stroke. Components of green tea, catechins, are polyphenols that exhibit antioxidant activity useful in the prevention of stroke-related cell death. Catechins were extracted from green tea and administered in various doses to young stroke-prone spontaneously hypertensive rats (SHRSP) for an extended period of time. In these rats, the onset of stroke was significantly delayed, a delay that was attributed to the ability of catechins to prevent a drastic increase in blood pressure that would precede a stroke event (Ikeda et al., 2007). Several reports have corroborated this study, where catechins have exhibited

neuroprotection at varying concentrations/doses (Hong et al., 2001) and this mode of protection has been largely attributed to the polyphenolic nature of the compound. Catechin-mediated neuroprotection largely reduced the number of apoptotic cells and lipid peroxidation products occurring as a result of oxidative damage (Hong et al., 2000).

Minocyline, a derivative of the popular antibiotic tetracycline, has also exhibited neuroprotective effects following stroke. Infarct size and incidence of apoptosis were markedly reduced in minocycline-treated animals. The proposed mechanism of minocycline action was attributed to its anti-apoptotic and anti-inflammatory properties (Tang et al., 2007). Other studies have corroborated these results as matrix-metalloproteinase (MMP) expression was significantly downregulated in minocycline-treated animals compared to the corresponding vehicle group (Nagel et al., 2008). MMPs are known products of inflammatory cascades that are activated as a response to cellular injury experienced during stroke (del Zoppo et al., 2007).

While these treatments have shown considerable promise as potential neuroprotective agents, there are a few points that need to be considered. First, the therapeutic window of tPA is limited (within 3 hours of stroke onset) and when administered for non-ischemic strokes, can exacerbate the condition, thereby worsening patient outcome. Although green tea catechins and minocycline do not exhibit platelet-cleaving properties, they must be taken on a daily basis for a lengthy period of time to be considered effective should a stroke occur. Furthermore, when various concentrations were administered to experimental subjects, the highest doses were found to be the most effective (Van Dyke

et al., 2000) as they completely abolished chemiluminescence (light photons emitted by the product of a reaction between luminol and peroxynitrite), whereas lower dosages showed minimal decreases in chemiluminescence. Lower dosages represent the concentration of catechins found in commercially-available green tea, therefore studies demonstrating the efficacy of low concentrations of catechins as effective free radical scavengers when administered immediately preceding a stroke would be desirable. Thus far, studies on green tea have suggested a chronic administration for weeks to be considered effective. Minocycline, while showing a neuroprotective effect in rodents subjected to stroke, has reported anti-inflammation properties. Minocyline is a derivative of Tetracycline, an antibiotic well known for its inhibitory properties on protein synthesis (Greenberger, 1967). Inhibition of protein synthesis also occurs during ischemia, a process that sets the stage for further cell damage during reperfusion (DeGracia et al., 2002). Minocycline, being an antibiotic, needs to be administered under a physician's supervision and could produce resistance to other organisms while conferring protection during stroke. Although the drug shows promise, further long-term studies of minocycline need to be conducted prior to prescribing/administering the drug as a stroke therapeutic.

1.5 Acetaminophen

History of Acetaminophen

Acetaminophen, also known as paracetamol, was widely accepted by the medical community when it was discovered by accident in the mid-to-late 1800s. At first, use of acetaminophen was strictly limited to an antipyretic, however, some physicians took note of the analgesic effect the drug had on arthritic patients. Other drugs with similar properties, such as aspirin, soon took precedence over acetaminophen and the drug was shelved for many years. In the late 1940s, acetaminophen took center stage again when it was rediscovered as a metabolite of acetanilide and phenacetin (two other common antipyretic drugs) and it was rapidly incorporated into American society as a non-prescription antipyretic and pain-reliever. Even though other countries were slow to accept acetaminophen as a potent antipyretic, by the late 1970s, the use of acetaminophen grew worldwide partly due to reports that aspirin, acetanilide and phenacitin produced highly undesirable side-effects when taken on a daily basis. These side-effects were not present in individuals who took similar doses of acetaminophen (Prescott, 2000).

Properties of acetaminophen

The molecular weight of acetaminophen is 151.2 g and the chemical formula is $C_8H_9NO_2$ (Figure 3). Its small size makes it an ideal candidate for a neuroprotectant, given the highly selective nature of the blood-brain barrier (Campbell et al., 1999). Large doses

(750-1250 mg/kg) can cause hepatotoxicity, but when administered within the therapeutic range (<50 mg/kg), the drug is safe while possibly conferring protection to injured tissue (Prescott, 2000). Acetaminophen is safe in the elderly population and does not require any dose-adjustment. Finally, acetaminophen is inexpensive, making it an ideal candidate for a neuroprotectant as it is readily-available to the general public.

Acetaminophen as a cytoprotectant

For many years, the use of acetaminophen in research was limited to its ability to induce hepatotoxicity when taken in hypertherapeutic doses (Prescott, 2000; Talbot, 1975). This model of hepatotoxicity is still being used today when examining mechanisms of liver damage (Bajt et al., 2008; Kon et al., 2007) or evaluating the efficacy of hepatoprotective compounds (Moffit et al., 2007; Oz and Chen, 2008). In the past decade, research with acetaminophen has expanded beyond hepatotoxicity research into cardioprotective studies. Acetaminophen has been shown to be safe when taken in therapeutic doses (Chan et al., 2006; Leshnower et al., 2006), and/or cardioprotective by significantly decreasing pathological events as a result of myocardial infarction or iron-overload (Walker et al., 2007; Zhu et al., 2006). Furthermore, oxidative modification of LDL – one of the key events leading to atherosclerosis – was significantly reduced in hypercholesterolemic animals after chronic administration of therapeutic doses (200 mg/day) of acetaminophen (Chou and Greenspan, 2002; Dinis et al., 1994; Nenseter et al., 1995; Ozsoy and Pabuccuoglu, 2007). We have previously reported on the cardioprotective nature of acetaminophen (*in vivo* and *in vitro*) following myocardial ischemia-reperfusion when

administered right before the ischemic insult (Golfetti et al., 2002; Hadzimichalis et al., 2007; Jaques-Robinson et al., 2008; Merrill and Goldberg, 2001; Merrill, 2002; Merrill et al., 2004; Merrill et al., 2007; Rork et al., 2004; Rork et al., 2006) and in a chronic setting (Golfetti et al., 2003). Myocardial function was markedly improved following acetaminophen treatment in our own studies. Our work has also shed light on the acetaminophen-mediated mechanism of cardioprotection. Our studies suggest that acetaminophen acts in the capacity of an antioxidant, since a significant reduction in oxidant-induced cell damage was observed in acetaminophen-treated hearts. These results are confirmed by our most recent study where the cardioprotective property of acetaminophen was evident following H_2O_2-mediated myocardial damage (Jaques-Robinson et al., 2008). Hydrogen peroxide participates in the Haber-Weiss reaction, and produces oxidants in the Fenton reaction (Koppenol, 2001). The main products of this reaction are hydroxyl radicals, damaging agents that interfere with normal cellular processes (Valko et al., 2007). Endogenous levels of hydrogen peroxide produce oxidants as well; however, these are scavenged by antioxidant mechanisms present within the cell. When the cell is inundated with hydrogen peroxide, the oxidants produced overwhelm cellular antioxidant mechanisms, and cell damage occurs. A few of our *in vivo* studies examined the effect of acetaminophen on MMP-2, one of the deleterious products generated by the actions of hydroxyl radicals, where acetaminophen-treated hearts showed a significant reduction in MMP-2 levels. Acetaminophen has also been shown to prevent mitochondrial damage in myocardial ischemia-reperfusion (Hadzimichalis et al., 2007; Jaques-Robinson et al., 2008; Rork et al., 2004). Specifically, the release of cytochrome *c* from the mitochondria was significantly reduced, suggesting a role for

acetaminophen in the inhibition of mitochondrial-mediated apoptosis. Additionally, electronmicrographs obtained from control, vehicle, and acetaminophen-treated hearts showed a marked increase in mitochondrial damage in vehicle hearts compared to control and acetaminophen-treated hearts. Damaged mitochondria were not as densely packed as control and exhibited incidence of swelling, an indication that the mitochondrial membranes and/or pores had been compromised by the cellular insult.

These studies and others have reported on the considerable role of the mitochondria in the cell death process as they are sensitive to the actions of pro-apoptotic stimuli. Thus, they are an important target for neuroprotection (Szeto, 2006a; Szeto, 2006b).

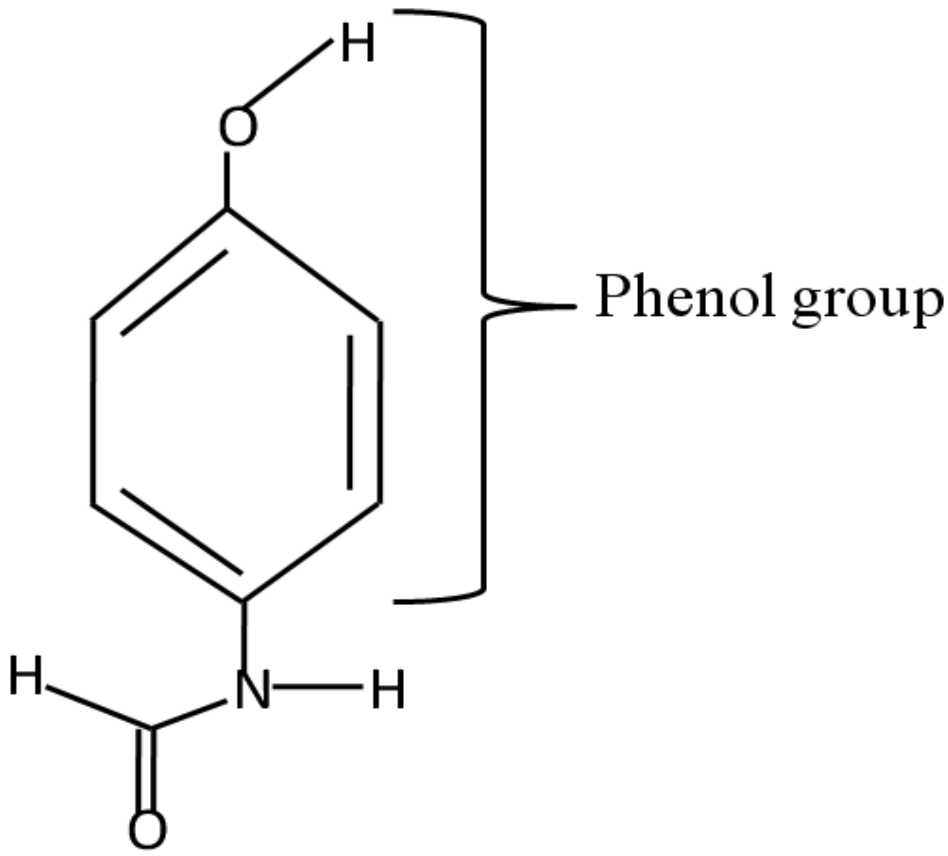


Figure 3. Chemical structure of acetaminophen. The phenol group confers some antioxidant capability to the drug.

Acetaminophen as a neuroprotectant

Research on the use of acetaminophen as a therapeutic has recently been extended to neuropathological settings. The first study reporting on the potential neuroprotective ability of acetaminophen suggested that the drug was capable of significantly reducing mitochondrial damage and oxidative stress sustained in a cellular model of Alzheimer's disease. Cells incubated with amyloid-β peptide aggregates known to trigger mitochondrial dysfunction or H_2O_2 – a participant in the Fenton reaction that produces oxidants – had significant reductions in apoptotis, mitochondrial dysfunction and lipid peroxidation levels (Bisaglia et al., 2002). Additionally, acetaminophen was successful in preventing neuronal loss due to glutamate toxicity (Casper et al., 2000b). The authors also reported a significant reduction in glioma cell growth - a highly lethal cancer primarily affecting the brain - by acetaminophen (Casper et al., 2000a). In *C. elegans*, low concentrations of acetaminophen (0.1mM) were effective in significantly reducing dopaminergic (DA) neuronal loss following a DA stress assay (Locke et al., 2008). Thus far, acetaminophen shows promise as a potential neuroprotective agent for stroke.

1.9 Experimental animal models for stroke

Experimental models of stroke have been developed in animals in an attempt to mimic the events of human cerebral ischemia. Some of the benefits of using rodent models in stroke research include smaller brain size, allowing more extensive and comprehensive evaluation of the entire brain without excess cost, time, and labor. Rats in particular are useful for stroke research because of their anatomic similarity to the human anatomy of cranial circulation as rats and humans have a complete Circle of Willis. Furthermore, commercially available inbred rodent strains are genetically homogeneous, allowing researchers to minimize variables due to a heterogeneous background. Investigators have begun to look at functional outcomes in experimental stroke by evaluating a battery of behavioral, cognitive, and sensorimotor tests. A number of neurosensory and motor behavior outcomes have been well described and standardized for rodents and can be applied to stroke models. However, there are a few disadvantages. Unlike humans, rodents have lissencephalic or unconvoluted brains. Due to size and blood volume limitations, physiologic monitoring can be more demanding in rodents. Lastly, concurrent and multiple measurements over time may be limited. Experimental studies involving rodents may have to include nonsurvival animal cohorts for physiologic measurements in addition to separate survival groups for infarct and functional assessments (Graham et al., 2004).

The goal of cerebral ischemia (focal and global) models is to reduce oxygen and glucose supply to brain tissue. Global ischemia occurs when cerebral blood flow (CBF) is

reduced throughout most or all of the brain, whereas focal ischemia is represented by a reduction in blood flow to a very distinct, specific brain region. With complete ischemia, global blood flow has ceased completely; whereas with incomplete ischemia, global blood flow is severely reduced but the amount of flow is insufficient to maintain cerebral metabolism and function. The focal model involves the transient or permanent occlusion of the mid-cerebral artery (MCA) to be used as a model of cerebral ischemia. This technique has been very popular since its inception in the late 1980s for studying mechanisms of both cellular injury and neuroprotection. It involves inserting a 4-0 nylon suture into the internal carotid artery of rats and then advancing the thread cranially to block the MCA. This thread can be passed and is usually advanced 17 to 20 mm from the origin of the internal carotid artery, thus occluding collateral circulation from the anterior communicating arteries (Traystman, 2003). Although this model is successful in generating cerebral infarct, it is also known to vary in the consistency of infarct produced when performed in different rat strains (Duverger and MacKenzie, 1988).

A global model has also been developed to mimic human cardiac arrest and involves the bilateral occlusion of the carotid and vertebral arteries (Crack and Taylor, 2005). The often-used 4-vessel occlusion (4-VO) model can be produced in awake, freely moving rats, but it involves a two-stage procedure to produce the ischemia. In the first stage, on the day before an experiment, atraumatic clasps are placed loosely around each common carotid artery and exteriorized in the neck of the animal. The vertebral arteries are then electrocauterized. Unfortunately, it is impossible to visualize these vessels directly, but this occlusion of the vertebral vessels is critical to the success of the model. On the

second day, the common carotid arteries are occluded while the animal is awake, and the ischemia is produced. This procedure must result in a complete loss of the righting reflex for the animal to be included in the study. The 4-VO model has been utilized by many investigators and has been well validated and well described. It can be utilized in either awake or anesthetized animals, which makes it extremely useful. However, it is not an easy model to use, and there has been much variability in results between laboratories. This 4-VO technique is successful in approximately 50 to 75% of the animals, but the effects of ischemia are quite variable between rat strains (Traystman, 2003). One advantage of the 4-VO model is the extensive damage experienced by the brain after brief ischemic periods (Pulsinelli and Buchan, 1988).

Bilateral carotid artery occlusion combined with systemic hypotension model

The bilateral carotid artery occlusion model was first introduced in the 1970s as a successful method for simulating stroke in experimental models. This model of forebrain ischemia was developed 25 years ago (Eklof and Siesjo, 1972a; Eklof and Siesjo, 1972b) and was initially used to characterize cerebral energy state following incomplete ischemia (Eklof and Siesjo, 1972a) (Nordstrom and Siesjo, 1978). The procedure described the clamping of the carotid arteries combined with systemic hypotension maintained during the length of the ischemic period. Measurements of CBF that the model is effective for producing severe ischemic conditions (Smith et al., 1984b). To support these conclusions, other studies have shown that cognitive deficits have been found to be associated with damage in the CA1 region of the hippocampus in studies using the bilateral common

carotid artery occlusion model. Furthermore, a severe state of ischemia is produced with the abrupt reduction of cerebral blood flow caused by the simultaneous occlusion of both common carotid arteries (Sarti et al., 2002). The major advantages of the 2VOHYP model over the 4-VO model in producing forebrain ischemia are that the 2VOHYP model requires a more simple surgical preparation, reperfusion can be readily accomplished, and this model is easily suitable for chronic survival studies.

2. PURPOSE

2.1 The effect of acetaminophen in brain following cerebral ischemia-reperfusion

The first part of this study investigated the effects of acetaminophen following cerebral ischemia-reperfusion. We have previously reported on the cardioprotective properties of acetaminophen (Hadzimichalis et al., 2007; Merrill and Goldberg, 2001; Merrill, 2002). In our studies and others (Leshnower et al., 2006; Zhu et al., 2006), acetaminophen has exhibited cardioprotective properties and is considered safe. It is well established that oxidants produced during cerebral ischemia-reperfusion (Chan, 1996; Chan, 2001; Crack and Taylor, 2005) are similar to the oxidants produced during myocardial ischemia-reperfusion (Dhalla et al., 2000; Warner et al., 2004). Thus, we were prompted to investigate the effect(s) of acetaminophen in reducing the damage experienced by the brain following transient global ischemia-reperfusion.

2.2 Mechanisms of Neuroprotection

The second part of this study examined the mechanisms of neuroprotection mediated by acetaminophen. Oxidants generated at the onset of reperfusion target the mitochondria (Christophe and Nicolas, 2006). Neuronal mitochondria are especially vulnerable to ischemia-reperfusion as they have higher metabolic rates than mitochondria in other organs (Szeto, 2006b; Warner et al., 2004). We have previously shown that acetaminophen confers cardioprotection by preventing mitochondrial dysfunction (Hadzimichalis et al., 2007), thus we sought to extend these findings to the brain as a potential mechanism for acetaminophen-mediated neuroprotection.

MATERIALS AND METHODS

1. EXPERIMENTAL PREPARATION

Materials

All chemicals were obtained from Sigma Aldrich, Inc., St. Louis, MO unless otherwise indicated. The OPN monoclonal antibody (mAb2A1) was kindly provided by Dr. David Denhardt.

1.1 Animal care and specification

Male Sprague-Dawley rats (325 – 375 g) were obtained from Ace Animals, Inc (Boyertown, PA) and were housed in AAALAC-accredited facilities at Rutgers University. Animals were fed standard laboratory rat chow and provided water *ad libitum* until the day of surgery.

1.2 Animal anesthetization

In accordance with National Institutes of Health/United States Department of Agriculture guidelines and after IACUC review and approval, animals were anesthetized using intraperitoneal (i.p.) administrations of ketamine/xylazine in an 80:12 mg/kg ratio,

respectively, and maintained with additional i.p. doses of ketamine (80 mg/kg) administered as necessary.

1.3 Statistical analysis

All data are presented ± S.E.M. Differences between three groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-test for multiple means comparisons. Differences between two groups was analyzed using Student's *t* Test. Significance was accepted at $P<0.05$ for all values.

2. EXPERIMENTAL PROTOCOLS

2.1 Surgical procedure

The 2-vessel occlusion combined with systemic hypotension (2VOHYP) procedure was used to induce stroke and was performed as previously described (Smith et al., 1984b) with a few modifications. Lidocaine was administered subcutaneously to the ventral and both inguinal regions prior to the surgical procedure to further alleviate potential pain induced during surgery. A ventral midline incision was made and the left jugular vein was cannulated for the purposes of administering heparin (250 U/kg) and acetaminophen (15 mg/kg). Next, the carotid arteries were isolated and marked with 3-0 suture. The left and right femoral arteries were cannulated for the purposes of recording blood pressure for the duration of the experiment and withdrawing blood to induce hypotension (Figure 4). Rats were mechanically ventilated using a small animal respirator (Model683, Harvard Apparatus, Holliston, MA). Respirator stroke rate was adjusted to maintain physiological blood pH.

In order to induce hypotension (~55 mmHg), blood was withdrawn from one femoral artery using a heparinized syringe until the desired blood pressure was attained. Ischemia was initiated by clamping the common carotid arteries for 15 min using bulldog clamps after induction of hypotension. Reperfusion was initiated with the removal of the bulldog clamps and lasted for 45 min. During the first few minutes of reperfusion, shed blood was returned to the animal (Figure 4).

2.2 Brain extraction

Extraction was performed by carefully making incisions on the base of the skull and using a Rongeurs to peel away the skull fragments. The brain was scooped out using a weigh spatula and placed in ice-cold 0.1M PBS. The cerebrum was rapidly excised from the cerebrum. In order to obtain the hippocampus, the cerebrum was divided into left and right hemispheres and the neocortex carefully peeled back to reveal the hippocampus. The hippocampus was removed using forceps and a scooped spatula. All brain extraction was performed in 4°C.

2.3 Treatment groups

Group sham-control: Rats were instrumented as above but were not subject to hypotension or carotid artery occlusion. Blood pressure and other hemodynamic variables were measured and the animal was maintained at physiological conditions for the duration of the experiment.

Group acetaminophen-ischemia: Rats were instrumented as above and subject to hypotension (~55 mmHg) followed by carotid artery occlusion for 15 min. Acetaminophen was intravenously administered (15 mg/kg) prior to carotid artery

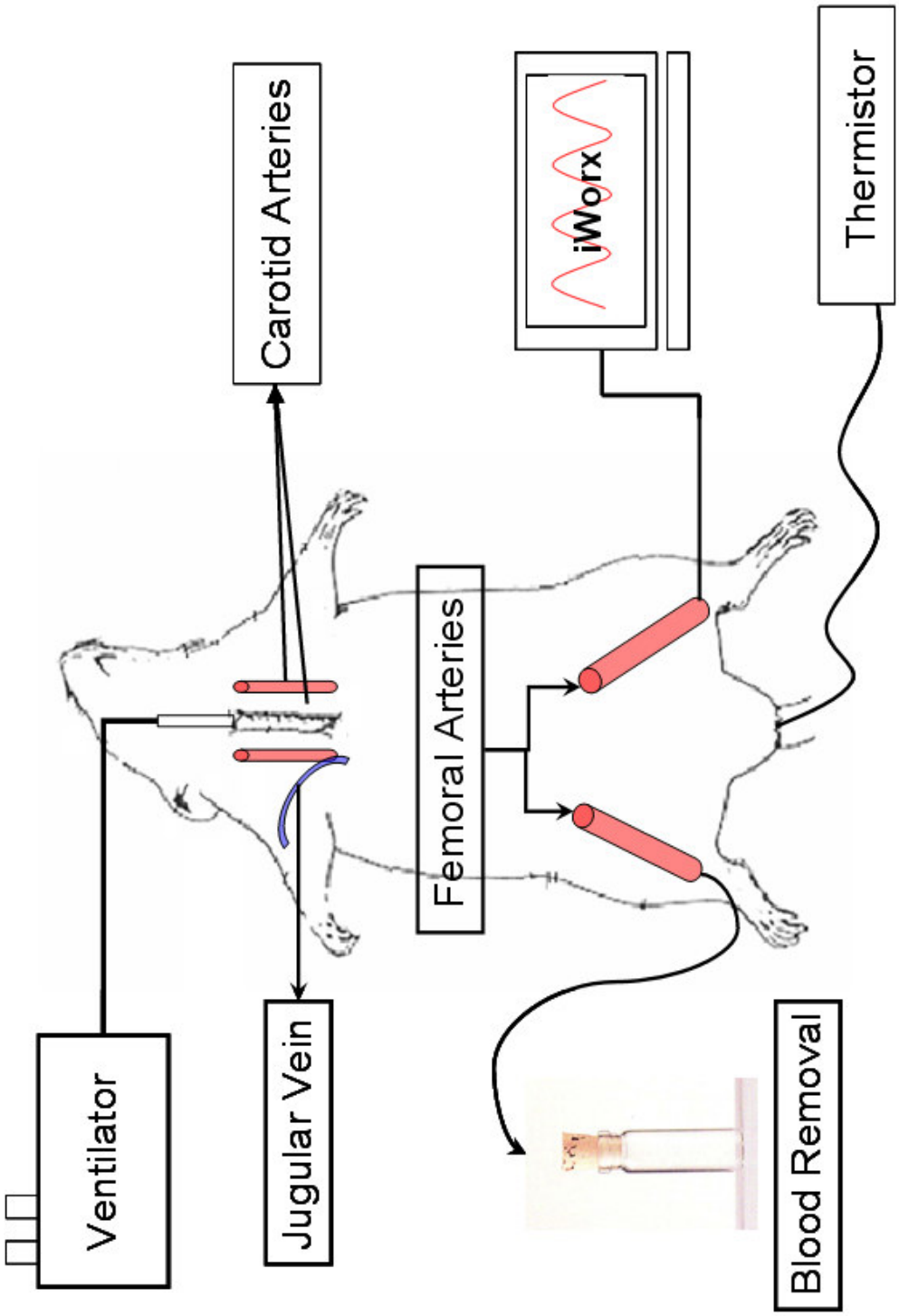


Figure 4. Surgical procedure. A schematic depicting the instrumentation of the rat prior to carotid artery occlusion.

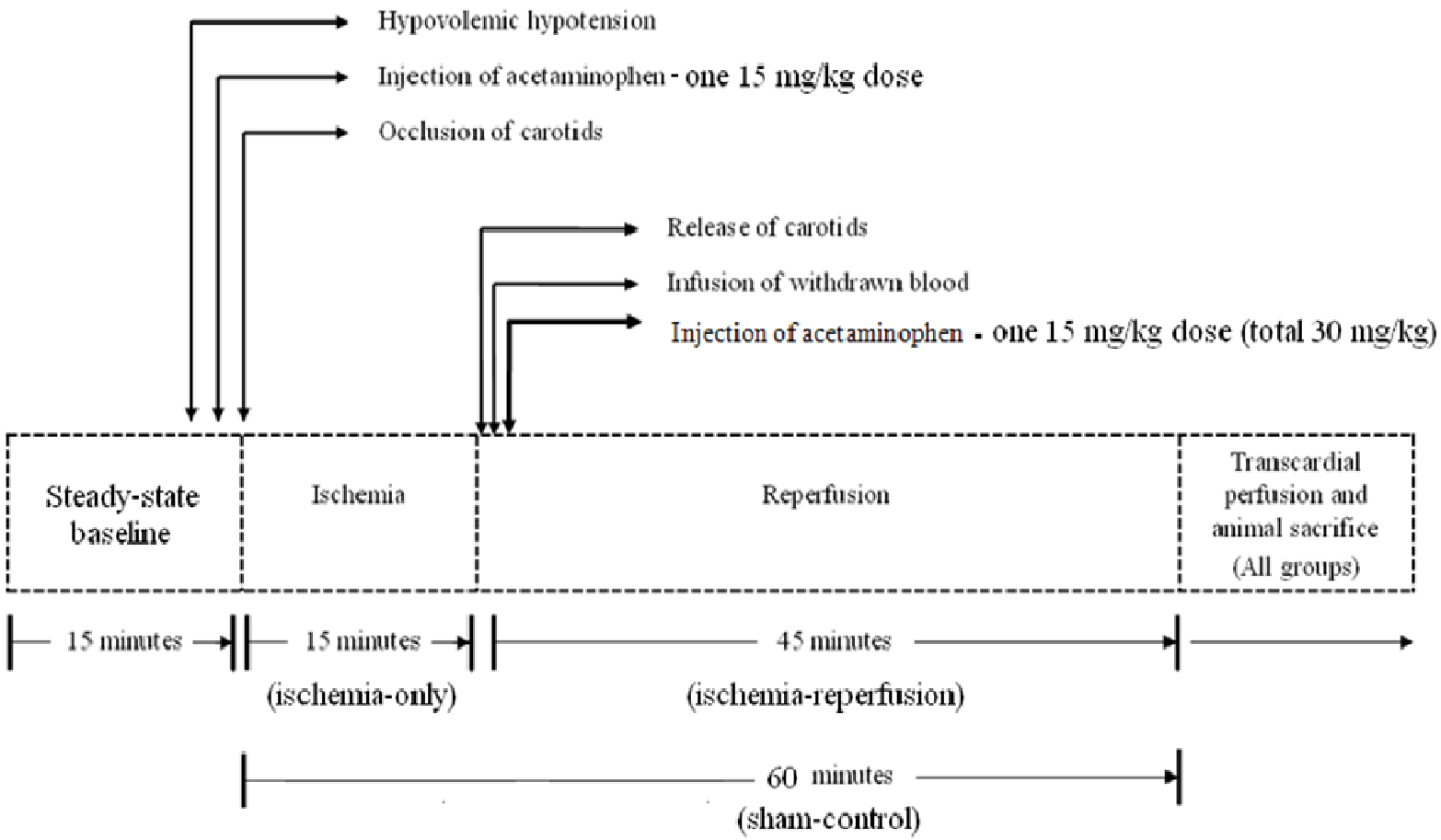


Figure 5. Treatment groups. Schematic depicting times of dose administration and experimental time points.

occlusion. At the end of the ischemic period, rats were transcardially perfused and brains were extracted for analysis.

Group vehicle-ischemia: Rats were instrumented as above and subject to hypotension (~55 mmHg) followed by carotid artery occlusion for 15 min. An equivalent dose of vehicle (0.2 – 0.3 mL, 0.9% saline) was intravenously administered prior to carotid artery occlusion. At the end of the ischemic period, rats were transcardially perfused and brains were extracted for analysis.

Group acetaminophen-ischemia-reperfusion: Rats were instrumented as above and subject to hypotension (~55 mmHg) followed by carotid artery occlusion for 15 min. Acetaminophen was intravenously administered (15 mg/kg) prior to carotid artery occlusion. At the end of the ischemic period, clamps were removed initiating 45 min of reperfusion. At the onset of reperfusion, shed blood was returned to the animal at a rate of 1 mL/min. An additional bolus of acetaminophen (15 mg/kg) was administered at this time. At the end of the reperfusion period, animals were transcardially perfused and brains were extracted for analysis.

Group vehicle-ischemia-reperfusion: Rats were instrumented as above and subject to hypotension (~55 mmHg) followed by carotid artery occlusion for 15 minutes An equivalent dose of vehicle (0.2 – 0.3 mL, 0.9% saline) was intravenously administered prior to carotid artery occlusion. At the end of the ischemic period, clamps were removed

initiating the 45 minute period of reperfusion. At the onset of reperfusion, shed blood was returned to the animal at a rate of 1 mL/min. An additional equivalent bolus of vehicle (0.9% saline) was administered at this time. At the end of the reperfusion period, animals were transcardially perfused and brains were extracted for analysis.

Group double dose acetaminophen-ischemia-reperfusion: Rats were instrumented as above and subject to hypotension (~55 mmHg) followed by carotid artery occlusion for 15 min. Acetaminophen (15 mg/kg) was intravenously administered prior to carotid artery occlusion and clamp removal. At the end of the ischemic period, the clamps were removed initiating the 45 minute period of reperfusion. At the onset of reperfusion, shed blood was returned to the animal at a rate of 1 mL/min. At the end of the reperfusion period, animals were transcardially perfused and brains were extracted for analysis.

Group double-dose-vehicle-ischemia-reperfusion: Rats were instrumented as above and subject to hypotension (~ 55 mmHg) followed by carotid artery occlusion for 15 min. An equivalent dose of vehicle (0.2 – 0.3 mL, 0.9% saline) was intravenously administered prior to carotid artery occlusion and clamp removal. At the end of the ischemic period, the clamps were removed initiating the 45 minute period of reperfusion. At the onset of reperfusion, shed blood was returned to the animal at a rate of 1 mL/min. At the end of the reperfusion period, animals were transcardially perfused and brains were extracted for analysis.

2.4 Physiological variables

Physiological data was obtained following 15 min of steady-state conditions (baseline), 15 min of ischemia, and 45 min of reperfusion. A small volume of arterial blood (0.2 mL) was withdrawn from one of the femoral arteries for blood gas analysis (Model NPT7, Radiometer America, Westlake, OH). Heart rate, systolic/diastolic values were obtained from blood pressure traces using a blood pressure transducer (BP-100, iWorx, Dover, NH).

2.5 TTC staining

2,3,5-Triphenyltetrazoliumchloride (TTC) is a dye commonly used to measure the region of infarct caused by an experimental procedure. In this study, TTC was used to determine whether 2VOHYP was sufficient in inducing a significant level of infarct in the animals. Furthermore, TTC was used to assay the efficiency of acetaminophen in significantly reducing infarct region after intravenous administration of 15 mg/kg and 30 mg/kg.

Following 2VOHYP, the animals from groups sham-control, acetaminophen-ischemia, vehicle-ischemia, acetaminophen-ischemia-reperfusion, vehicle ischemia-reperfusion, acetaminophen double dose-ischemia-reperfusion, and vehicle double dose-ischemia-reperfusion (n = 4/ group) were euthanized by transcardial perfusion of ice-cold 0.1M PBS. The cerebrum was separated from the cerebellum and the brainstem and immediately sliced into 2 mm thick slices and placed in a 0.1% TTC solution (dissolved

in distilled water) for 30 min at 37°C using a water bath. After incubation, the brain slices were removed and placed in 4% paraformaldehyde (dissolved in 1X PBS) overnight (Peng et al., 2007; Wang et al., 2007a). Following paraformaldehyde incubation, brain slices were scanned using a flatbed scanner (Brother MFC-7420). Scanned images were converted to grayscale in order to differentiate colorless regions (infarct) from undamaged regions. Infarct area within each brain slice was carefully traced and measured by Image J (NIH, public domain software) and expressed as a percentage of the surface area of the entire brain slice.

2.6 Subcellular Fractionation

Rats from groups sham-control, acetaminophen-ischemia-reperfusion and vehicle-ischemia-reperfusion (n = 4/group) were transcardially perfused with ice-cold 0.1M PBS following termination of reperfusion. Brains were extracted as above and placed in homogenization buffer (10 ml/g) containing (in mM): 210.0 mannitol, 7.0 sucrose, and 5.0 4-morpholinopropanesulfonic acid, pH 7.4, 25°C, with 1mM PMSF and protease inhibitor cocktail (Sigma Aldrich Inc, St. Louis, MO) (Hadzimichalis et al., 2007). Tissue was homogenized with 10 – 15 strokes using a Dounce Homogenizer (Fisher Scientific, Waltham, MA). Separation of mitochondrial and cytosolic fractions was obtained as previously described (Tokarska-Schlattner et al., 2005) with a few modifications. Briefly, the homogenate was centrifuged at 4000 *g* for 10 m at 4°C and the resulting supernatant was centrifuged at 7000 *g* for 10 m at 4°C. The pellet from the second centrifugation represented the mitochondrial fraction and was resuspended in 10 mM sodium phosphate

buffer, pH 9.0. The supernatant from the second centrifugation represented the cytosolic fraction. Cytosolic and mitochondrial fractions were stored at -20°C until use.

2.7 Measurement of mitochondrial swelling

Total protein concentration was measured in mitochondrial suspensions from Groups sham-control, acetaminophen-ischemia-reperfusion and vehicle-ischemia-reperfusion (n = 4 per group) using the Bradford assay. Mitochondrial suspensions were subject to spectrophotometric analysis (540 nm) at 25°C with a spectrometer (SpectraMax plus384; Molecular Devices, Toronto, Canada) as previously described (Bosetti et al., 2004; Gadelha et al., 1997). Light absorbance values were expressed as a percentage of control values.

2.8 Mitochondrial morphometry

Electron microscopy was used to evaluate and compare changes in mitochondrial features in hippocampal tissue obtained from groups sham-control, acetaminophen-ischemia-reperfusion and vehicle-ischemia-reperfusion (n = 2/group). Damaged mitochondria would exhibit marked changes in membrane and cristae morphology. In this study, electron microscopy was used to corroborate results obtained from spectrophotometric analysis and provide a means of visualizing the extent of tissue damage experienced by hippocampal tissue following 2VOHYP and treatment with acetaminophen.

Following 2VOHYP, the animals were euthanized by transcardial perfusion of ice-cold 0.1M PBS followed by perfusion using ice-cold Trump's fixative (pH ~7.2). Brains were extracted as above and placed in Trump's fixative. The cerebrum was excised and the hippocampus extracted as above and 1 mm3 were obtained and submerged in Trump's fixative. Blocks were postfixed with 1% osmium tetroxide and subsequently dehydrated in graded ethanol. Samples were embedded in Epon-Araldite cocktail, sectioned with a diamond knife ultramicrotome (model LKB-2088, LKB, Bromma, Sweden), and viewed with an electron Nikon Diaphot 3000 (model JEM-100CXII, JEOL USA, Peabody, MA) using standard protocols. Mitochondria were identified and digital photomicrographs were acquired at a magnification of 1 μm. Each mitochondrion was outlined and the total mitochondrial and cytosolic areas were measured as previously described (Hirai et al., 2001) using image analysis software (Scion Image, public domain software developed at the NIH and available at http://www.scioncorp.com/). Cytosolic area excluded the nucleus. The total mitochondrial areas were expressed as a percentage of the total cytosolic areas.

2.9 FACS analysis

Changes in mitochondrial membrane potential were measured with 5,5',6,6'-tetrachloro-1,1',3,3'- tetraethylbenzimidazolylcarbocyanine iodide (JC-1) using FACS in groups sham-control, acetaminophen-ischemia-reperfusion and vehicle-ischemia-reperfusion. Mitochondrial size, shape, and density do not influence mitochondrial membrane potential, making JC-1 a reliable means of determining and comparing mitochondrial

membrane potential between samples. Cell suspensions for FACS analysis were generated according to Campanella et al (Campanella et al., 2002). Following 2VOHYP and brain extraction, cerebral tissue was homogenized in Hank's Balanced Salt Solution (HBSS) using a Dounce Homogenizer (Fisher Scientific, Waltham, MA), passed through a 40 μm nylon mesh strainer, and centrifuged at 1000 *g* for 10 min. The resulting pellet was subjected to a Percoll density gradient (ρ= 1.095 and ρ=1.03), overlayered with HBSS and centrifuged at 1000 *g* for 20 min. Cells were collected from the interface of the two layers, washed with 10% FBS and centrifuged at 400 *g* for 10 min. Cells were then treated with JC-1 according to manufacturer's instructions and run through the flow cytometer (Beckman-Coulter, Fullerton, CA). Excitation wavelength was set at 488 nm. Quantification of changes in mitochondrial membrane potential was performed and was obtained by calculating the ratio of the percentage of cells fluorescing red to the percentage of cells fluorescing green.

2.10 Western blot

Protein concentration in cytosolic and mitochondrial fractions was determined using Bradford reagent (Bio-Rad, Hercules, CA). Cytosolic and mitochondrial proteins (10 μg) were resolved on a 12% SDS-polyacrylamide gel at 100V for 2 h. Protein was then transferred to a PVDF membrane (Immobilon-P, Millipore, Billerica, MA) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 330 mA for 90 m. The membrane was then blocked with 2% BSA for 1 h, and hybridized with primary antibody against cytochrome *c* (1:1000, rabbit polyclonal IgG, Santa Cruz Biotechnology, Santa

Cruz, CA) for 2 h at room temperature. After four washes with TBS-T (100 mM tris–base, 154 mM NaCl, 0.1% Tween-20, pH 7.5), the membrane was then incubated with horseradish peroxidase conjugated secondary antibodies (1:2000; goat anti-rabbit IgG HRP) for 45 m. Membranes were washed four times in TBS-T and then developed on X-ray film using ECL+ method (enhanced chemiluminisence reagents, Amersham Biosciences, Piscataway, NJ). Membranes were then washed two times in stripping buffer (186.5 mM Glycine, 3.5 mM SDS, 0.01% Tween-20, pH 2.2), followed by two 10 m washes in PBS and by two 5 m washes in TBS-T. Membranes were then blocked in 2% BSA for 2 hours and hybridized with primary antibody against β-tubulin (1:1000, rabbit polyclonal IgG, Santa Cruz Biotechnology, Santa Cruz, CA) or voltage dependent anion channel (VDAC, 1:800, goat polyclonal IgG, Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature for cytosolic and mitochondrial fractions respectively. After four washes with TBS-T (100 mM tris–base, 154 mM NaCl, 0.1% Tween-20, pH 7.5), the membrane was then incubated with horseradish peroxidase conjugated secondary antibodies (1:2000; goat anti-rabbit, donkey anti-goat IgG HRP) for 45 m at room temperature. Membranes were washed four times in TBS-T and then developed on X-ray film using ECL+ method (enhanced chemiluminisence reagents, Amersham Biosciences, Piscataway, NJ). Experiments were done at least two times.

2.11 Tissue sectioning

Following 2VOHYP, animals from groups sham-control, acetaminophen-ischemia-reperfusion and vehicle-ischemia-reperfusion were perfused with ice-cold 0.1M PBS. Brains were extracted as above and the cerebrum was excised. The cerebrum was incubated for 24 h in 4% paraformaldehyde/PBS, 10% sucrose, 20% sucrose and 30% sucrose at 4°C. Prior to sectioning, brains were divided into left and right hemisphere and placed in a 1:1 30% sucrose/OCT solution for 2 h followed by incubation in OCT for 1 h at 4°C.

Cerebral hemispheres were embedded in OCT over dry ice and 10-micron thick sections were obtained using a cryostat (Model CM1900, Leica Microsystems, Bannockburn, IL). Sections were mounted onto room-temperature glass slides (Fisherbrand SuperFrost Plus, Fisher Scientific, Waltham, MA) and then warmed for 2 h at 37°C. Slides were stored at -80°C until use.

2.12 Hematoxylin & Eosin staining

Slides were prewarmed for 10 min at 37°C. Sections were dehydrated using a series of alcohols. Slides were washed twice in Xylenes for 5 min and 2 min respectively, twice in 100% Ethanol for 2 min each, once for 2 min in 95%, 70% and 50% ethanol. Slides were then immersed in tap water for 2 min followed by a 20 min incubation in Harris-modified Hematoxylin. Excess dye was removed by running cold tap water over slides for 2 min.

Slides were then placed in 70% ethanol for 2 min, followed by 10 dips in acid alcohol (1.25 mL HCl in 250 mL 50% ethanol), washed in 70% ethanol for 2 min, incubated in alkaline alcohol (1.25 mL Ammonium hydroxide in 250 mL 50% ethanol) for 2 min and briefly immersed in Eosin B for 15 – 20 s. Slides were then dehydrated by immersing them in 95%, 100% for 2 min followed by immersion in Xylenes I and II for 2 min each. A few drops of mounting medium were placed on the section followed by a coverslip. Sections were then observed under 20X magnification using (Nikon Diaphot 3000), regions of interest were identified based on the staining pattern and images were digitally acquired.

2.13 Immunohistochemistry

Slides were prewarmed for 10 min at 37°C and washed two times in 1X PBS. Determination of cleaved caspase-9 expression was carried out using the following protocol, as previously described (Henshall et al., 2001) with a few modifications: Sections were fixed in pre-cooled acetone for 5 min at room temperature, followed by three rinses in 1X PBS. Endogenous hydrogen peroxidase activity was quenched by incubation in hydrogen peroxide blocking solution (1:9 hydrogen peroxide: methanol) for 30 min at room temperature. After one rinse in 1X PBS, sections were blocked in universal blocking solution (1% BSA, 0.1% cold fish gelatin, 0.5% NaN_3 in 1X PBS, pH 7.2-7.4) for 1 h at room temperature. Slides were washed once in 1X PBS and incubated in goat anti-cleaved caspase-9 (1:100, goat polyclonal IgG, Santa Cruz Biotech, Santa Cruz, CA) overnight at 4°C. After 3 washes in 1X PBS, sections were incubated with

HRP-conjugated secondary antibody (1:100, donkey anti-goat IgG, Santa Cruz Biotech, Santa Cruz, CA) in 1% BSA for 45 min at room temperature. Slides were dehydrated in a series of alcohols, and viewed using a microscope (Nikon Diaphot 3000).

2.14 *In-situ* detection of DNA fragmentation

Apoptotic cell death was assayed in brain tissue sections as previously described (Wang et al., 2007b) using the TACS·XL *In Situ* Apoptosis Detection kit (Trevigen Inc, Gaithersburg, MD). The steps were as follows: Slides were prewarmed for 2 h at 37°C, dehydrated in a series of alcohols (100% ethanol, 95% ethanol and 70% ethanol), and washed in 1X PBS for 5 min. Sections were fixed by incubation in 3.7% buffered formaldehyde for 10 min at room temperature, followed by a 10 min wash in 1X PBS. Sections were permeabilized by incubation in Cytonin™ for 30 min at room temperature followed by 2 washes in 1X PBS. An incubation in Quenching Solution (1:9 dilution 30% H_2O_2: methanol) for 5 min was followed by a 1 min wash in 1X PBS at room temperature. Slides were immersed in 1X TdT Labeling Buffer for 5 min followed by incubation in Labeling Reaction Mix (B-dNTP, TdT enzyme, TdT labeling buffer) for 1 h at 37°C in a humid chamber. The reaction was stopped by immersion in 1X TdT Stop Buffer (0.5M EDTA) for 5 min at room temperature. Sections were washed in 1X PBS for 2 min prior to incubation with anti-BrdU (1:50) for 30 min at 37°C in a humid chamber. Excess antibody was removed by two 2 min washes in 1X PBST (1:2 dilution, Tween-20: 1X PBS). Color development was achieved by incubation in DAB solution for 7 min at room temperature. Excess DAB was removed by several 2 min washes in

deionized water. Sections were then counterstained by immersion in Methyl Green for 5 min followed by dehydration alcohols (two washes in 70% ethanol, 95% ethanol, 100% ethanol, Xylenes). After mounting medium was placed on the slides, they were coverslipped and allowed to harden overnight. Viewing was achieved at 20X with a microscope (Nikon Diaphot 3000).

RESULTS

1. Physiological variables

1.1 *Hemodynamic variable*s

No significant differences were observed in groups sham-control, acetaminophen-ischemia-only (15 mg/kg), vehicle-ischemia-only, acetaminophen-ischemia-reperfusion (15 mg/kg) and vehicle-ischemia-reperfusion regarding hemodynamic variables taken at the end of baseline (15 m post-surgical instrumentation). Hypotension was induced during ischemia (~55 mmHg) which resulted in a significant decrease in blood pressure for groups acetaminophen-ischemia-only (15 mg/kg), vehicle-ischemia-only, acetaminophen-ischemia-reperfusion vehicle-ischemia-reperfusion, acetaminophen double dose-ischemia-reperfusion and vehicle double dose-ischemia-reperfusion compared to baseline values. Upon reperfusion, there was a return to normotension (~100 mmHg), due to the re-infusion of shed blood. This was denoted as a significant increase for groups acetaminophen-ischemia-reperfusion, vehicle-ischemia-reperfusion, acetaminophen double dose-ischemia-reperfusion and vehicle double dose-ischemia-reperfusion compared to ischemic values. No significant differences were observed for blood pressure between baseline and reperfusion time points in groups sham-control, acetaminophen-ischemia-reperfusion, vehicle-ischemia-reperfusion, acetaminophen double dose-ischemia-reperfusion, and vehicle double dose-ischemia-reperfusion.

The effects of acetaminophen on heart rate, systemic arterial blood pressure, pH, blood oxygen (PO_2), and carbon dioxide (PCO_2) were evaluated in Groups acetaminophen-ischemia-only (15 mg/kg), vehicle-ischemia-only, acetaminophen-ischemia-reperfusion and vehicle-ischemia-reperfusion. All respiratory and cardiovascular parameters remained within baseline values in groups acetaminophen-ischemia-only (15 mg/kg), vehicle-ischemia-only, acetaminophen-ischemia-reperfusion and vehicle-ischemia-reperfusion. In addition, the core body temperature also did not differ significantly between groups, thus ruling out hypothermia-mediated effects on the results (Table 1).

	Baseline	Ischemia	Reperfusion
Mean arterial pressure, MAP (mmHg)			
Vehicle	110.7±5.1	61.6±2.9*	90.0±3.3
Acetaminophen	109.3±5.1	61.3±4.3*	95.0±6.1
Heart rate (beats per minute)			
Vehicle	266.5±7.6	271.7±17	252.8±10
Acetaminophen	260.2±8.7	255.0±12	271.5±11
PCO_2 (mmHg)			
Vehicle	26.0±1.4	30.3±1.9	26.8±1.5
Acetaminophen	28.8±1.6	31.7±2.9	27.8±2.6
PO_2 (mmHg)			
Vehicle	84.7±4.3	70.7±2.8	88.3±6.2
Acetaminophen	91.7±7.6	77.3±3.9	88.0±5.3
pH			
Vehicle	7.43±0.007	7.39±0.01	7.38±0.008
Acetaminophen	7.44±0.009	7.40±0.01	7.40±0.01
Body temperature (°C)			
Vehicle	37.0±0.3	37.1±0.3	36.7±0.2
Acetaminophen	37.0±0.5	36.9±1.1	37.0±0.5

Table 1. Comparison of hemodynamic and metabolic parameters between vehicle- and acetaminophen-treated groups

2. Detection of cell damage

2.1 *Evaluation of cerebral damage due to mitochondrial dysfunction*

No infarct size was observed in control animals, whereas a significant increase in infarct area (colorless regions) was observed in corresponding vehicle following ischemia and ischemia-reperfusion (n = 4 in each group). A significant decrease in infarct area was observed in acetaminophen-treated (15 mg/kg) animals following ischemia-reperfusion compared to corresponding vehicle groups; however these results were not significant in acetaminophen-treated animals following ischemia only (n = 4 in each group, Figure 6).

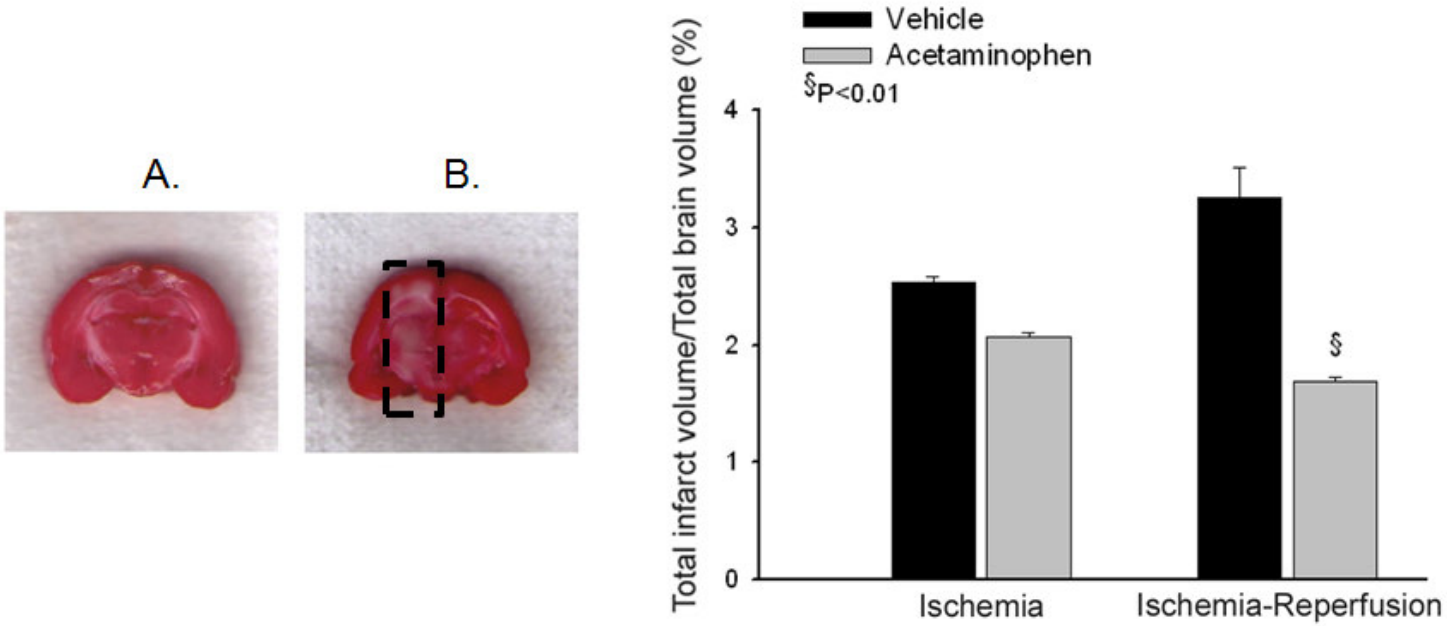


Figure 6. Acetaminophen significantly reduces cerebral damage. Left: Representative acetaminophen-treated (A, 15 mg/kg dose) and vehicle (B) slices. Rectangle indicates infarct (colorless regions). Right: Infarct volumes were measured and expressed as a percentage of the total volume of the brain slice. *P<0.05 determined by ANOVA followed by Tukey's post -test compared to control. §P<0.05 determined by ANOVA followed by Tukey's post-test compared to vehicle. Error bars indicate S.E.

2.2 *Changes in mitochondrial membrane potential*

Analysis of mitochondrial membrane potentials revealed that following acetaminophen treatment (15 mg/kg) in ischemia-reperfused animals, higher ratios of red fluorescence over green fluorescence were obtained compared to vehicle ischemia-reperfused animals. The higher ratio of red to green fluorescence corresponded to significantly higher mitochondrial membrane potential values (0.72 ± 0.23 vs. 0.01 ± 0.003, $P<0.05$, $n = 4$ in each group, Figure 7) in animals subjected to ischemia-reperfusion with acetaminophen-treatment (15 mg/kg) compared to corresponding vehicle.

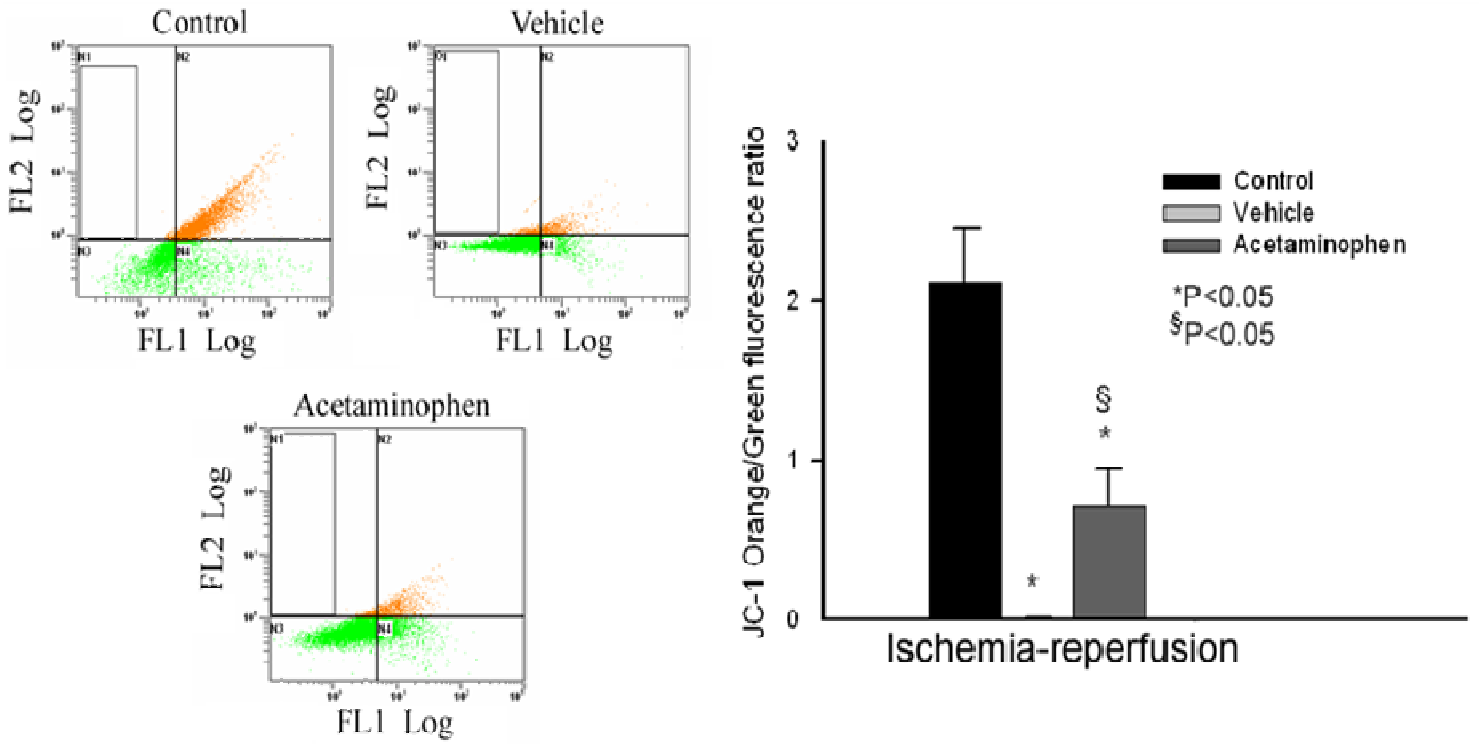


Figure 7. Acetaminophen preserves mitochondrial membrane potential. Left. Higher values for mitochondrial membrane potential were observed in control and acetaminophen-treated brains, whereas a near complete abolishment of membrane potential was noted in mitochondria of vehicle-treated brains. Right. Acetaminophen-treated (15 mg kg) brains exhibited higher ratios of red over green fluorescence compared to vehicle-treated brains. *P · 0.05, determined by ANOVA followed by Tukey's post-test, compared with vehicle-treated brains. §P ·0 05 determined by ANOVA followed by Tukey's post-test compared to vehicle-treated group. Error bars indicate S.E.

2.3 *Effect of acetaminophen on mitochondrial swelling*

Brains obtained from vehicle animals subject to ischemia-reperfusion had a higher occurrence of mitochondrial swelling compared to brains from acetaminophen-treated (15 mg/kg) ischemia-reperfused animals when expressed as a percentage of control absorbance values. A significant decrease in light absorbance was evident in vehicle groups, whereas acetaminophen-treated (15 mg/kg) exhibited higher light absorbance values (0.22±0.17 vs. 1.27±0.18. $P<0.05$, n = 4 in each group). A similar trend was observed in animals treated with a double-dose of acetaminophen (30 mg/kg) compared to corresponding vehicle (0.77±0.23 vs. 1.71±0.47. $P<0.05$, n = 4 in each group, Figure 8).

2.4 *Acetaminophen and mitochondrial structure*

The mitochondria in acetaminophen-treated rats following ischemia-reperfusion were smaller in size compared to corresponding vehicle rats. Quantitative analysis confirmed significantly smaller mitochondrial to cytosolic area ratios in acetaminophen versus vehicle [(1.24± 0.02) $X10^8$ vs. (3.11± 0.1) $X10^8$, respectively. $P<0.05$, n = 2 in each group; Figure 9)]. Additionally, cristae in acetaminophen-treated mitochondria were well-defined compared to vehicle brains.

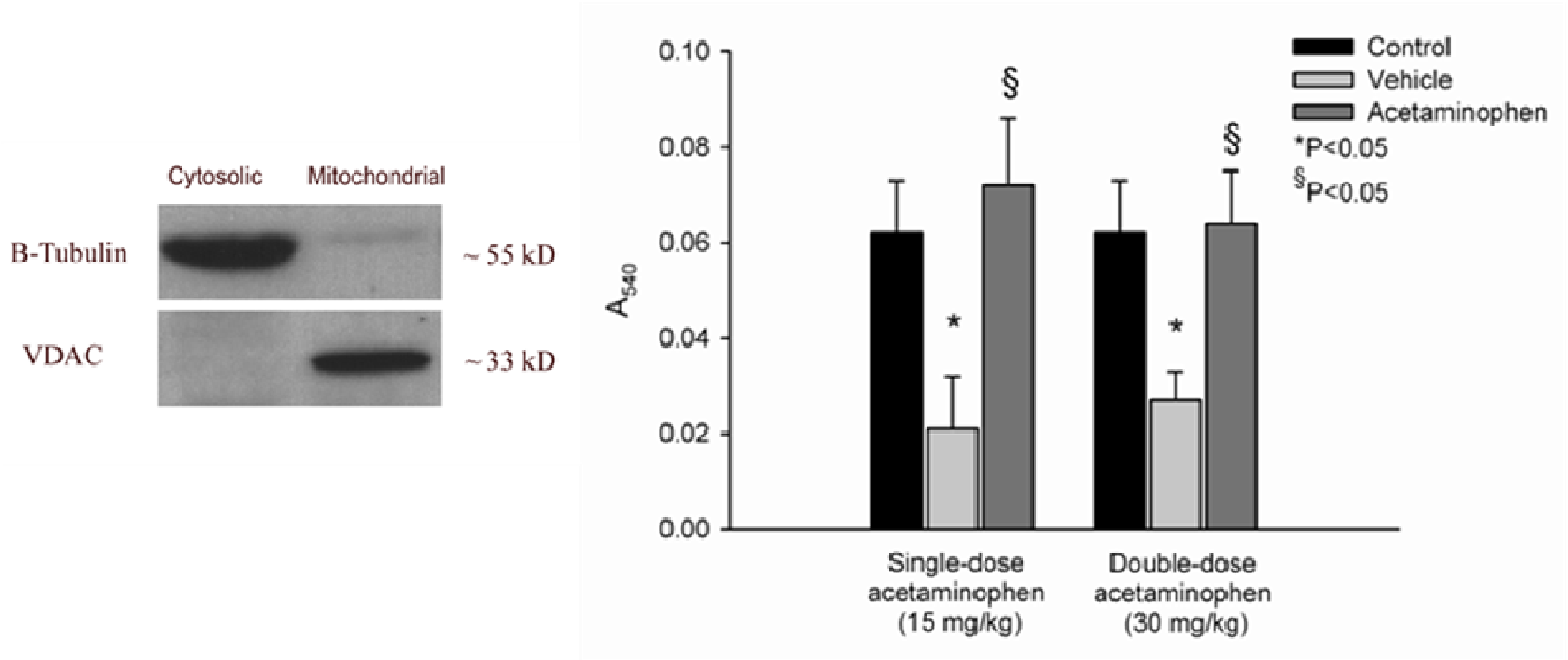


Figure 8. Acetaminophen prevents mitochondrial swelling. Left: Representative Western blots showing a lack of β-tubulin and voltage-dependent anion channel protein (VDAC) in mitochondrial and cytosolic fractions respectively. Right: Spectrophotometric analysis of mitochondrial swelling comparing changes in light scattering (A_{540}) between acetaminophen-treated (15 mg/kg and 30 mg kg) and vehicle compared to control. *P 0.05 as determined by one way ANOVA with Tukey's post test compared to control. §P 0.05 as determined by one way ANOVA with Tukey's post test compared to vehicle. Error bars indicate S.E.

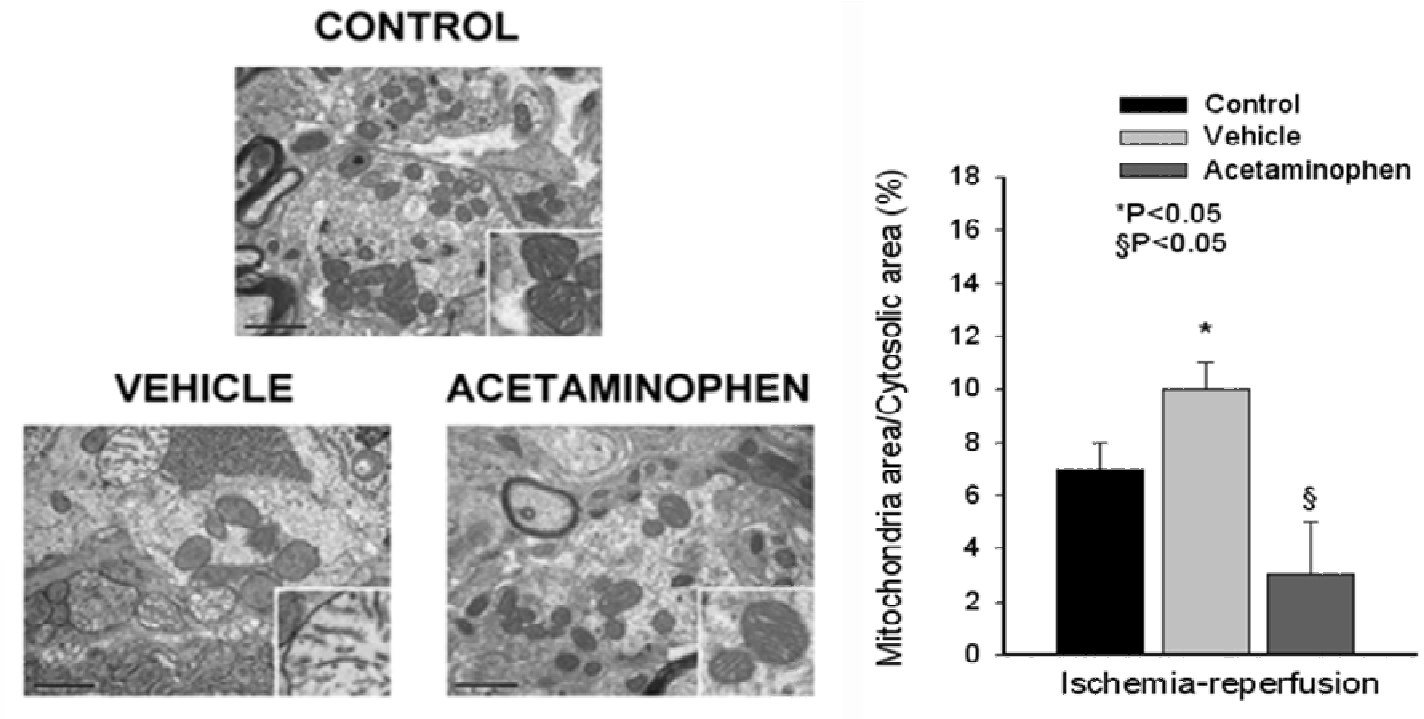


Figure 9. Acetaminophen preserves mitochondrial structure. Left: Representative electron microscopy images of mitochondria from hippocampal neurons showed well-defined cristae in control and acetaminophen-treated (15 mg/kg) animals, with a loss of cristae in vehicle animals (insets). Right: Morphometric analysis of the percent coverage of cytoplasmic area by mitochondria in neurons between acetaminophen-treated and vehicle groups. *P .0.05 as determined by ANOVA followed by Tukey's post-test compared to control. §P .0.05 as determined by ANOVA followed by Tukey's post-test compared to vehicle. Error bars indicate S.E.

2.6 *Effect of Acetaminophen on cytochrome c release*

Cytochrome *c* content was measured in cytosolic and mitochondrial fractions obtained from the hippocampus, left and right cortices of control, acetaminophen-treated (15 mg/kg and 30 mg/kg) and in the corresponding vehicle tissue. A significant increase in cytosolic cytochrome *c* content was observed in both vehicles compared to control. Interestingly, no significant differences were observed between vehicle and acetaminophen-treated groups (15 mg/kg and 30 mg/kg, n = 4 in each group, Figure 10). However, analysis of mitochondrial cytochrome *c* content revealed interesting results. Acetaminophen-treated groups (15 mg/kg and 30 mg/kg) exhibited significantly higher mitochondrial cytochrome *c* content compared to vehicle in hippocampal, left and right cortical tissue. No significant differences in mitochondrial cytochrome *c* content were observed between control and acetaminophen-treated (15 mg/kg and 30 mg/kg) groups (n= 4 in each group, Figure 11).

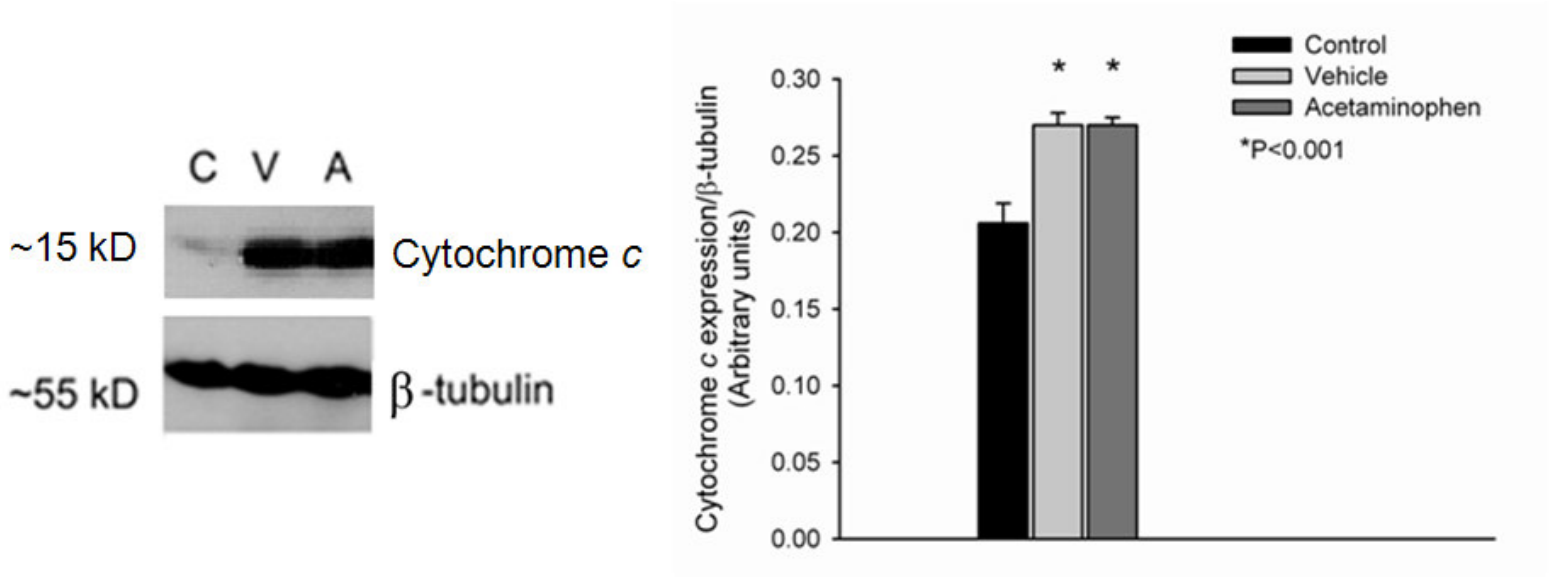


Figure 10. Cytosolic release of cytochrome *c*. Left: Representative Western blots showing an increase in cytochrome *c* release in vehicle (V) and acetaminophen treatment (A) compared to control (C). Right: Quantitative analysis of cytosolic cytochrome *c* expression normalized to β-tubulin expression in control, vehicle and acetaminophen (15 mg/kg and 30 mg/kg) groups. *P<0.05 as determined by ANOVA followed by Tukey's post-test compared to control. §P<0.05 determined by ANOVA followed by Tukey's post-test compared to vehicle. Error bars indicate S.E.

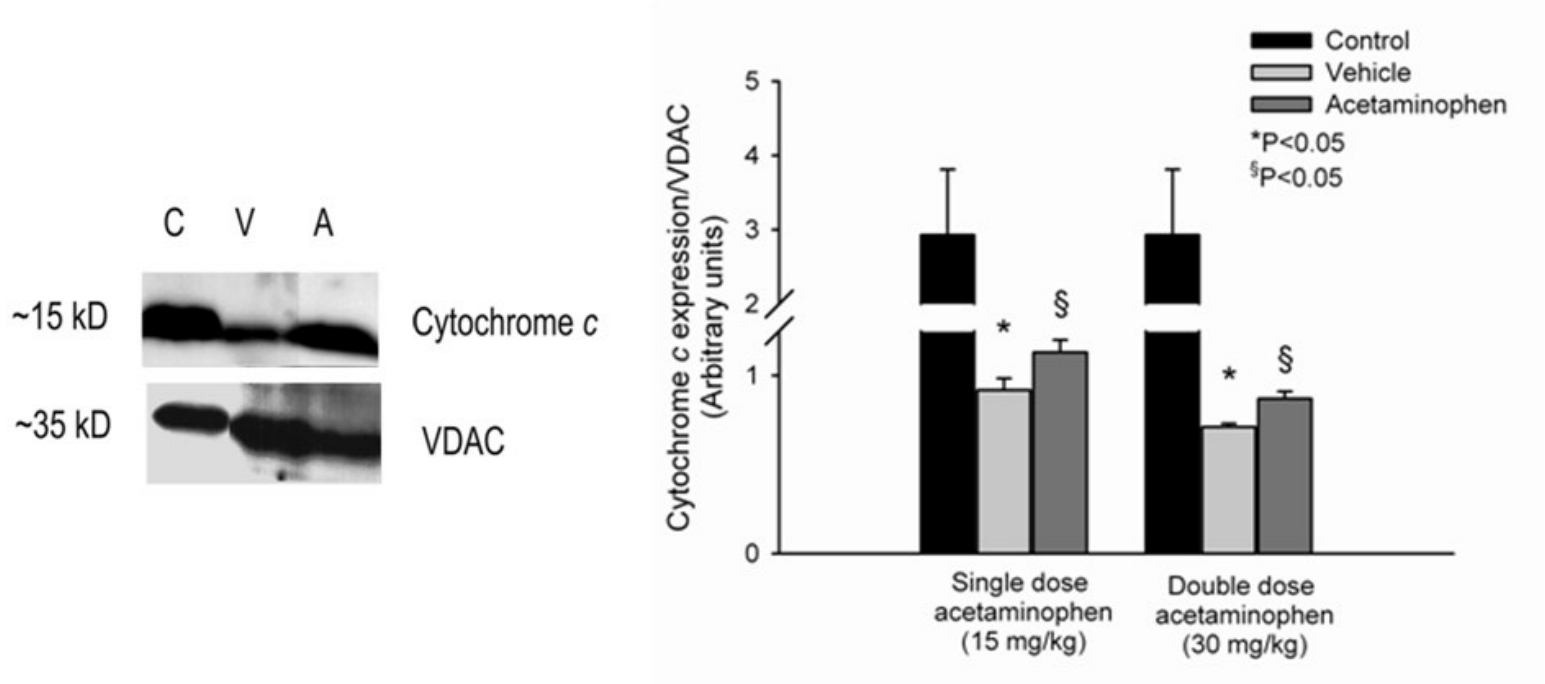
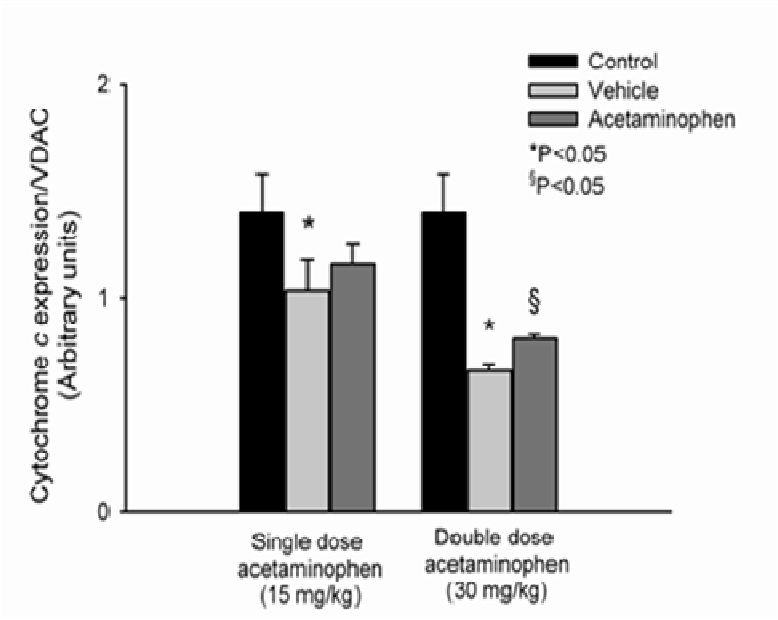


Figure 11. Mitochondrial cytochrome *c* content. Left: Representative Western blots showing a decrease in cytochrome *c* content in vehicle (V) compared to control (C) and acetaminophen (A). Similar trends were observed in hippocampus, left and right cortexes. Right: Quantitative analysis of mitochondrial cytochrome *c* content normalized to voltage-dependent anion channel (VDAC) expression in hippocampal tissue obtained from control, vehicle and acetaminophen (15 mg/kg and 30 mg/kg) groups. *P<0.05 as determined by ANOVA followed by Tukey's post-test compared to control. §P<0.05 determined by ANOVA followed by Tukey's post-test compared to vehicle. Error bars indicate S.E.



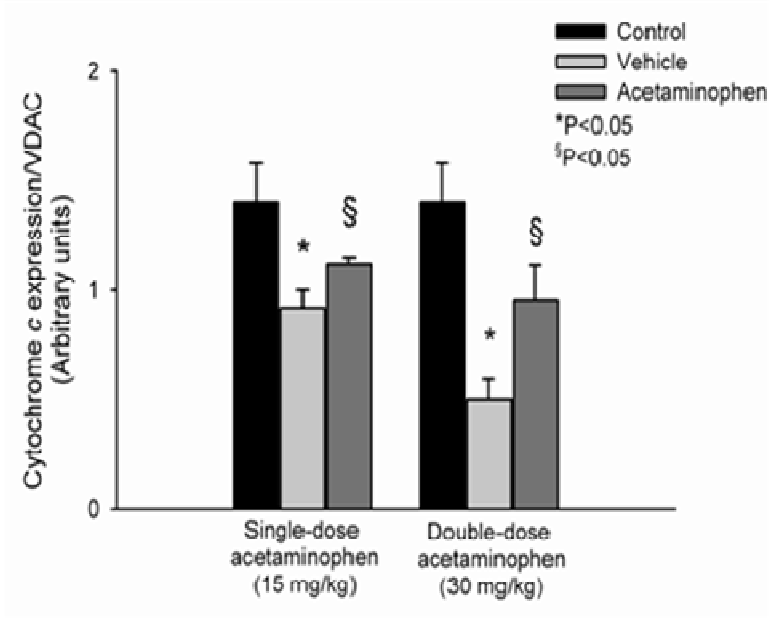


Figure 12. Mitochondrial cytochrome *c* content in the cortexes. Left: Quantitative analysis of cytosolic cytochrome *c* content normalized to voltage-dependent anion channel (VDAC) expression in left cortical tissue obtained from control, vehicle and acetaminophen (15 mg/kg and 30 mg/kg) groups. Right: Quantitative analysis of mitochondrial cytochrome *c* content normalized to voltage-dependent anion channel (VDAC) expression in right cortical tissue obtained from control, vehicle and acetaminophen (15 mg/kg and 30 mg/kg) groups. *P< 0.05 as determined by ANOVA followed by Tukey's post-test compared to control. §P< 0.05 determined by ANOVA followed by Tukey's post-test compared to vehicle. Error bars indicate S.E.

2.7 *Effect of acetaminophen on tissue morphology following stroke*

Sections obtained from the cerebrum of control, acetaminophen-treated (15mg/kg) ischemia-reperfused animals and vehicle rats revealed an increase in edema in vehicle, whereas control and acetaminophen-treated (15 mg/kg) did not exhibit extensive tissue damage (n = 4 in each group, Figure 15). A significant decrease was noted in the cortices and the CA3 hippocampal region between control and vehicle animals, however no significant differences were observed between vehicle and acetaminophen-treated animals in the same areas. Interestingly, no significant differences in cell number were observed between control, vehicle and acetaminophen-treated tissue in the CA1 region of the hippocampus (n= 4 in each group, Figure 16).

2.8 *Acetaminophen and cleaved caspase-9 expression*

Sections obtained from the cerebrum of acetaminophen-treated (15 mg/kg) and corresponding vehicle animals revealed an increase in cleaved caspase-9 expression in vehicle animals compared to control and acetaminophen-treated groups. No observable differences in cleaved caspase-9 expression were noted between acetaminophen-treated (15 mg/kg) and control animals (n = 4 in each group, Figure 15).

2.9 *In-situ detection of DNA fragmentation*

Sections obtained from the cerebrum of control and acetaminophen-treated (15 mg/kg) animals revealed low incidence of DNA fragmentation, whereas corresponding vehicle tissue exhibited high incidence of DNA fragmentation, indicating apoptosis had occurred at a higher rate in vehicle ischemia-reperfused animals compared to control and acetaminophen-treated groups (n = 4 in each group, Figure 15).

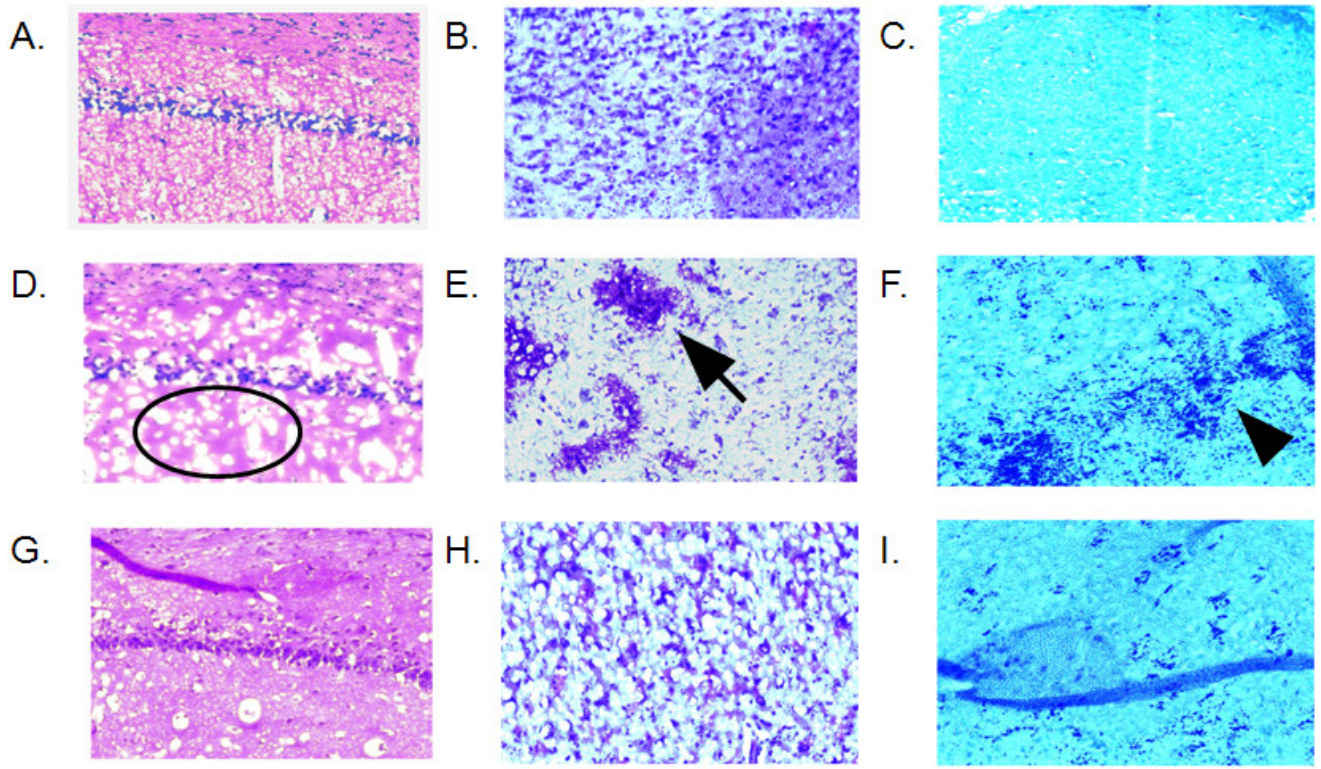


Figure 13. Analysis of tissue morphology. Representative tissue sections (10 µm) stained for morphological changes (H&E, A, D, G), cleaved caspase-9 expression (B, E, H) and in situ detection of DNA fragmentation (C, F, I) from control, acetaminophen-treated (15 mg/kg) and vehicle. Vehicle sections exhibited severe edema (circle, D) in the hippocampus (similar results seen in cortices), increased expression of cleaved caspase-9 (arrow, E) and increased DNA fragmentation (arrowhead, F) compared to control (A, B, C, respectively) and acetaminophen-treated tissue (G, H, I) in the cerebral cortices.

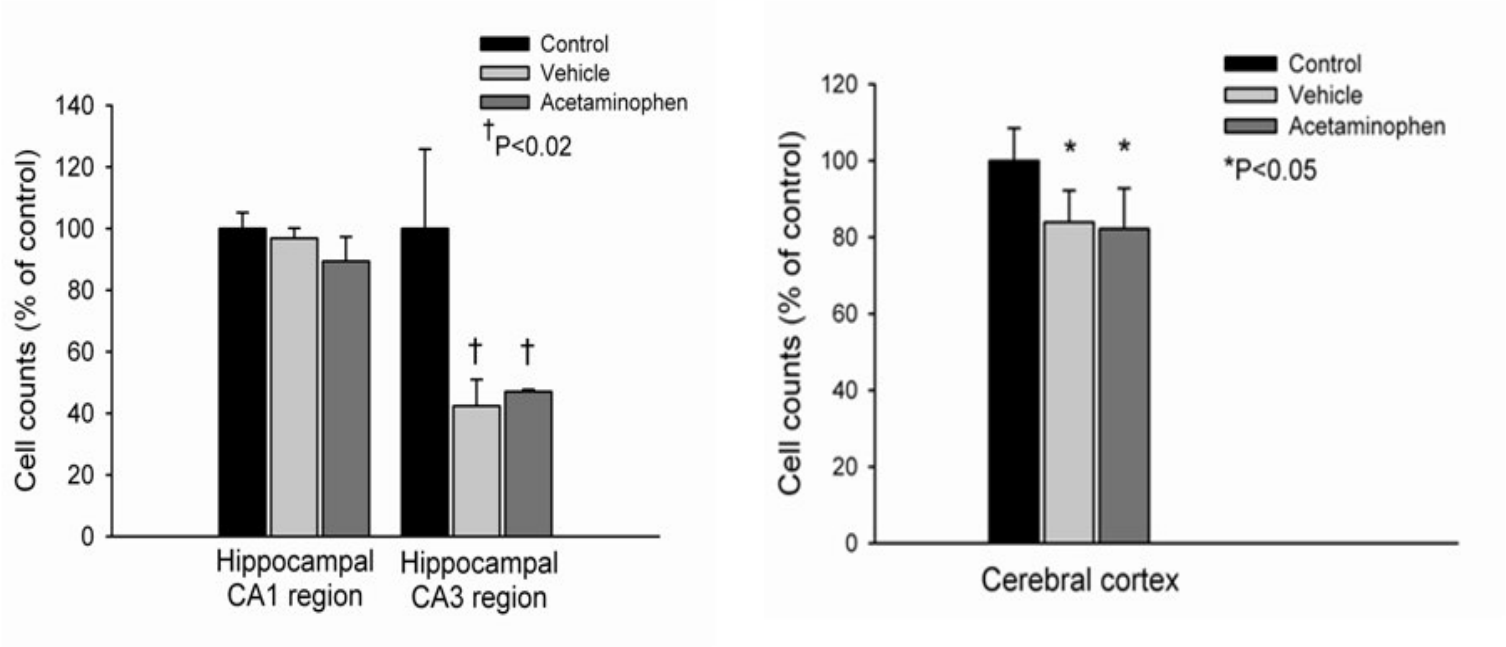


Figure 14. Hippocampal and cortical cell counts. Top: Quantitative analysis of cell counts in hippocampal tissue obtained from control, vehicle and acetaminophen-treated (15 mg/kg) groups. Bottom: Quantitative analysis of cell counts in cortical tissue obtained from control, vehicle and acetaminophen-treated (15 mg/kg) groups. *P<0.05 as determined by ANOVA followed by Tukey's post-test compared to control. Error bars indicate S.E.

CHAPTER 2

INTRODUCTION

1.1 Osteopontin

Osteopontin (OPN) was originally discovered in rat osteosarcoma lines where it was characterized as an extracellular matrix protein due to its binding affinity to the RGD peptide sequence (Oldberg et al., 1986). This binding capability suggested a role in cell migration and adhesion (Yokosaki et al., 2005). OPN is a highly acidic, O-glycosylated phosphoprotein generally secreted by cells, however an intracellular form of the protein exists as well (Zohar et al., 1997).

The molecular weight of OPN can vary from 45 - 75 kDa due to glycosylation and phosphorylation of the serine residues. There is a highly conserved sequence within the structure of OPN (GRDGS) as well as a highly conserved thrombin-cleavage site. Upon thrombin cleavage, a cryptic integrin binding site (SVVYGLR) is exposed. OPN also has a limited number of MMP-3 and MMP-7 cleavage sites, a few of which are located close to the thrombin cleavage site. Cleavage by thrombin or MMPs results in a fragment with an enhanced ability to promote cell adhesion and migration (Kazanecki et al., 2007).

Integrins that bind to the RGD peptide can serve as OPN receptors as the structure of OPN contains an RGD binding site located a few residues away from the thrombin

cleavage site. Some of these integrin receptors include, but are not limited to, $\alpha_V\beta_5$, $\alpha_V\beta_1$ and $\alpha_V\beta_3$, where $\alpha_V\beta_3$ was established as the primary receptor. Cleavage by MMP-3 eliminates OPN binding to $\alpha_V\beta_3$ and $\alpha_V\beta_3$, where both integrin receptors are involved in cell adhesion. An additional receptor for OPN is CD44, a multifunctional family of cell surface adhesion molecules expressed by both normal and malignant cells. CD44 receptors are primarily associated with hyaluronan-mediated adhesion and degradation of hyaluronan. Multiple CD44 isoforms (splice variants) exist, and some of these isoforms have increased ability to promote metastasis more than others. Studies have suggested that CD44 isoforms play a major role in cancer metastasis (Tanabe and Saya, 1994). Since OPN binds to some of these variants, a role for OPN in the promotion of cancer metastasis has been reported (Yang et al., 2008).

The role of OPN following myocardial insults has been investigated with interesting results. Although OPN was found to be upregulated immediately following myocardial ischemia in wildtype (WT) mice, expression returned to baseline levels within days of myocardial injury (Murry et al., 1994; Trueblood et al., 2001). OPN knockout (KO) mice were also evaluated for other indices of cell damage, including apoptotic cell death and collagen content. While cell death rates did not differ between KO and WT mice, collagen content was markedly increased in WT mice, suggesting a role for OPN in matrix remodeling following cardiovascular injury (Schellings et al., 2004; Trueblood et al., 2001; Xie et al., 2003).

Although cerebral ischemia is characterized by leukocyte infiltration and tissue remodeling (Barone et al., 1995; Hallenbeck et al., 1986), the role of OPN following cerebral insults has not been as fully explored as compared to the heart. Thus far, a marked increase in OPN expression was noted following ischemia, with peak levels of OPN appearing 5 d post-ischemia (Wang et al., 1998a). The increase in OPN suggests a role in tissue remodeling following ischemic injury. While most studies correlate the role of OPN with pro-inflammatory properties early on after ischemia, some studies suggest a neuroprotective role for OPN. The neuroprotective ability of OPN is thought to be a direct result of Akt inhibition, where Akt is associated with inhibition of apoptosis (Meller et al., 2005).

The expression of OPN in the early stages of reperfusion following cerebral ischemia has yet to be studied. The results obtained from this study will help shed more light on the role OPN plays in the brain following ischemia-reperfusion-induced injury by comparing the incidence of apoptotic cell death and OPN protein expression following transient global ischemia and reperfusion.

2. PURPOSE

2.1 The effect of stroke and/or acetaminophen on osteopontin expression

The first part of this study investigated the effects of acetaminophen following cerebral ischemia-reperfusion. While many studies have looked at the expression of osteopontin following myocardial ischemia/reperfusion (Murry et al., 1994; Schellings et al., 2004; Trueblood et al., 2001; Xie et al., 2003), few have analyzed the changes in osteopontin expression in the brain (Kang et al., 2008; Meller et al., 2005; Wang et al., 1998a). Thus, we were prompted to investigate the changes in osteopontin expression following cerebral ischemia/reperfusion. Additionally, we sought to determine the effect(s), if any, of acetaminophen on osteopontin expression following transient global ischemia-reperfusion.

MATERIALS AND METHODS

1. EXPERIMENTAL PROTOCOLS

1.1 Western blot

Protein concentration in cytosolic and mitochondrial fractions was determined using Bradford reagent (Bio-Rad, Hercules, CA). Cytosolic proteins (10 µg) were resolved on a 12% SDS-polyacrylamide gel at 100V for 2 h. Protein was then transferred to a PVDF membrane (Immobilon-P, Millipore, Billerica, MA) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 330 mA for 90 m. The membrane was then blocked with 2% BSA for 1 h, and hybridized with primary antibody against OPN (1:2000, mouse monoclonal IgG) for 2 h at room temperature. After four washes with TBS-T (100 mM tris–base, 154 mM NaCl, 0.1% Tween-20, pH 7.5), the membrane was then incubated with horseradish peroxidase conjugated secondary antibodies (1:2000; goat anti-mouse IgG HRP) for 45 m. Membranes were washed four times in TBS-T and then developed on X-ray film using ECL+ method (enhanced chemiluminisence reagents, Amersham Biosciences, Piscataway, NJ). Membranes were then washed twice in stripping buffer (186.5 mM Glycine, 3.5 mM SDS, 0.01% Tween-20, pH 2.2), followed by two 10 m washes in PBS and by two 5 m washes in TBS-T. Membranes were then blocked in 2% BSA for 2 hours and hybridized with primary antibody against β-tubulin (1:1000, rabbit polyclonal IgG, Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature.

After four washes with TBS-T (100 mM tris–base, 154 mM NaCl, 0.1% Tween-20, pH 7.5), the membrane was then incubated with horseradish peroxidase conjugated secondary antibodies (1:2000; goat anti-rabbit, donkey anti-goat IgG HRP) for 45 m at room temperature. Membranes were washed four times in TBS-T and then developed on X-ray film using ECL+ method (enhanced chemiluminisence reagents, Amersham Biosciences, Piscataway, NJ). Experiments were performed at least two times.

RESULTS

1.1 *Acetaminophen and osteopontin expression during stroke*

Osteopontin expression was measured in cytosolic fractions of hippocampus, left cortex and right cortex obtained from control, acetaminophen-treated (15 mg/kg and 30 mg/kg) and vehicle rats. In the hippocampus, a significant increase in OPN expression was seen following acetaminophen-treatment (15 mg/kg and 30 mg/kg) compared to control, whereas a significant increase in OPN expression was only noted in the corresponding double-dose vehicle ischemia-reperfused group (n = 4 in each group, Figure 15). The left cortex and right cortex from acetaminophen-treated (30 mg/kg) and corresponding vehicle ischemia-reperfused animals were similar in that both saw significant increases in OPN expression compared to control. A significant decrease in OPN expression was observed in the acetaminophen-treated (30 mg/kg,) ischemia-reperfusion group compared to corresponding vehicle (n = 4 in each group, Figure 16). The left cortex from acetaminophen-treated (15 mg/kg,) ischemia-reperfused animals did not exhibit significant changes in OPN expression compared to control, however a significant increase in OPN expression was noted in corresponding vehicle ischemia-reperfused animals. In the right cortex, acetaminophen-treatment (15 mg/kg) significantly lowered OPN expression, however no changes in OPN expression were noted in corresponding vehicle tissue (n = 4 in each group, Figure 16).

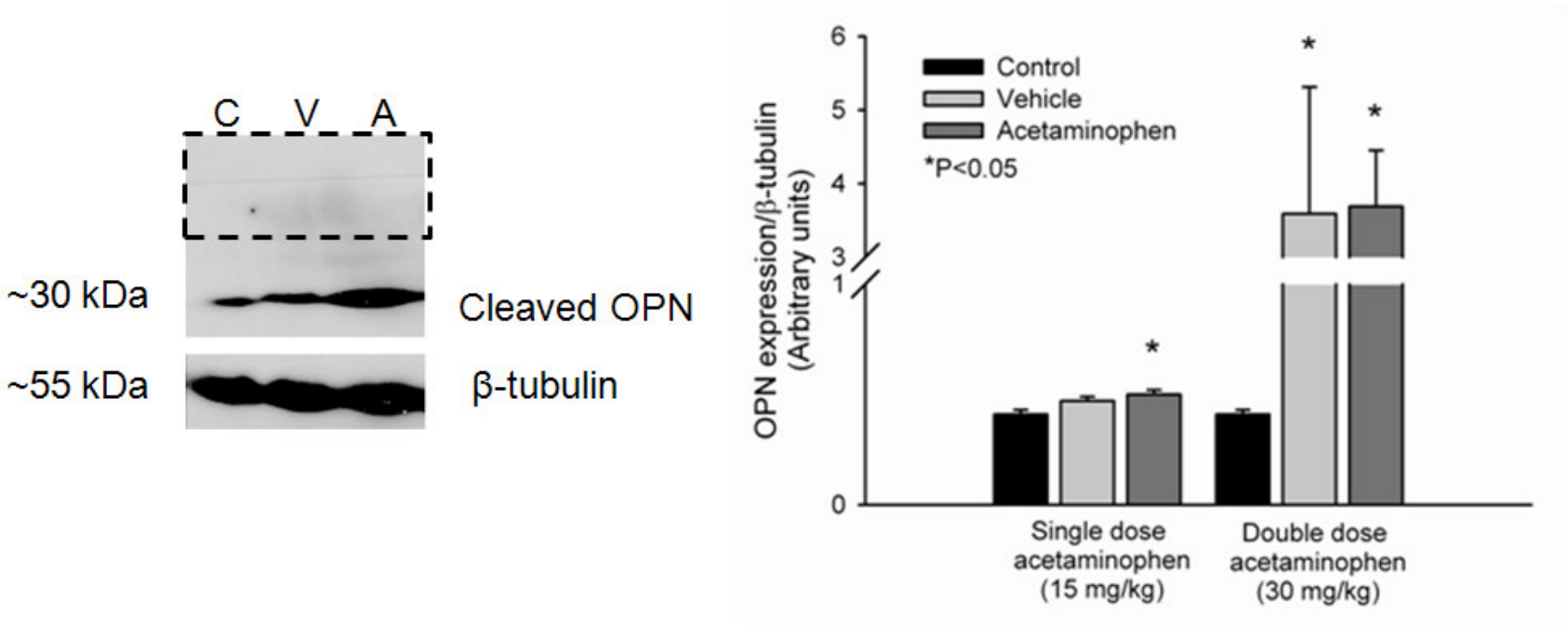


Figure 15. OPN expression in the hippocampus. Left: Representative Western blots showing increased OPN cleavage in acetaminophen (A) compared to control (C) and vehicle (V) following treatment with 15 mg/kg of acetaminophen. Box indicates lack of detection of full-length OPN. Right: Quantitative analysis of OPN cleavage normalized to β-tubulin expression. *P<0.05 as determined by ANOVA followed by Tukey's post-test compared to control. Error bars indicate S.E.

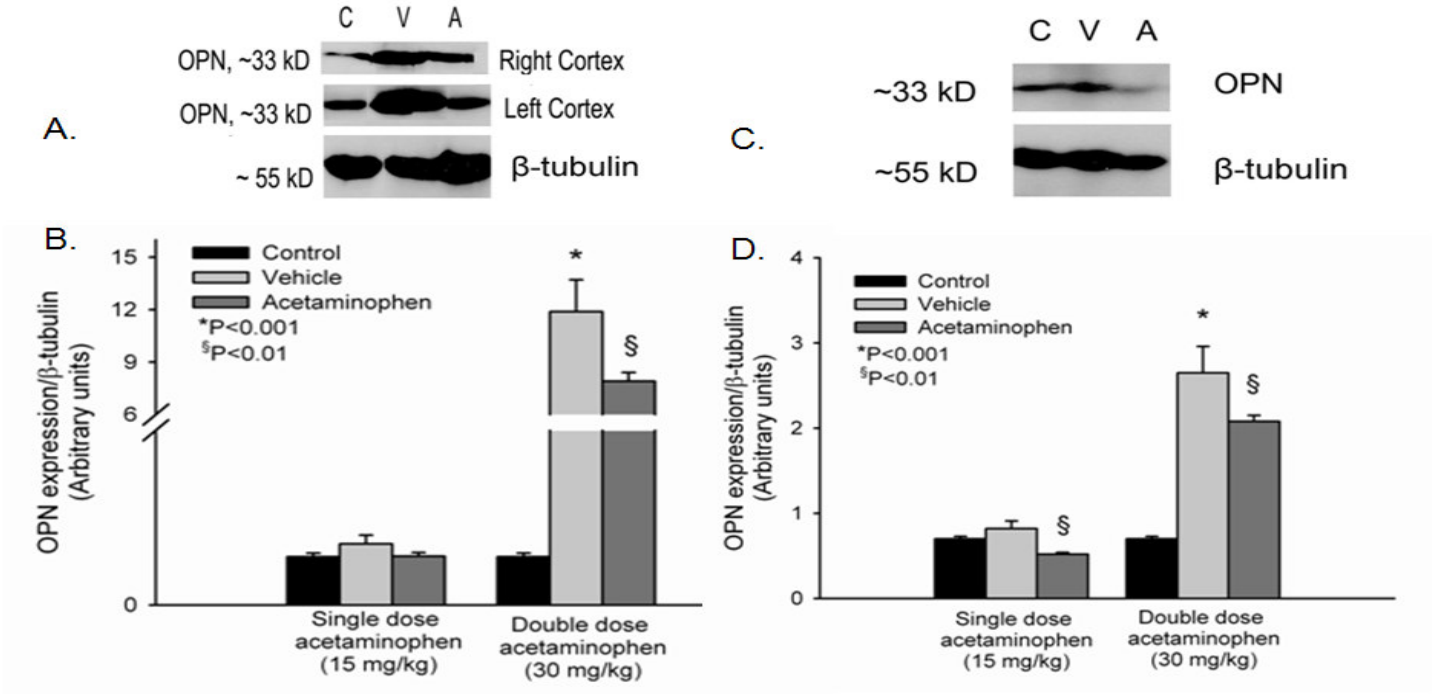


Figure 16. OPN expression in the cortexes. A. Representative Western blots from control (C), vehicle (V) and acetaminophen (A)-treated brains showing an increase in OPN expression with 30 mg/kg acetaminophen. B. Quantitative analysis of OPN expression normalized to β-tubulin expression in the left cortex following treatment with 15mg/kg and 30 mg/kg of acetaminophen. C. Representative Western blots from control (C), vehicle (V) and acetaminophen (A) showing a significant decrease in OPN expression in the right cortex. D. Quantitative analysis of OPN expression normalized to β-tubulin expression in the right cortex following treatment with 15 mg/kg and 30 mg/kg of acetaminophen. *P<0.05 determined by ANOVA followed by Tukey's post-test compared to control. §P<0.05 determined by ANOVA followed by Tukey's post-test compared to vehicle. Error bars indicate S.E.

DISCUSSION

Each year about 795,000 people experience a new or recurrent stroke. About 610,000 of these are first attacks, and 200,000 are recurrent attacks. Blockage of a cerebral artery results in interruption of the blood flow and supply of nutrients, glucose and oxygen to the brain. Blood flow levels are important in determining infarct size by supplying the conditions essential for maintenance of cellular energy hemostasis. Decreased blood flow leads to a reduction in phosphocreatinine and ATP, resulting in severe impairment of cellular function by disruption of ATP-dependent processes. Since neurons do not have the ability to regenerate on a mass scale, neuronal cell death can result in deleterious consequences for a victim of stroke with crucial motor and speech abilities unable to be fully recovered (Barone and Feuerstein, 1999). The mitochondria have been identified as targets of cytoprotection for several disease states, mainly due to the high susceptibility of the organelle to cellular insults. In the brain, the mitochondria are especially vulnerable due to the high metabolic activity of the brain that plays a key role in producing significant levels of cell-damaging oxidants (Szeto, 2006a; Szeto, 2006b).

Research on the use of acetaminophen as a therapeutic has recently been extended to neuropathological settings. The first study reporting on the potential neuroprotective ability of acetaminophen suggested that the drug was capable of significantly reducing mitochondrial damage and oxidative stress sustained in a cellular model of Alzheimer's disease. Cells incubated with amyloid-β peptide aggregates known to trigger mitochondrial dysfunction or H_2O_2 – a participant in the Fenton reaction that produces

oxidants) had significant reductions in apoptotis, mitochondrial dysfunction and lipid peroxidation levels (Bisaglia et al., 2002). Additionally, acetaminophen was successful in preventing neuronal loss due to glutamate toxicity (Casper et al., 2000b). The authors also reported a significant reduction in glioma cell growth - a highly lethal cancer primarily affecting the brain - by acetaminophen (Casper et al., 2000a). These results were further corroborated when low concentrations of acetaminophen (0.1mM) were effective in significantly reducing dopaminergic (DA) neuronal loss following a DA stress assay in C. elegans (Locke et al., 2008). Thus far, acetaminophen shows promise as a potential neuroprotective agent for stroke. Here we show, for the first time, that acetaminophen prevents mitochondrial dysfunction and confers neuroprotection to the brain following transient global cerebral ischemia-reperfusion.

Effectiveness of surgical procedure in inducing cerebral tissue damage

The bilateral carotid artery occlusion model was first introduced in the 1970s as a successful method for simulating stroke in experimental models. This model of forebrain ischemia was developed 25 years ago (Eklof and Siesjo, 1972a; Eklof and Siesjo, 1972b) and was initially used to characterize cerebral energy state following incomplete ischemia (Eklof and Siesjo, 1972a) (Nordstrom and Siesjo, 1978). The procedure described the clamping of the carotid arteries combined with systemic hypotension maintained during the length of the ischemic period. Measurements of CBF that the model is effective for producing severe ischemic conditions (Smith et al., 1984b). To support these conclusions, other studies have shown that cognitive deficits have been found to be associated with

damage in the CA1 region of the hippocampus in studies using the bilateral common carotid artery occlusion model. Furthermore, a severe state of ischemia is produced with the abrupt reduction of cerebral blood flow caused by the simultaneous occlusion of both common carotid arteries (Sarti et al., 2002).The major advantages of the 2VOHYP model over the 4-VO model in producing forebrain ischemia are that the 2VOHYP model requires a more simple surgical preparation and that reperfusion can be readily accomplished. Furthermore, the damage experienced by animals subjected to 2VOHYP is more pronounced than animals subjected to 4VO within a short relatively short period of time (15 – 25 s) (Lipton, 1999).

The mitochondria are the primary site of cellular respiration, participating in oxidative phosphorylation that produces the majority of ATP for the cell. Without a doubt, they are important organelles within the cell. During in vitro cellular respiration, the mitochondria convert 1 – 2% of supplied oxygen into oxidants (Finkel and Holbrook, 2000). When oxidants are generated under certain stress conditions, some of them act as signaling molecules by regulating cellular metabolism (Nemoto et al., 2000; Nishikawa et al., 2000). However, during post-ischemia reperfusion periods, the generation of oxidants can threaten cell survival. Reperfusion inundates the mitochondria with oxygen, causing them to undergo higher rates of metabolism in an effort to restore ischemia-mediated ATP depletion. The sheer number of oxidants produced as a result of the high metabolic rate of the mitochondria overwhelms endogenous antioxidants. Under these conditions, oxidants are free to inflict damage on the cell by binding to DNA and proteins, inhibiting their functions (Chan, 1996). Oxidants can attack lipids as well since Sprague-Dawley

rats subjected to physiological stress had significantly higher levels of lipid peroxidation in the hippocampus and cerebral cortex (Liu et al., 1996). Oxidants produced upon reperfusion bind to DNA bases and induce “lesions” that can be repaired via base excision and other DNA repair mechanisms (Ames et al., 1993; Marnett, 2000) unless oxidant activity exceeds the ability of the repair mechanisms.

Oxidant damage can trigger several pathways culminating in cell death. Most importantly, the oxidants produced by the mitochondria attack the organelle by disrupting mitochondrial dynamics. Additionally, the accumulation of Ca^{2+} during post-ischemia reperfusion triggers the opening of the mitochondrial permeability transition pore, a tightly regulated pore located in the inner membrane of the mitochondria. The pore is Once MPTP activation occurs, cytochrome c is released, oxidative phosphorylation is inhibited and eventually the mitochondrion ruptures due to the influx of solutes. While the inner mitochondrial membrane can accommodate modest increases in mitochondrial volume due to extensive folding, the outer membrane is not as resistant and will rupture once osmotic swelling occurs (Matsumoto et al., 1999). Mitochondrial rupture accelerates the release of cytochrome *c*, which binds to cytosolic components eventually activating apoptotic caspases (Chan, 2001).

Effect of acetaminophen on cerebral injury due to mitochondrial dysfunction

2,3,5 triphenyl tetrazolium chloride (TTC) is often used to verify or detect the extent of cerebral infarct following cerebral ischemia-reperfusion. The principle underlying the

mechanism of TTC is based on the number and activity of mitochondrial enzymes in the tissue (Goldlust et al., 1996). The dye has also been used to effectively evaluate infarct area in myocardial studies (Vanhaecke et al., 1986). Our data supports the efficacy of the 2VOHYP procedure in inducing significant cerebral tissue damage as the colorless regions denoting infarct were present in vehicle-treated rats, compared to control, where infarct regions were negligible. Although a significant reduction in infarct size was noted in acetaminophen-treated rats following ischemia-reperfusion, acetaminophen-treatment following ischemia did not result in a significant decrease in infarct region (Figure 6). This can be attributed to the different mechanisms underlying ischemia-induced damage compared to reperfusion-mediated damage. Ischemic cell death is characterized by a loss of cell function directly resulting from inhibition of oxidative phosphorylation that will significantly reduce available ATP. Lack of ATP production halts many ATP-dependent cellular processes, thus pushing cell death mechanisms into play. Additionally, protein synthesis is severely inhibited during ischemia (Raley-Susman and Lipton, 1990). Inhibition of proteins synthesis has been attributed to the accumulation of cytosolic Ca^{2+} content, free radical production, and depletion of endoplasmic reticulum (ER) Ca^{2+} content. Although our results suggest that acetaminophen might be protective following ischemia, the trend did not prove to be significant, suggesting that acetaminophen might not be effective against all the cell death pathways responsible for initiating programmed cell death.

Histopathological assessment of cell death

A significant decrease in cell count was observed in vehicle (group vehicle-ischemia-reperfusion) tissue compared to control, indicating that our surgical procedure was effective in inducing stroke damage. Interestingly, no significant differences were found between vehicle and acetaminophen-treated samples (15 mg/kg). This can be attributed to the contribution of both apoptosis and necrosis occurring in the brain during cerebral ischemia/reperfusion, where both types of cell death have been shown to equally contribute to overall cell death (Nakka et al., 2008). Also, an increase in edema was observed in our vehicle samples compared to control and acetaminophen-treated (15 mg/kg) animals further corroborating the efficacy of surgical procedure. Histological assessment of edema has been associated with oxidants produced at the onset of reperfusion (Chan and Yu, 2000; Matsuo et al., 1996). Cell death cannot be attributed to apoptosis or necrosis using H&E staining; therefore we assayed alternate slides for DNA fragmentation occurring as a result of apoptosis. The apoptotic kit labeled 3'-OH DNA ends, specific to apoptosis (Li et al., 1995). A reduction in apoptotic cell death was seen with acetaminophen treatment (15 mg/kg), similar to control, whereas an observable increase in apoptotic cell death was seen in vehicle tissues, suggesting that apoptotic cell death was prevented by acetaminophen treatment.

Effect of acetaminophen on mitochondrial structure and function

Mitochondrial morphometry, an analysis tool used to assess mitochondrial damage (Hirai et al., 2001), was used to compare the extent of damage to hippocampal tissue in control, vehicle and acetaminophen-treated rats. One visible feature of mitochondrial dysfunction is swelling. The swollen phenotype can be readily distinguished from surrounding, undamaged mitochondria by their increased size and lack of cristae folding. The inner membrane of the mitochondria is extensively folded into cristae and becomes more permeable to solutes 1500 kDa and above as the cell sustains longer periods of damage. Mitochondrial area per sample was measured to obtain a quantitative aspect of swelling. A significant increase in mitochondrial area was noted in vehicle brains, with a corresponding decrease in acetaminophen-treated brains following ischemia-reperfusion (Figure 9). Mitochondria obtained from the acetaminophen-treated rats had conserved cristae folding and were similar in morphology to those obtained from group sham-control. While these results show that acetaminophen protects mitochondrial structure in hippocampal tissue, the area being quantified is a fraction of the area that was subject to transient global ischemia. We also assayed the cortices from control, vehicle and acetaminophen-treated (15 mg/kg) animals and found a significant decrease in light absorbance in vehicle mitochondria compared to control and acetaminophen-treated (15 mg/kg) samples. In theory, the mitochondria from the double-dose acetaminophen-treated (30 mg/kg) ischemia-reperfused animals should exhibit higher light absorbance values compared to single-dose acetaminophen-treated ischemia-reperfused mitochondria. However, no significant differences were observed between these groups.

Spectrophotometrical analysis of mitochondrial light absorbance is not a definitive test, and should be coupled with more specific assays. The spectrophotometrical analysis provided a means of reference as to the degree of damage (swelling) experienced by one group compared to another. The only conclusion that can be taken from the spectrophotometrical analysis is that acetaminophen prevents mitochondrial damage compared to vehicle. In order to corroborate and expand on these results, cortices from all three groups were treated with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) and analyzed using flow activated cell sorting (FACS). JC-1 is an effective dye to use in comparing the mitochondrial membrane potential between groups since the size, density, and shape of mitochondria do not affect the activity of JC-1. Dye molecules easily penetrate the mitochondria and form aggregates that fluoresce red/orange when the membrane potential is high. A significant reduction in membrane potential will cause the dye molecules to stay in monomeric form and fluoresce green. A ratio of red over green fluorescence can then indicate the state of mitochondrial function in a sample. Our results revealed a significant drop in mitochondrial membrane potential in vehicle rats compared to sham-controls. Acetaminophen treatment (15 mg/kg) appeared to preserve mitochondrial membrane potential as the values in this group were significantly higher than the corresponding vehicle. A decrease in mitochondrial membrane potential is accompanied by loss of MPTP (Kroemer and Reed, 2000). Our data suggest that loss of MPTP is prevented to some degree by acetaminophen treatment.

Effect of acetaminophen on mitochondrial release of cytochrome c

The cytochrome *c* from somatic cells is a water-soluble 13 kDa heme-containing protein that is encoded by a nuclear gene. It normally resides in the spaces within the cristae of the inner mitochondrial membrane and is effectively sequestered by narrow cristae junctions. Within the inner mitochondrial membrane, cytochrome *c* participates in the mitochrondrial electron-transport chain as a redox intermediate to shuttle electrons between Complex III and Complex IV. However, when the cell detects an apoptotic stimulus, such as DNA damage, metabolic stress or the presence of unfolded proteins, the intrinsic apoptotic pathway is triggered and mitochondrial cytochrome *c* is released into the cytosol. In the cytosol, cytochrome *c* engages the apoptotic protease-activating factor-1 (APAF1), and forms the apoptosome (Ow et al., 2008). Western blot analysis of mitochondrial cytochrome *c* content was significantly higher in control and acetaminophen-treated (15 mg/kg and 30 mg/kg), with a significant reduction in cytochrome *c* content in corresponding vehicle brains. It would stand to reason that cytosolic cytochrome *c* content would resemble mitochondrial data in that a significant reduction of cytochrome *c* release would occur with acetaminophen treatment (15 mg/kg and 30 mg/kg) compared to corresponding vehicle. Surprisingly, there was no significant difference in the cytosolic cytochrome *c* content between these two groups. A plausible explanation for this discrepancy could be attributed to the types of cell death experienced by brain tissue following global ischemia reperfusion. Rats that were subjected to 2 – 10 min of transient global ischemia (2VOHYP procedure) exhibited necrosis in the neocortex after just 4 min of ischemia. The extent of necrosis is directly related to the

duration of ischemia, where longer periods of global ischemia result in higher numbers of cells undergoing necrosis (Smith et al., 1984a). Necrotic cell death is characterized by inflammation, stimulating cell death in surrounding cells. Recently, the role of apoptosis as a cell death modulator has been explored, where DNA fragmentation indicative of apoptosis was found to occur after 10 min of ischemia (Li et al., 1995). As with necrotic cell death, a similar trend of apoptotic cell death and ischemic duration has been observed (Schumer et al., 1992). Based on these studies, our ischemic period of 15 min was sufficient in inducing apoptotic cell death; however necrosis could have occurred at a higher rate than apoptosis, thus resulting in a significant increase in cytochrome *c* release into the cytosol. The most promising aspect of our study is that acetaminophen treatment protected the mitochondria during the early stages of reperfusion, when the oxidant-mediated damage is the worst (Chan, 1996).

Effect of a higher dose of acetaminophen on mitochondrial release of cytochrome c

Our results above suggest that a low dose of acetaminophen (15 mg/kg) was sufficient in conferring neuroprotection by preventing mitochondrial release of cytochrome *c* in the right cortex. We increased the dosage of acetaminophen to 30 mg/kg to verify that the neuroprotection exhibited by acetaminophen was not due to endogenous cellular responses and to determine whether a higher dose of acetaminophen would exhibit neuroprotection in the left cortex. Interestingly, we found that there was an appreciable increase in the mitochondrial cytochrome *c* content in the right and left cortex of rats

treated with 30 mg/kg of acetaminophen. These data strongly suggest that acetaminophen confers neuroprotection following cerebral ischemia-reperfusion.

Interestingly, a marked increase in cerebral tissue damage was observed in rats subjected to the double-dose treatment with acetaminophen. Two 15 mg/kg doses were delivered to the animal at different time points. One dose was administered prior to ischemia and the second dose was administered prior to the onset of reperfusion. This procedure was employed to determine whether a higher dose of acetaminophen would increase the neuroprotection observed in the single-dose group (15 mg/kg). We proposed that since the onset of reperfusion produces significant amounts of damage (Chan, 1996), acetaminophen administered immediately before the onset of reperfusion would be more effective in reducing cerebral tissue injury. The subsequent increase in cell damage might be explained by the increase in volume experienced by the blood vessels during reperfusion. With the addition of the bolus of acetaminophen, the rats were re-infused with shed blood (taken prior to induction of ischemia to induce hypotension). It is conceivable that the increase in volume due to the bolus of acetaminophen or vehicle would have increased the supply of blood to formerly ischemic tissue, thus increasing the oxygen available to affected cerebral vasculature (Buxton and Frank, 1997).

Effect of acetaminophen on osteopontin expression

Control, vehicle ischemia-reperfused and acetaminophen-treated (15 mg/kg and 30 mg/kg) ischemia-reperfused hippocampal and cortical tissue was assayed for OPN

expression using the monoclonal antibody 2A1. The target sequence for this antibody is located toward the C-terminus of the protein. Additionally, the target site is on the C-terminal end of the thrombin cleavage site. In theory, the antibody would reveal the extent of thrombin cleavage of OPN as well as the relative levels of full-length OPN. Interestingly, our Western blots only revealed one band with the approximate molecular weight of 33 kDa, with no bands of a higher molecular weight. Once thrombin cleavage of OPN takes place, a cryptic integrin binding site is revealed, which might explain the absence of a higher molecular weight band. Marginal increases in OPN expression were seen in the hippocampus in acetaminophen-treated (15 mg/kg) and vehicle compared to control. No significant difference in OPN expression was seen in the left cortex in acetaminophen-treated (15 mg/kg) and corresponding vehicle compared to control. The right cortex exhibited no significant changes in OPN expression between control and vehicle; however acetaminophen-treatment (15 mg/kg) had significantly lower OPN expression, although further study is required to determine the extent of the effect of acetaminophen on OPN expression. Considerable changes in OPN expression were noted in the double-dose acetaminophen-treated (30 mg/kg) groups and the corresponding vehicle ischemia-reperfused group. In the hippocampus, left and right cortices, OPN expression was significantly higher following stroke damage. Interestingly, the left cortex exhibited a significant increase in OPN expression compared to the right cortex. This can be attributed to the biochemical differences between the two hemispheres, where the left hemisphere is considered to be more vulnerable to cerebral insults compared to the right hemisphere (Robinson, 1979; Robinson and Coyle, 1980). A concomitant decrease in OPN expression with acetaminophen treatment (30 mg/kg) was observed in both cortices.

A significant increase in OPN receptor expression following cerebral ischemia-reperfusion has been reported in the rat hippocampus and other areas of the brain (Choi et al., 2007). The two most studied receptors for OPN are CD44 and integrin, in line with the cytokine and extracellular matrix activities of OPN (Wang and Denhardt, 2008). Expression of CD44 was found to be higher in astroglia and neurons that sustained significant damage, where OPN expression peaked 3 days post-ischemia and returned to basal levels 7 days post-ischemia (Kang et al., 2008). The function of OPN has been linked to tissue remodeling in the brain following cerebral ischemia reperfusion (Wang et al., 1998a). Although OPN offers no immediate benefits to neurons and astroglia, it may lead to remodeling in an attempt to recover full neuronal function.

OPN expression was significantly reduced in acetaminophen treated groups (30 mg/kg) in the cortices, however no significant reduction was observed in the hippocampus of acetaminophen-treated (30 mg/kg) compared to corresponding vehicle tissue. *In situ* detection of DNA fragmentation revealed an observable decrease in cortical apoptotic cell death in the acetaminophen-treated group (15 mg/kg). Based on these results, our data might suggest a correlation between OPN expression and apoptosis in the cortex. A few forms of cancer, associated with apoptosis deregulation, have shown increased OPN expression, where OPN stimulated pro-survival factors. Here, we have shown that OPN expression is directly correlated with incidence of cell death, and a decrease in seen in acetaminophen-treated groups (15 mg/kg and 30 mg/kg), suggesting that in the brain, OPN protein expression is governed to a certain degree by pro-apoptotic signals.

Alternatively, acetaminophen - by preventing the incidence of apoptosis- could have inhibited upregulation of OPN translation.

Further study on OPN expression in these regions of the brain need to be determined using an antibody that will target the N-terminal region of the protein. This will help further clarify the action of OPN in the cell following transient global ischemia-reperfusion as well as corroborating our results above that show an increased level of thrombin-mediated OPN cleavage in vehicle tissue.

Proposed model

Based on our data as well as published reports on the actions of acetaminophen in the brain, we propose a model where acetaminophen prevents the opening of the mitochondrial pores by upregulating the bcl-2 protein. This main function of this protein is to prevent the action of other proteins that promote the opening of the pore, thus facilitating the release of the pro-apoptotic compound, cytochrome *c*. Our data strongly suggests that acetaminophen prevents the release of cytochrome *c*, whereas another study has shown a significant upregulation of bcl-2 (Tripathy and Grammas, 2009).

Conclusions

This study shows for the first time, the efficacy of acetaminophen in conferring neuroprotection following transient global cerebral ischemia-reperfusion. Although

acetaminophen did not show significant recovery of mitochondrial function following ischemia, the drug was able to prevent mitochondrial damage during post-ischemia reperfusion. Mitochondrial release of cytochrome *c* was markedly reduced with treatment of 15 mg/kg in the hippocampus and right cortex. Treatment with 30 mg/kg of acetaminophen resulted in the prevention of cytochrome *c* release in hippocampus, left and right cortex, suggesting that a high dose of acetaminophen is sufficient in overcoming damage experienced by the left cortex. The left cortex has been reported to be especially sensitive to cerebral insults. This statement is corroborated by our data showing a substantial increase in OPN expression in the left cortex compared to the right cortex and hippocampus. An increase in OPN expression is regarded as a marker of cell damage, although studies have shown that increases in OPN expression can lead to tissue remodeling in the brain (Ellison et al., 1999). Surprisingly, we found that OPN expression was significantly diminished by acetaminophen treatment (15 mg/kg) in the right cortex of animals subjected to ischemia-reperfusion, however further study is required. In vehicle and acetaminophen-treated (15 mg/kg) ischemia-reperfused animals, a significant increase in OPN expression was observed in the hippocampus compared to control. A dose of 30 mg/kg of acetaminophen exhibited more pronounced OPN expression in the hippocampus, although significant reductions of OPN expression were observed in the cortices. These data suggest that OPN expression might be differentially regulated in certain regions of the brain. It is plausible that an increase in OPN expression in the hippocampus might occur to promote tissue remodeling in an effort to recover lost cerebral activity.

Our data do not take into consideration the response of the vasculature to cerebral ischemia/reperfusion and, possibly, the effects of acetaminophen. Since ischemia and reperfusion are both dependent on blood volume, it is reasonable to assume that the cerebral and surrounding vasculature could have had an effect on the cells, thus producing the results we obtained. One study reported that acetaminophen significantly reduces coronary vasodilation induced by arachidonic acid and prostaglandins, suggesting that acetaminophen might act as a vasoconstrictor (Shaffer et al., 1981). Another study reported on the diminished response of cerebral arterioles to nitric oxide, a potent vasodilator, following cerebral ischemia (Cipolla and Bullinger, 2008). The altered behavior of cerebral blood vessels could have a significant impact on the response of the brain to cerebral ischemia/reperfusion, and acetaminophen could have acted upon the vasculature as well. These findings need to be further corroborated in future studies.

Future directions

The data obtained in this study suggest an anti-apoptotic role for acetaminophen as mitochondrial activity was preserved. Future studies with acetaminophen might involve a longer reperfusion period to determine whether the protection exhibited during the early period of reperfusion can be extended to hours following ischemic injury. A study where acetaminophen conferred neuroprotection following hours, even weeks, after ischemia would be most beneficial to clinical studies and would improve the chances of a translational study being successful. Most compounds deemed neuroprotective in rodent models of stroke rarely make a successful transition to clinical applications. However, acetaminophen has been clinically evaluated in different settings, where the drug was

reported to reduce headache and induce hypothermia, suggesting a role as a neuroprotectant (den Hertog et al., 2007; Dippel et al., 2003; van Breda et al., 2005). These studies hold promise that acetaminophen, if effective after long periods of reperfusion, might be successful in clinical trials either by inducing hypothermia or by mediating mitochondrial damage.

The role of acetaminophen in affecting OPN expression can be further studied by using OPN knockout animals. This experiment will further elucidate the role of OPN in stroke as a pro-inflammatory cytokine or a pro-survival factor responsible for tissue remodeling in the brain. Changes in MMP-2 and 9 expression, upregulated during cerebral ischemia-reperfusion (Liu et al., 2009; Yang et al., 2007), should also be assayed in this study as OPN can induce MMP activity (Kohan et al., 2009).

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PUBLICATIONS

1. **Baliga SS**, Jaques-Robinson KM, Hadzimichalis, NM, Golfetti R, Merrill GF. Acetaminophen is neuroprotective in cerebral ischemia-reperfusion. (In review).

2. Jaques-Robinson KM, Golfetti R, **Baliga SS**, Hadzimichalis NM, Merrill GF. Acetaminophen is cardioprotective against H2O2-induced injury in vivo. Experimental Biology and Medicine (Maywood), 233(10):1315-22, November 2008.

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