# Effect of Protein-Energy Malnutrition on Nuclear Factor Kappa B Activation Following Global Ischemia

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## ABSTRACT

Our laboratory previously found that protein-energy malnutrition (**PEM**) existing prior to brain ischemia impaired functional outcome measured in an open field test, and one-third of animals showed a marked increase in reactive gliosis. It was hypothesized that PEM worsened stroke outcome by increasing inflammation via increased activation of the transcription factor, nuclear factor kappa B (**NFκB**). Mongolian gerbils (11-12 wk old) were randomly assigned to a control diet (12.5% protein) or a protein-deficient diet (2%) for 28 days. The control group on average gained 4.9g and the PEM group lost 7.4g. PEM gerbils had significantly decreased food intake ($P<0.001$; unpaired *t*-test). Animals were then subjected to global ischemia or sham surgery, resulting in four experimental groups. Global ischemia was achieved by a 5 min bilateral common carotid artery occlusion with tympanic temperature regulated at 36.5 ± 0.2°C. PEM independently increased hippocampal NFκB activation by three times higher than control diet animals at 6hr after surgery ($p=0.014$; 2-factor ANOVA) detected by electrophoretic mobility shift assay (**EMSA**). There was no significant effect of ischemia on NFκB activation and there was no interaction of diet and ischemia. Serum glucose and serum cortisol were also measured since both variables can be affected by PEM and can influence stroke outcome, but there was no significant effect of diet or ischemia. Because of the increased NFκB activation observed in PEM-Sham animals, a second experiment investigated if PEM also increased NFκB activation in the absence of surgery. Gerbils of the same age were randomly assigned to either control diet or PEM for 28 days but did not receive any surgery. PEM consistently increased NFκB activation. Since PEM exists in 16% of elderly stroke patients at admission, the data suggest that PEM may worsen stroke

outcome through increased activation of NFκB. Because increased NFκB activation was also observed in PEM independent of ischemia, the data also have implications for the inflammatory response of protein-energy malnourished elderly in general.

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# DEDICATION

**This Thesis Is Dedicated to**

**My Grandparents (Ji, Peikun and Yu, Shuiying)**

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | analysis of variance |
| BBB | blood-brain barrier |
| BCA | Bicinchoninic acid |
| BCAO | bilateral common carotid artery occlusion |
| CA1 | cornu-ammonus |
| CNS | central nervous system |
| COX-2 | cyclooxygenase-2 |
| DNA | deoxyribonucleic acid |
| EMSA | electrophoretic mobility shift assay |
| FOOD trial | Feed Or Ordinary Diet trial |
| GC | glucocorticoid |
| GR | glucocorticoid receptor |
| GPx | glutathione peroxidase |
| GSH | glutathione |
| ICAM-1 | intracellular adhesion molecule-1 |
| IFN-γ | Interferon gamma |
| IL-1α | interleukin-1α |
| IL-1β | interleukin-1β |
| IL-6 | interleukin-6 |
| IL-10 | interleukin-10 |
| iNOS | inducible nitric oxide synthase |
| IKK | IκB kinase |

| | |
|---|---|
| IκB | inhibitory component kappa B |
| LPS | lipopolysaccharide |
| MCAO | middle cerebral artery occlusion |
| MDA | malondialdehyde |
| MMPs | matrix metalloproteinase family enzymes |
| MnSOD | manganese superoxide dismutase |
| NADH | Nicotinamide adenine dinucleotide (reduced form) |
| NADPH | nicotinamide adenine dinucleotide phosphate (reduced form) |
| NFκB | nuclear factor kappa B |
| $NO^{\bullet}$ | nitric oxide radical |
| $ONOO^{-}$ | Peroxynitrite |
| OTC | L-2-oxothiazolidine-4-carboxylic acid |
| PEM | protein-energy malnutrition |
| PMN | polymorphonuclear neutrophil |
| RNS | reactive nitrogen species |
| ROS | reactive oxygen species |
| SAA | sulphur amino acid |
| SD | standard deviation |
| SOD | superoxide dismutase |
| TBARS | Thiobarbituric acid reactive substances |
| TNF-α | tumour necrosis factor alpha |
| Trx | Thioredoxins |
| VCAM-1 | vascular cell adhesion molecule 1 |

# CHAPTER 1

# INTRODUCTION

Stroke is the fourth leading cause of death, and the leading cause of disability in adults in Canada (1). The pathophysiological mechanisms of stroke are still not fully understood. Two major mechanisms, oxidative stress and inflammation, play important roles in secondary damage following stroke (2, 3). The immediate reduction of blood supply to the ischemic brain initiates a series of cellular and molecular events. These events include depletion of glucose, rapid decrease of ATP production, membrane depolarization, glutamate excitoxicity, and calcium overload, leading to increased production of reactive oxygen species, other free radicals, and lipid peroxidation (2). Ultimately, these result in oxidative stress. Inflammation can be triggered by oxidative stress (4). Neutrophils infiltrate the brain immediately following stroke (5). Endothelial and peripheral leukocytes accumulate in the region of cerebral ischemia and are activated. Infiltrated inflammatory cells (leukocytes and microglia) induce toxic free radicals, reactive oxygen species and protease production and cause increased production of pro-inflammatory molecule gene expression.

Nuclear factor kappa B (NFκB) is a key transcription factor in the inflammatory response, and its activation can trigger upregulation of a number of pro-inflammatory genes, including chemokines, adhesion molecules, cytokines, acute phase proteins, growth factors, and oxidant generation, promoting activity of enzymes such as cyclooxygenase-2 and inducible nitric oxide synthase. NFκB activation is critical for neurotoxic damage following stroke (6, 7). There is clear evidence that NFκB is activated immediately for a prolonged period in several ischemic models (8-11).

Protein-energy malnutrition (PEM) exists in approximately 16% of stroke patients at admission to hospital (12-14). Nutritional status often worsens following the stroke. Rates of malnutrition can be as high as 49% at the time of transfer to rehabilitation services in Canada (15). PEM increases risk of morbidity and mortality following stroke. In a study entitled the FOOD (Feed or Ordinary Food) Trial Collaboration, 3012 stroke patients were investigated in 16 countries (16). The findings suggested that being undernourished immediately after stroke was associated with reduced survival, functional ability, and living circumstances six months later. A previous study in our laboratory showed that PEM gerbils subjected to global ischemia did not habituate as well in an open field test, indicating impaired functional outcome (17). Reactive gliosis demonstrated in one-third of PEM gerbils also suggested an increased inflammatory response.

The mechanisms by which PEM worsens stroke outcome may involve increased oxidative stress and inflammation in brain. Although alterations in brain glutathione (GSH) concentration were not found, the previous study in our laboratory suggested that PEM was increasing oxidative stress in ischemic hippocampus (17). In support of the idea that PEM is increasing the inflammatory response, increased plasma concentrations of several inflammatory mediators including interleukin-6 (IL-6), C-reactive protein, and soluble receptors of tumour necrosis factor alpha (TNF-α) have been reported in PEM children (18). Another study showed plasma cysteinyl leukotrienes increased in PEM (19). NFκB activation and production of TNF-α and interleukin-1β (IL-1β) production were increased in the liver of protein malnourished mice when induced with lipopolysaccharide; this was correlated with decreased GSH concentration (20). The GSH delivery agent, N-acetylcysteine, restored liver GSH and normalized NFκB activation and

cytokine production. The effect of PEM on the inflammatory response in brain following ischemia has not been explored and could be a critical factor affecting stroke outcome.

Therefore, the hypothesis of this thesis was that PEM worsens stroke outcome by increasing activation of NFκB in global ischemia. The main objective then was to investigate whether PEM increased activation of NFκB in gerbil hippocampus following global ischemia. Since PEM may affect serum glucose (21-23) and serum glucocorticoid (GC) concentrations (24) and both factors have been demonstrated to influence ischemic brain injury (25, 26), a second objective was to investigate if these factors were influenced by PEM or ischemia in the models used for this study. GCs were of additional interest because they can directly regulate NFκB activation (27).

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Stroke as a Health Issue

Stroke is the fourth leading cause of death in Canada and it costs the Canadian economy $2.7 billion a year (1). Each year, between 40,000 and 50,000 reported strokes occur in Canada. About 16,000 Canadians die from stroke and it also causes a high rate of disabilities. In the UK and the USA, approximately 100,000 and 500,000 reported strokes occur every year, respectively (28). Stroke in the developing world is less well documented (29). A consensus statement from the Asia-Pacific Consensus Forum on Stroke Management predicts that in the next 30 years the burden of stroke will grow most in developing countries rather than in the developed world (29). In China, for example, stroke is becoming the leading cause of death, and about 2 million new reported strokes occur each year (30).

The rate of stroke survivors returning home has increased since 1970 (28, 31). The stroke mortality rate has declined in the USA, and similar declines have also occurred in most other western countries because of improved public medical care, hygiene, and nutrition (31). Despite this decreased rate of mortality, there are still no therapeutic effective treatments for stroke. Dietary intervention is one modifiable factor that could not only influence the prevalence of stroke but also its course and outcome (28). Finestone and coworkers (15) found that 49% of stroke patients had PEM at the time of admission to rehabilitation units, suggesting that immediate post-injury poor nutritional status could damage the antioxidant defense system (15, 32). The FOOD Trial Collaboration also demonstrated that stroke patients who are undernourished immediately after stoke had reduced survival, functional ability, and living circumstances six months

later (16). Therefore, this thesis focuses on the impact of PEM on ischemic brain injury, and specifically on the inflammatory response by evaluating a key transcription factor, NFκB.

## 2.2 Mechanisms of Ischemic Brain Damage

### 2.2.1 Membrane depolarization, glutamate excitotoxicity, and intracellular calcium overload

During ischemia, the cerebral blood flow (CBF) reduction is greatest in the center of the ischemic area, also called the core. The peripheral region of the ischemic territory, called the penumbra, is the region in which the ischemic damage is potentially reversible (31, 33). Brain tissue has a relatively high consumption of oxygen and glucose and depends mostly on oxidative phosphorylation for energy production (4). Restriction of the delivery of oxygen and glucose during brain ischemia impairs the energy requirement of maintaining ionic gradients. When blood flow reaches 50% of its normal rate, oxidative energy production falls severely and anaerobic energy production leads quickly to lactate production (34). Energy failure is probably the predominant mechanism of cell death in the ischemic core because failure of the sodium-potassium ATPase and other energy-dependent ionic pumps leads the neuron to lose its ionic gradients (33, 35, 36). With energy depletion, membrane potential is lost and neurons and glia depolarize.

Once the membrane is depolarized, voltage-dependent $Ca^{2+}$ channels become activated (37, 38). $Ca^{2+}$ moves into the cell and excitatory amino acids are released to the extracellular space (2, 39).The extracellular $Ca^{2+}$ concentration is relatively high compared to the cytoplasm $Ca^{2+}$ concentration (40). The influx of $Ca^{2+}$ to the cell initiates a wide variety of harmful events that cause neuron death. It overactivates numerous

enzyme systems, such as proteases, lipases, and endonucleases to produce toxic free radicals (3, 38). Free radicals can induce the formation of inflammatory mediators which activate microglia and lead to the invasion of blood-borne inflammatory cells (leukocyte infiltration) via upregulation of endothelial adhesion molecules (41, 42). When free radicals are generated, they can damage the membrane, mitochondria and DNA, in turn triggering apoptosis.

When the membrane is depolarized, another damaging event is that the glutamate-sodium transporter cannot work efficiently, and glutamate accumulates extracellularly (2, 4, 33, 37). Activation of specific ionotropic and metabotropic glutamate receptors dramatically increases intracellular $Ca^{2+}$, $Na^{+}$, and $Cl^{-}$ levels, while $K^{+}$ released into the extracellular space can propagate a series of spreading waves of depolarization (3, 4, 33). Water shifts to the intracellular space via osmotic gradients and causes edema.

### 2.2.2 Oxidative damage

Oxidative stress has been suggested to be involved in the pathogenesis of cerebral ischemia and reperfusion injury (2, 43, 44). The brain is particularly vulnerable to oxidative stress due to its relatively high consumption of oxygen, high concentration of iron and lipids with unsaturated fatty acids, and its relatively low concentration of antioxidants compared to the liver or kidney (45). At reperfusion after ischemic insult, several molecular events such as phospholipase activation, lipid peroxidation and dysfunction of the mitochondrial respiratory chain all have been shown to lead to the production of free radicals (44).

2.2.2.1 Oxidant generation system

A main event during cerebral ischemia is the generation of free radicals and other oxidant species; due to their high reactivity, they provoke damage to lipids, DNA, and proteins, leading to neuronal death (44). They also contribute to the breakdown of the blood brain barrier (BBB). These reactive species include reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, hydroxyl radical, hypochlorite and singlet oxygen, and reactive nitrogen species (RNS), such as nitric oxide radical ($NO^{\bullet}$) and peroxynitrite ($ONOO^{-}$) (2, 33, 37).

Superoxide anion can be produced at several sites in the cell. The most important source of superoxide anion in normal healthy aerobic cells is the mitochondrial electron-transport chain (2). The mitochondrial respiratory chain consists of a series of electron carriers and is organized into four complexes. Some of the electrons (approximately 2 to 5%) passing through these carriers consume $O_2$ to produce superoxide instead of being fully reduced to water (2).

During ischemia, the above-mentioned $Ca^{2+}$ influx activates phospholipase $A_2$ (37, 38). This enzyme liberates the unsaturated fatty acid arachidonic acid and then initiates the formation of free radicals via the cyclooxygenase and lipoxygenase pathways in the presence of NADH or NADPH (2, 37). Other prooxidant enzymes that generate superoxide anion are NADPH oxidase and xanthine oxidase (2).

Among ROS, superoxide is directly toxic to neurons (46). In addition, evidence suggests that superoxide contributes indirectly to tissue damage by enhancing vasogenic edema and BBB disruption after brain ischemia (47). The role of superoxide in exacerbating BBB is supported by the evidence that endothelial cells are the cellular constituents of BBB, and also the major source of superoxide production (47).

Although superoxide anion itself is a weak oxidant, it can react with a variety of molecules to produce stronger oxidants. It reacts with thiols, thus causing the release of arachidonic acid, which initiates an arachidonate metabolic cascade resulting in more superoxide anion production (2). Superoxide anion can also react with $NO^{\bullet}$ to produce the strong oxidant, $ONOO^{-}$(2). Superoxide anion is converted by the enzyme superoxide dismutase (SOD) to hydrogen peroxide (2).

Superoxide anion can reduce $Fe^{3+}$ to $Fe^{2+}$. $Fe^{3+}$ is normally bound to ferritin, a multimeric iron-carrying protein. Under the hypoxia-ischemia condition, in which the environment is acidic, superoxide anion is converted to the peroxyl radical and converts ferritin bound $Fe^{3+}$ to free iron $Fe^{2+}$. Free $Fe^{2+}$ reacts with hydrogen peroxide and generates strong oxidants, the hydroxyl radicals. These are also called the Haber-Weiss reaction. Hydroxyl radical is particularly reactive in the cell. Once formed, it can abstract an electron from most macromolecules, including thiol-containing enzymes, such as GSH reductase and GSH peroxidase, two important enzymes in antioxidant defense system. It also reacts with DNA, causing DNA strands to break or causing increased potential for mutagenesis through the hydroxylation of DNA bases. In addition, it can attack the methylene carbon of the polyunsaturated fatty acids leading to a lipid peroxidation chain reaction (reviewed in (2)).

Singlet oxygen can be formed by several reactions, including two superoxide anions interacting spontaneously, hypochlorite interacting with hydrogen peroxide, $ONOO^{-}$ interacting with hydrogen peroxide and the Haber-Weiss reaction (48). Singlet oxygen can oxidize polyunsaturated fatty acids and generate lipid hydroperoxide, a powerful oxidant that is able to initiate the propagation of lipid peroxidation chains with transition ions, inactivate SOD and catalase, and also produce DNA-damaging singlet oxygen (2).

$NO^{\bullet}$ is a physiological messenger in the central nervous system (CNS) that is formed from L-arginine and $O_2$ in a reaction catalyzed by nitric oxide synthase. Although $NO^{\bullet}$ seems to be implicated in important functions in the CNS such as regulation of cerebral blood flow or memory (48-50), it can also have damaging effects. Both leukocytes and glial cells can synthesize $NO^{\bullet}$ via different mechanisms involoving inducible nitric oxide synthase (iNOS) (37, 48), whereas endothelial cells can produce $NO^{\bullet}$ by activation of endothelial nitric oxide synthase (eNOS) (37). $NO^{\bullet}$ can interact with superoxide anion to produce $ONOO^-$, which can oxidize thio-containing proteins, DNA bases and polyunsaturated lipids. $ONOO^-$ can also inhibit mitochondrial electron transport, leading to decreased ATP production and increased production of ROS (37, 48).

$NO^{\bullet}$ levels have been found to be elevated in rat middle cerebral artery occlusion (MCAO), an experimental model of stroke. Several sources for $NO^{\bullet}$ overproduction caused by cerebral ischemia have been reviewed (51-53). The noted elevation in intracellular $Ca^{2+}$ after ischemic insult is a likely trigger of $NO^{\bullet}$ production via neuronal nitric oxide synthase (nNOS) activation.

Lipid peroxidation is initiated when a hydrogen atom is abstracted from a methylene carbon in a polyunsaturated lipid, resulting in a lipid radical (2). This lipid radical can interact with $O_2$ molecules to generate a peroxyl radical. The peroxyl radical can be converted to a lipid hydroperoxide resulting in the formation of a new lipid radical (54). This chain of peroxidation has the ability of self-perpetuation. Lipid peroxidation is one of the most damaging events since it greatly alters the fluidity of the membrane and affects a variety of membrane functions including increasing the permeability of the membrane to ions and decreasing various membrane ATPase activities (4, 55). It is also dangerous because hydroperoxides can interact with transition element ions and give rise

to lipid alkoxyl and lipid peroxyl radicals (2, 54). In turn, this can initiate new rounds of lipid peroxidation chains.

2.2.2.2 Antioxidant defense systems

Antioxidants together act as a defense system by inhibiting free radical production, scavenging free radicals, and increasing their degradation (56). Antioxidant defense systems include two categories, non-enzymatic and enzymatic antioxidants. Some of the major non-enzymatic antioxidants include tocopherol (Vitamin E), ascorbic acid, uric acid and reduced-GSH. Enzymatic antioxidants include the thioredoxin system, SOD, catalase and the family of glutathione peroxidases (GPx) (56). Thioredoxins (Trx) are a class of small 12-kDa redox proteins known to be present in all eukaryotic and prokaryotic organisms. They are characterized by a highly conserved active site that contains two cysteine residues which are reduced from the oxidized form by the flavoenzyme Trx reductase and NADPH. Therefore, Trx can scavenge free radicals and is able to protect cells against oxidative stress.

Both catalase and GPx can scavenge peroxides and convert them to water and molecular oxygen (48, 56). However, catalase is mainly located in a cell organelle called the peroxisome, while GPx widely exists in the cells. Moreover, the GPx can scavenge both hydrogen peroxides and organic peroxides. GPx requires GSH as an electron donor (56).

In the antioxidant defense system, GSH plays a central role. It not only enables GPx to scavenge all peroxides, but is also important in the regeneration of tocopherol from the tocopheroxyl radical (56). The importance of GSH in the antioxidant defense system can also be supported by the fact that supplementation of a GSH delivery agent dramatically

decreases free radicals following ischemia. N-acetylcysteine has been reported to effectively decrease ROS, such as hydroxyl radicals, hydrogen peroxide, and peroxyl radicals, and also to increase the endogenous GSH synthesis following ischemia (57).

#### 2.2.2.3 Oxidative stress after ischemia

To measure free radical levels in the brain is rather difficult due to their low concentration and short half-life. In most studies, indirect techniques, such as irreversible damage in biological macromolecules or increased consumption of endogenous antioxidants, are used to evaluate oxidative stress (45). Early oxidative stress has been shown as soon as 30-60 min after ischemia. This oxidative stress is characterized by a drop in concentrations of GSH, Vitamin C (58), Vitamin E, and ubiquinol-10 , associated with an increase in malondialdehyde (MDA) and lipid-conjugated diene levels (54). Other studies have found production of hydroxyl radicals and superoxide anions immediately after ischemia as well as from several hours up to 21 days after ischemia (59-61). Candelario-Jalil et al (62) have demonstrated the time course of oxidative damage in different brain regions following ischemia in gerbils. Sustained lipid peroxidation and decreased antioxidant enzyme activity were shown in the gerbil hippocampus up to 7 days following ischemia.

Several studies have demonstrated increased levels of lipid peroxidation markers in the plasma, erythrocytes, and blood of stroke patients, by using an assay to measure total thiobarbituric acid-reactive substances (TBARS) (63), or MDA (64).

### 2.2.3 Inflammation

Inflammation is a series of responses of vascularized tissues of the body to injury (41).

The primary objective of inflammation is to localize and eliminate the irritant and repair the surrounding tissue. For the survival of the host, inflammation is a necessary and beneficial process (41). The acute inflammatory response involves three major stages: first, dilation of capillaries to increase the blood flow; second, microvascular structural changes and escape of plasma proteins from the blood stream; and third, leukocyte transmigration through the endothelium and accumulation at the site of injury (41, 42). Brain inflammation during ischemia-reperfusion is believed to play an important role in the development of secondary damage through its ability to increase the accumulation of inflammatory cells and microvascular dysfunction (5, 41).

Inflammation is mediated by a complex array of mechanisms involving both cellular and molecular components (5). A large amount of evidence has been accumulated that numerous brain cells have an immediate or prolonged inflammatory response following ischemia.

2.2.3.1 Peripheral leukocytes

Leukocyte infiltration into brain infarction is a widely documented histopathological event in experimental models of stroke, but also is routinely observed in patients suffering from stroke. Leukocytes are considered to be key mediators of the host response in reperfusion injury in the brain (65). They are the main cellular components of the immune system. Granulocytes, which can be further divided into three lineages of cells, neurophils, eosinophils and basophils, are the first hematogenous cells that appear in the brain in response to ischemia. In a MCAO model, granulocytes have been shown to accumulate in cerebral vessels as early as 30 minutes after the onset of insult, prior to the infiltration to the infarction core and penumbra (65-67). The highest number of

intravascular neutrophils was found at 12 hr after occlusion, whereas the number of granulocytes peaked in ischemic parenchyma 24 hr after occlusion (66).

Evidence suggests that leukocytes may play a key role leading to secondary brain damage after ischemia. First of all, a correlation has been reported between poly-morphic neutrophil (PMN) accumulation in the ischemic zone and the expansion of cerebral damage in human brain (68). Secondly, neutropenia (decreased circulating neutrophils) has a beneficial effect on reperfusion injury after ischemia (69, 70). Thirdly, genetic manipulations or treatments that prevent PMN recruitment and adhesion to microvessel endothelia are protective against cerebral ischemia (71, 72). Moreover leukocytes produce proteolytic enzymes, ROS, and other inflammatory mediators that may contribute to additional damage (43). For example, iNOS has been shown to be active and to produce toxic $NO^{\cdot}$ in infiltrating neutrophils (51, 52). Other free radical producing mechanisms in neutrophils include NADPH oxidase (73).

2.2.3.2 Microglia

Microglia are the resident macrophage cell population and represent the primary immunocompetent cells within the CNS. Microglia comprise 10-20% of the total glial cell population in the CNS and act in concert with astrocytes and neurons to maintain normal brain homeostasis (74). They respond to several neurotransmitters and have a capacity to release regulatory molecules involved in inflammation, such as cytokines (75). Moreover, together with astrocytes, they release trophic factors such as nerve growth factor and basic fibroblast growth factor.

Microglia are involved in several pathological situations. They react to invasion by infectious agents and participate in removing cellular debris after traumatic or ischemic

injury. Upon stimulation, microglia transform into the ameboid shape characteristic of activated microglia (76). In focal cerebral ischemia, microglial activation occurs very rapidly within hours after the onset of ischemia and persists for weeks (77, 78). For example, in MCAO models, increased immunoreactivity for phosphotyrosine or isolectin (markers for microglia) can be detected as early as 3-6 hr after the onset of ischemia (79, 80). Moreover, activated microglia with ameboid shapes and retracted processes are clearly seen 24 hr after the ischemia.

Several inflammatory mediators are produced by microglia in ischemic insults. Classical inflammatory mediators produced by microglia are cytokines such as IL-lα, IL-lβ, IL-6 and TNF-α (75). Moreover, lipid derivatives such as arachidonic acid and prostaglandins, as well as free radicals such as superoxide and $NO^{\bullet}$, are produced. Free radicals or ROS produced by brain microglia promote abnormal phagocytic activity of microglia leading to further damage of ischemic tissue (81). Enzymes catalyzing the production of several pro-inflammatory mediators are also produced in microglia. These include, for example, cyclooxygenase 2 (COX-2), matrix metalloproteinase (MMPs), caspase-l, and iNOS (82-85).

Microglia have also been shown to produce beneficial and anti-inflammatory mediators that may protect from the ischemic insult. These mediators include trophic factors like transforming growth factor-β and cytokines such as interleukin10 (IL-I0) and the antiapoptosis protein Bcl-2 (86-88). Moreover, phagocytosis of cellular debris from degenerated neurons has been shown to support wound healing and peripheral neuronal regrowth (89), and the secretion of beneficial molecules can support neuronal recovery (90).

2.2.3.3 Vascular endothelium and the blood-brain barrier

Loss of vascular integrity is a widely described phenomenon in cerebral ischemia. There are anatomical and functional barriers that maintain vascular integrity as well as the transport and transmigration of circulating blood cells. The BBB is a membrane that controls the passage of substances from the blood into the central nervous system. It enables fluid retention within plasma space and prevents cellular extravasation during normal conditions. Several factors and mechanisms affect the integrity of the BBB. Ischemia, inflammation and inflammatory mediators (cytokines, adhesion molecules), free radicals, and proteases (MPPs, plasminogen activators, thrombin) have been suggested to increase BBB permeability and subsequent vascular edema in several experimental stroke settings (91). For example, a 3 -hr transient MCAO occlusion followed by a 3 -hr reperfusion period in rats has been shown to cause a significant increase in permeability of the BBB (92). A dysfunctional BBB and brain edema appear to play an important role in the development of brain damage in animal models of brain ischemia. Moreover, clinical manifestations of ischemic brain edema as a result of impaired BBB function is one of the most important causes of clinical deterioration and death within the first 2-5 days after stroke (93).

2.2.3.4 Neurons

There are reports showing that neurons themselves can produce inflammatory mediators and enzymes that may contribute to tissue injury progression. For example, Nagayama et al (94) showed that COX-2 is produced in neurons after ischemia. In addition to COX-2, neurons are able to produce cytokines such as IL-6, IL-1 and TNF-α in response to injury (95-97).

Summary

There is abundant evidence that inflammation contributes to the development of secondary brain damage following stroke. The increased free radical generation in ischemia induces the expression of a wide variety of pro-inflammatory molecules, including a number of cytokines and chemokines, by triggering the activation of transcription factors, including NFκB, interferon regulatory factor 1, and hypoxia inducible factor 1 (3). Cytokines such as TNF-α, IL-1β and chemokines can upregulate the expression of adhesion molecules on endothelial cells, leukocytes and platelets, leading to infiltration of leukocytes into the brain parenchyma (5). Cell adhesion molecules, including intercellular adhesion molecule 1 (ICAM-1), P-selectins and E-selectins, interact with complementary surface receptors on neutrophils, leading to neutrophil infiltration. Cytokines can also activate microglia, while chemokines directly guide the migration of bloodborne cells towards their targets. Inflammatory cells (leukocytes and microglia) contribute to brain damage by producing free radicals and other toxic inflammatory molecules that lead to blood brain barrier dysfunction, edema, and cell death (5).

The mechanisms of inflammation causing brain damage are characterized by the accumulation of inflammatory cells and inflammatory mediators in the ischemic brain. These inflammatory mediators also include enzymes that promote the production of reactive oxygen and nitrogen species, which can damage tissues by disrupting cell membranes, altering DNA and ultimately leading to apoptosis. Two important regulatory enzymes being generated in this process are iNOS and COX-2. iNOS is an enzyme that converts L-arginine to toxic amounts of NO. It is not normally found in most cells. Associated with inflammation in ischemia, iNOS is induced in neutrophils infiltrating the

brain, cerebral blood vessels (53, 98) and in activated microglia (99). Ischemic neurons also express COX-2, an enzyme that mediates ischemic injury by producing superoxide and toxic prostanoids (3).

### 2.2.4 The transcription factor, NFκB

Under ischemic conditions, the increased oxidative stress and production of pro-inflammatory substances can trigger activation of a key transcription factor, NFκB. NFκB is a ubiquitous transcription factor composed of a complex of proteins which are critical regulators of a variety of responses, especially in inflammation. Indeed, NFκB was originally identified from the B-cells of the immune system, being a nuclear factor that bound to the site in the immunoglobulin κ enhancer (100). Since then, NFκB has been shown to have a central role in the inflammatory response and in several diseases.

#### 2.2.4.1 Molecular properties of NFκB

NFκB is a DNA binding protein which has five known NFκB/Rel/Dorsal (NRD) family proteins, p65 (also called RelA), p50, cRel, RelB and p52 (101, 102). The active forms of NFκB are dimers most frequently composed of the two DNA binding subunits, p50 and p65. NFκB is widely expressed in numerous tissues including the CNS (102).

NFκB, when activated, can quickly upregulate gene expression without the delay required for protein synthesis. This ability for a quick response to stimulation is because it preexists in the cytoplasm of most cells in an inactive form non-covalently bound to the inhibitor IκB (101, 102). There are six known members of the IκB family, including the two most studied ones, IκBα and IκBβ. The N-terminal region of IκB proteins contains both phosphorylation and ubiquitination sites, both vital for NFκB activation and

subsequent IκB protein degradation by proteosome (103). IκBα is a 37-kDa protein and it regulates NFκB activity by a rapid process (102). IκBα contains a κB promoter which can be transcriptionally upregulated by NFκB. This dramatically decreases the time required for IκB to re-inhibit NFκB. The persistent activation of NFκB is regulated by IκBβ, a continuing inducing agent which causes NFκB to be maintained in the nucleus.

2.2.4.2 Activation of NFκB

A wide variety of stimuli can activate NFκB, including cytokines such as TNF-α, IL-1β, bacteria such as lipopolysaccharide (LPS), growth factor, stress, and UV light. Many ischemia-induced factors, such as inflammatory cytokines, increased intracellular $Ca^{2+}$, and glutamate excitotoxicity, also can cause NFκB activation (104, 105). These stimuli activate the phosphorylation of IκB kinase (IKK), the key step in IκB protein phosphorylation. Indeed, the extent to which IKKs are activated seems to dictate the extent of IκB degradation and subsequent NFκB activation (106, 107). Three different IKK-kinases have been described; IKKα and IKKβ are functional kinases (108), whereas, the third IKKγ, is suggested to link the IKK- complex to upstream activators. Following phosphorylation, IκB is targeted for destruction by the ubiquination-proteosomal degradation pathway, allowing the translocation of NFκB to the nucleus. Once translocated, NFκB dimers bind to a specific DNA motif and regulate transcription of target genes containing NFκB consensus sequences in their promoter region (109). Figure 2.1 shows the NFκB activation signaling pathway as well as some of the target genes.

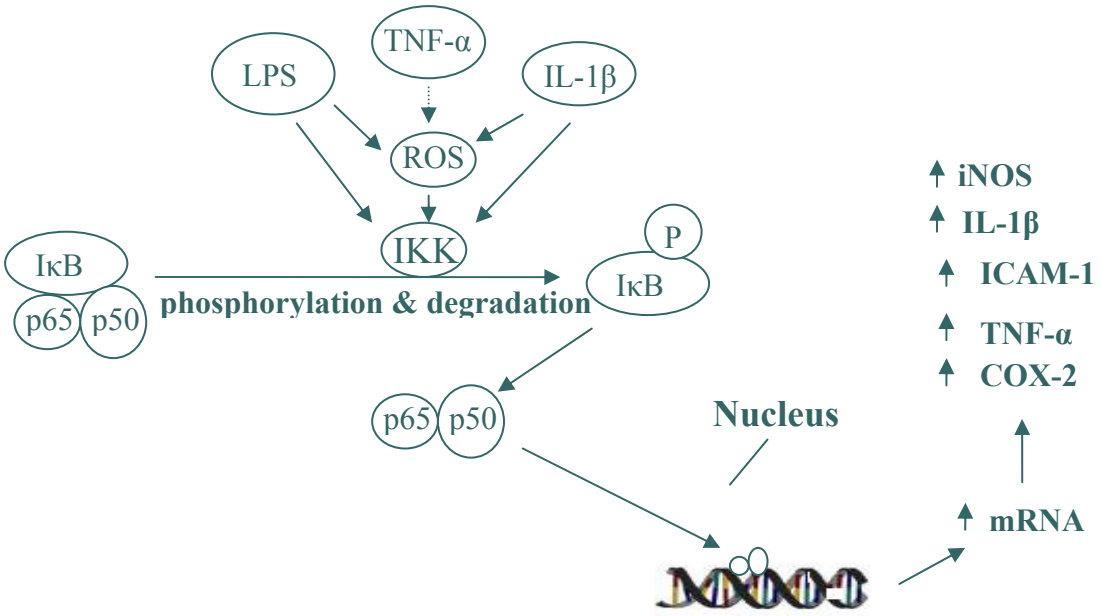



Figure 2.1 NFκB activation signaling pathway. Inactivated p50/p65 NFκB dimer binds to its inhibitor IκB in cytoplasm. Stimuli such as LPS, TNF-α and IL-1β can activate the enzyme, IKK, and promote ROS production. IKK, which can also be activated by ROS, activates IκB phorsphorylation which leads to IκB degradation. This allows p65 and p50 to translocate to the nucleus and bind to the target gene. This causes increased mRNA expression of a number of pro-inflammatory molecules, including iNOS, TNF-α, IL-1β, ICAM-1, and cycloxygenase-2 (COX-2).

2.2.4.3 Inflammatory gene expression regulated by NFκB

NFκB is a key regulator of inflammation because its activation can trigger upregulation of a number of pro-inflammatory genes, including cytokines, chemokines, adhesion molecules, acute phase proteins, growth factors, immunoreceptors, and enzymes such as COX-2 and iNOS (Table 2.1). These pro-inflammatory genes all share the common NFκB binding sequences in their promoters.

Table 2.1 Major NFκB responsive inflammatory genes

| ***Gene*** | ***Reference*** |
|---|---|
| TNF-α | (110) |
| Interleukins (IL-1, -2, -6, -8, -10, -12) | (111-116) (117-119) |
| COX-2 | (120, 121) |
| iNOS | (122) |
| ICAM-1 | (123) |
| VCAM-1* | (124) |
| E-selectin | (125) |
| MnSOD* | (126) |

* VCAM-1: vascular cell adhesion molecule 1; MnSOD: manganese superoxide dismutase.

2.2.4.4 NFκB activation in stroke models

Activation of NFκB appears to be critical for neurotoxic damage following stroke, and there is clear evidence that NFκB is rapidly activated in several ischemia models. Salminen et al (9) demonstrated a time-dependent change in transcription factor binding activities, including NFκB, in a transient focal cerebral ischemia model. Focal and global ischemia are two types of experimental models of stroke, based on the reduction of blood flow in a defined brain area and blood flow to the whole brain stopped, respectively. Subsequently, immunohistochemistry in the human brain was used to demonstrate that there is an enhancement of NFκB staining in glial cells in the infarct areas (127). Based on recent reports, the most prominent NFκB activation after brain ischemia is seen in neurons in both focal and global ischemia (10, 11, 128, 129). Two key investigations in 1997 by Clemens et al (10, 11) illustrated details of the time course and the cell types in which NFκB is upregulated. Using a transient forebrain global ischemia model, increases

of NFκB p50 and p65 immunoreactivity were mainly located in neurons in the hippocampal CA1 region (10). The p50 subunit of NFκB showed an increase as early as 6 hr following global ischemia with an increasing intensity over 12 and 48 hr followed by a decreased intensity at 96 hr (11). However, one limitation of this study is that the results are reliant solely on western blot, which has lower specificity compared with the electrophoresis mobility shift assay (EMSA) in analyzing NFκB activation. Although these studies of cerebral ischemia had relatively short time frames, there is some evidence that the increase in NFκB may be quite prolonged in some cells. Domanska-Janik et al (8) have also shown NFκB activation in the dorsal part of the hippocampus as early as 3 hr after global ischemia and for up to 7 days following the insult. Importantly, Schneider et al (130) showed that genetic disruption of inducible NFκB activity in p50 knock-out mice was able to prevent neuronal cell death significantly in a focal transient ischemia model, supporting a direct cell death promoting role for NFκB in ischemia. However, causality between NFκB activity and neuronal cell death has not been established well enough in brain disease models. Transient NFκB activation in ischemic injury may also have some benefit, whereas more prolonged activation may be detrimental. NFκB DNA-binding activity decreased in hippocampus of mice lacking p50 has also been reported to enhance neurodegeneration in both hippocampus and striatum following focal ischemia (131). Whether NFκB activation is neurotoxic or neuroprotective still needs to be further investigated.

#### 2.2.4.5 Redox regulation of NFκB activation

Cell redox status has been demonstrated to have a clear role in regulating NFκB activation. This is supported by three types of evidence. First, as reviewed by Christman

(132), most of the stimuli of NFκB activation that can activate IKK, such as lipopolysaccharide, TNFα, IL-1β and hydrogen peroxide, produce oxidative stress in the cell. Secondly, treatment with oxidants increases NFκB activation; hydrogen peroxide has been shown to directly activate NFκB activation (133). Overexpression of SOD, which causes increasing production of hydrogen peroxide, enhances the TNFα-induced activation of NFκB (128). Third, the cysteine prodrug, N-acetylcysteine, has been demonstrated to inhibit NFκB activation and protect against ischemia (134). Shen et al (135) also demonstrated that N-acetylcysteine and pyrrolidine dithiocarbamate reduce neuronal loss in rat hippocampus after global ischemia through inhibition of NFκB activity.

## 2.3 Protein-Energy Malnutrition

### 2.3.1 PEM as a clinical problem and its determinant role in stroke outcome

The elderly are both a group at high risk for stroke (136) and they are vulnerable to poor nutritional status due to factors such as eating disorders, poor dental status, drug side effects, decreased physical activity and metabolism, and chronic diseases (137-139). There are two lines of protein-energy deficiency, called kwashiorkor and marasmus. In kwashiorkor, the diet is low in protein but provides enough Calories. Serum albumin is decreased, skin and hair show dyspigmentation, and edema and fatty liver also occur (139). In marasmus, there is general insufficient energy intake with protein deficiency. Patients suffer with muscle wasting and loss of subcutaneous fat, but do not present with edema, hypoalbuminemia, or fatty liver (139). In North America, the most commonly seen protein deficiency in hospitalized patients is PEM, which is a combination of both conditions (140).

Compromised protein-energy deficiency has been reported for stroke at the time of admission to hospital (13, 14, 16, 93, 141, 142) and during the hospital stay (13, 14, 16, 93, 141, 142) using anthropometric, haematological and biochemical characters for assessment of nutritional status. In a study in an Australian private hospital, 19.2% of stroke patients were malnourished on admission and this was associated with a significantly greater patient-generated subjective global assessment score (a global assessment for malnutrition) and lower body weight (143). Forty-nine% of Canadian stroke patients at the time of admission to rehabilitation units had PEM, with dysphagia reported in 47% (15, 32). They suggested there was inadequate nutritional intervention immediately post-injury which could compromise antioxidant defense mechanisms (15, 32). Nyswonger et al (144) demonstrated that the length of time to start of feeding after stroke varies, and stroke patients fed within seventy-two hours of injury have a shorter hospital stay. Among the various markers of nutritional status, low serum albumin concentration is the only one that showed a significant and independent association with various outcomes such as longer lengths of hospital stay and higher mortality rates in stroke patients (12-14, 32, 136, 141, 142, 145). Albumin synthesis initially decreases in PEM. With severe protein deficiency, the synthesis of albumin and other serum protein decreases dramatically, resulting in decreases in osmotic pressure and extravasation of water and edema (146). However, serum albumin has limitations as an indicator of protein status because it is also influenced by many other conditions as well as by liver and renal disease (14, 142, 147).

In a recent study entitled the FOOD Trial Collaboration, 3012 stroke patients were investigated in 16 countries (16). It was found that being undernourished immediately after stroke was associated with reduced survival, functional ability, and living

circumstances six months later. This study suggests that PEM influences stroke outcome, but a major limitation of this study was the absence of standardization of assessments of nutritional status. A recent study done by our laboratory in a global ischemia model in gerbils (17) suggested that the association shown by the FOOD Trial is causal. Ischemic animals subjected to PEM did not habituate as well in an open field test, which is a functional measure of brain injury. A subgroup also displayed thigmotaxis (a preference for the outer perimeter of the open field) and marked reactive gliosis. The reactive gliosis suggested that PEM might be influencing the inflammatory response in stroke and may be a critical determinant of stroke outcome.

2.3.2 PEM and ischemic brain damage: mechanisms

2.3.2.1 PEM and redox status, inflammation and NFκB activation

Although impaired immune function (T-cell subsets, lymphocyte proliferation and cytokine production) has been observed in elderly patients with PEM (148, 149), an abnormal or prolonged inflammatory response can also occur in PEM. In one study in PEM children, plasma concentrations of several inflammatory mediators, including IL-6, C-reactive protein, and soluble receptors of TNF-α, were found to be greater (18). Increased endogenous cysteinyl leukotrienes leading to increased edema has also been reported in kwashiorkor patients (19). The age of onset of PEM may influence effects on the inflammatory response since the production of a number of cytokines has been reported to be decreased by PEM in adolescent animals but increased in middle-aged animals (150).

Previous work in our laboratory suggests that PEM may increase the inflammatory response in brain ischemia. In this study, it was reported that one-third of PEM gerbils

exposed to global ischemia showed dramatic increases in reactive gliosis in the hippocampus at 10 days after the insult (17). Although the glial cell type was not identified, it is known that both microglia (151) and astrocytes (152) are activated after global ischemia. The reactive gliosis suggested an increased inflammatory response because reactive astrocytes can produce and release inflammatory mediators such as cytokines and chemokines (153), and microglia can produce toxic molecules including pro-inflammatory cytokines and also reactive species including NO, oxygen radicals and arachidonic acid derivatives (154).

An important question is how PEM could enhance the inflammatory response in the post-ischemic period. Li et al (20) have reported that protein malnutrition causes increased NFκB activation in liver in response to lipopolysaccharide as well as increasing mRNA for its target genes, TNFα and IL-1β. This has never been studied in brain, but given the key role of NFκB in promoting inflammation following brain ischemia (8, 10, 11, 155), this is a potential mechanism by which PEM enhances inflammation and worsens functional outcome. Effects of PEM on NFκB could be mediated by an alteration in GSH concentration and redox state. In liver, supplementation of the GSH delivery agent, N-acetylcysteine, normalized lipopolysaccharide-induced NFκB activation, restored liver GSH level, and decreased TNFα and IL-1β mRNA (20).

GSH is an important antioxidant for scavenging peroxides (2). As well as playing a central role in the antioxidant defense system, it is important in redox regulation of NFκB activation (20, 133). Cellular GSH concentration is determined by several factors including consumption by formation of conjugates via GSH S-transferase, extent of oxidation, *de novo* synthesis, and reduction of oxidized GSH by GSH reductase (55).

Activity of the γ-glutamylcysteine synthetase and availability of cysteine are the rate limiting factors for GSH synthesis (55). The most important source of cysteine is the diet. If dietary cysteine is decreased, GSH may be used to supply cysteine for critical proteins, limiting the amount of GSH available for antioxidant defense. Liver GSH responds to the protein, cysteine, and energy content of the diet (156). When mice are fed a low-protein diet, hepatic GSH is depleted and after supplementation with a cysteine precursor, hepatic GSH can be increased beyond the physiological maximum (157). GSH concentration is also reduced in lungs of protein-energy malnourished rats but can be increased with the cysteine prodrug, L-2-oxothiazolidine-4-carboxylate (OTC) (158).

PEM may increase NFκB activation by decreasing brain GSH and enhancing ischemia-induced oxidative stress. However, brain GSH concentration appears to be more tightly regulated by mechanisms in the brain and less responsive than other tissues to diet although acute, severe sulfur amino acid deficiency decreases GSH in a number of brain regions (159). It was hypothesized in a previous study from our laboratory that PEM would exacerbate a global ischemia-induced depletion of GSH due to decreased substrate for synthesis in the face of increased demand (17). Although this was not found in hippocampus or neocortex, the limitation of that study is that only one timepoint (12 hr) was assessed post-ischemia, and there was considerable variability in ischemic damage. It is also possible that PEM increases NFκB activation through an increase in oxidative stress that is independent of a change in GSH concentration. This is supported by the study of Bobyn et al. (17) which showed independent PEM- and ischemia-induced decreases in hippocampal protein thiols and a trend for GSH reductase to decrease with ischemia only when the animals were also PEM. These serve as markers of oxidative stress because of the susceptibility of thiol groups to oxidation (160). Thus, PEM may

affect redox status following brain ischemia, but more sensitive indicators are needed in future investigations.

2.3.3 PEM and other mechanisms

PEM may influence stroke outcome by influencing blood glucose (21-23) and GC concentrations (24). PEM, GC, and blood glucose may interact in a complicated manner to affect ischemic brain injury. Figure 2.2 shows the potential interactions.

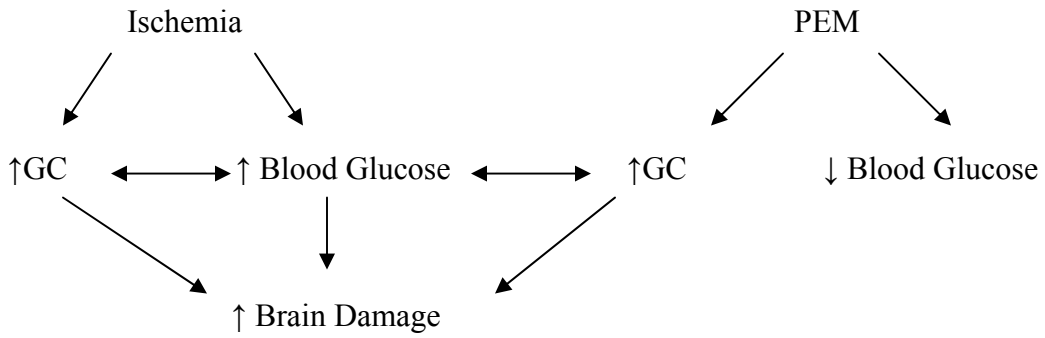



Figure 2.2 The relationship of ischemia, PEM and brain damage.

For three decades, numerous studies have shown that elevated pre-ischemic glucose levels lead to exacerbated post-ischemic brain damage (161-163). Hyperglycemia has been reported in several brain ischemia models and is correlated with poor prognosis in ischemic stroke because elevated glucose levels lead to elevated intra-ischemic lactic acid levels and lactic acidosis, thus exacerbating brain damage (164). The incidence and degree of hyperglycemia has been related to the severity of acute stroke, higher hospital mortality as well as poor functional outcomes (162, 165-167). The underlying mechanisms by which hyperglycemia affects stroke outcome may involve inducing a pro-oxidative and pro-inflammatory state that can cause direct neuronal toxicity. PEM has been shown in several studies to decrease serum glucose level (21, 23), while insulin has

been reported to be decreased to promote glucose homeostasis (24). Ischemia- induced hyperglycemia could also be a stress response with activation of the hypothalamo-hypophyseal-adrenal axis, which leads to an increase in cortisol and catecholamines (166). Hyperglycemia is also known to be associated with inflammation and oxidative stress (166). Glucose treatment results in a direct increase in NFκB binding activity in mononuclear cells contrary to water or alcohol (168).

PEM may also influence ischemic brain injury by increasing GC concentration. A high level of GC is seen in response to acute deficits of protein and energy (24). Blood cortisol in acutely protein-energy malnourished children (169) and serum corticosterone concentration in acutely protein energy malnourished young adult rodents (170, 171) range from 1.3 to 6-fold the concentration found in matched healthy subjects. Mice on a protein-free diet for 7 days also showed significantly impaired macrophage function mediated by elevated serum GC levels (171). Increased GC concentration in PEM may be detrimental to stroke outcome. High GC levels have been associated with cognitive dysfunction and severity of hemiparesis after stroke, and may predict mortality (172-174). GCs increase neuron loss (25), and expression of many cytokines receptors are potently upregulated by GCs. Membrane bound receptors for IL-1, IL-2, IL-4, IL-6, IFN-γ, and TNF-α are induced by GCs on several cell types (25, 175-177). These studies had not provided evidence that increased GCs was due to PEM, and thus further studies should be investigated in the context of stroke and PEM.

GCs are well known to affect NFκB activation. The hippocampus contains a high density of GC receptors (GR) (178), which can bind to activated NFκB in the nucleus. GR can also switch on IκBα gene expression (179). Increased production of IκBα binds to NFκB in the cytoplasma, preventing its translocation and activation. In contrast to my

hypothesis, decreased peritoneal macrophage NFκB translocation in response to lipopolysaccharide stimulation has been demonstrated in protein-deficient mice and suggested to be linked to increased circulating GCs levels (179).

## 2.4 Animal Models of Stroke

The literature describes a vast number of different approaches to studying cerebral ischemia. These experimental models are designed to mimic the cellular and molecular events that take place in the brain after ischemic insults. An ideal animal model would closely mimic human stroke from all aspects, including complex physiology. These elements are needed to ensure reproducibility of ischemic insults and to make correct and statistically valid conclusions (180). Rodents are the most widely used animals. They share many features of brain vascular anatomy and physiology in common with the human, and they are relatively inexpensive and easy to work with. The two most widely used types of animal models of cerebral ischemia are focal ischemia and global ischemia.

### 2.4.1 Focal ischemia

Focal ischemia is a model resulting in reduction of local blood flow in a defined area of the brain. Focal ischemia is divided into techniques that allow for reperfusion of the ischemic tissue (transient focal cerebral ischemia) and those without reperfusion (permanent focal cerebral ischemia) (181). MCAO is the model mostly widely used and can be achieved in many ways including embolism, endovascular filament (transient or permanent), and permanent and transient transcranial MCAO (181, 182). Focal ischemia is popularly used, since it mimics the majority of ischemic strokes in people (182, 183). The severity of the insult is controlled by varying the occlusion time. Another essential

feature of this model is that ischemic damage, rather paradoxically, progresses for days and even weeks after the restoration of the blood flow (183). The reperfusion injury is also well characterized for this model. However, this model relies on invasive and traumatic surgical procedures, requiring skillful techniques to reproduce the ischemic damage, and thus it often produces variable results (181).

### 2.4.2 Global ischemia

In glocal ischemia, in contrast to focal ischemia, blood supply to the whole brain is stopped. It could be either transient or permanent (182). Global brain ischemia most often occurs in the clinical setting as a result of cardiac arrest, which results in cessation of systemic blood circulation, hypotension and hypoperfusion of the brain (37). While experimental global ischemia models have been criticized for mimicking cardiac arrest rather than clinical ischemic stroke, they share many of the same pathophysiological mechanisms and cause more consistent neuronal damage (182). The pathophysiological mechanisms of interest in this thesis, oxidative stress (17, 62, 184), NFκB activation (8), and inflammation (8) are all present in global ischemia. Models of global ischemia have been applied in several mammals such as cats (185, 186), rats (187) and gerbils (188) as well as in primates (161). There are several ways to produce global cerebral ischemia. The major ones in rodents are the two-vessel occlusion (hypotension and bilateral common carotid artery occlusion) and four-vessel occlusion (common carotid and vertebral artery occlusion) in the rat, decapitation, and either unilateral or bilateral common carotid artery occlusion (BCAO) in the gerbil (182, 189-191).

2.4.3 BCAO in Mongolian gerbils

The Mongolian gerbil has been used widely because of its unique vasculature which lacks a circle of Willis (183). Due to the absence of posterior communicating arteries between vertebral and internal carotid arteries, global ischemia is produced simply by BCAO (192). Delayed neuronal cell death occurs selectively in highly vulnerable brain regions such as neocortical layers, dorsomedial striatum and, perhaps most importantly, in the CA1 region of the hippocampus (188, 193-195). Well-characterized both histologically and functionally, with behavioral testing, and without any complicated procedures, BCAO has typically resulted in reproducible and consistent ischemic brain damage (196-200). When the brain temperature is controlled at normothermic levels during ischemia, the BCAO gerbil model results in 75%-90% delayed CA1 neuron death with 3 and 5 min occlusions respectively without any sign of post-ischemic seizures (199, 201, 202). Functional abnormalities have been well established following global ischemia in the gerbil model (181, 197, 200, 201, 203). In keeping with the present thesis, oxidative stress, inflammation, and NFκB activation have been shown in the brain by this model of ischemia (8, 17, 62, 184).

However, two important recent studies have demonstrated that a significant proportion of gerbils from the major North American suppliers no longer have an incomplete circle of Willis, causing variability in neuronal damage after occlusion. Using both histological and behavioral testing, gerbils from Charles River showed a high incidence (22.8% with bilateral and 38.6% with unilateral anastomoses) of posterior communicating arteries. Behavioral indices of CA1 ischemic injury, including increased locomotion and habituation deficits, were also significantly attenuated in the Charles River gerbils (204). Seal et al (205) also demonstrated that ischemic injury was markedly

attenuated in the hippocampus of gerbils when posterior communicating arteries were greater than 50μm in diameter. These posterior communicating arteries, ranging from 19 to 125 μm in diameter, were reported in 90% of gerbils from Charles River and Harlan Sprague-Dawley, the only two North American vendors of Mongolian gerbils for laboratory use. This evidence is consistent with higher than expected variability in neuronal damage in the gerbil global ischemia model in a previous study from our laboratory (17).

The high prevalence of posterior communicating arteries and their profound effect on attenuating hippocampal injury is causing investigators to adopt other models or modify this model. Since complete ischemia causes persistent hyperactivity apparent for even three days postischemia (198, 199), monitoring post-ischemic activity can be used as an indicator of complete ischemia. This strategy was used during the course of this thesis.

# CHAPTER 3

# METHODOLOGY

## 3.1 Experiment 1

### 3.1.1 Animals and Diets

Adult male Mongolian gerbils (Charles River Canada, QC, Canada), age 11-12 weeks, were acclimated for 4-7 days, and randomly assigned to adequate protein (CON, containing 12.5% protein as casein) or low protein diet (PEM, containing 2% protein as casein) (Dyets Inc., Bethlehem, PA, USA). The diet was modified from the AIN-93M rodent diet (206) and does not contain the antioxidant tertiary-butylhydroquinone (Table 3.1). Gerbils fed a 2% protein diet voluntarily decrease their food intake, which results in a mixed PEM (17). Animals were housed in groups of three at 22°C with a 12 hr light/dark cycle in shoebox cages with CareFRESH (Absorption Corp, Bellinghem, WA, USA) bedding and with free access to food and water. Weekly body weights and daily food intakes were monitored during 28 days of feeding time. All animal care and procedures followed the Canadian Council on Animal Care guidelines and were approved by the University of Saskatchewan Committee on Animal Care and Supply.

### 3.1.2 Surgical Procedures

After four weeks on their diets, the animals were subjected to either global ischemia or sham surgery, resulting in four experimental groups: control diet with sham surgery (CON-Sham, n=3), control diet with global ischemia (CON-Isch, n=6), PEM with sham surgery (PEM-Sham, n=3), PEM with global ischemia (PEM-Isch, n=6). Global ischemia was achieved with five minute bilateral carotid artery occlusion under 1.5%-2% isoflurane anesthetic with 1L/min $O_2$. The common carotid arteries were isolated through

Table 3.1 Modified AIN-93M Rodent Diet

| Component | Adequate protein[§] (Control, CON) g/kg | Low protein[§§] (PEM) g/kg |
|---|---|---|
| Vitamin free casein | 140 | 22.4 |
| L-cystine | 1.8 | 0.29 |
| Sucrose | 100 | 100 |
| Cornstarch | 465.7 | 543.049 |
| Dextrinized Cornstarch | 155 | 181.001 |
| Soybean oil | 40 | 40 |
| Cellulose | 50 | 50 |
| Mineral mix* | 35 | 35[¶] |
| Calcium phosphate dibasic | 0 | 12.4 |
| Calcium carbonate | 0 | 3.36 |
| Vitamin mix[†] | 10 | 10 |
| Choline bitartrate | 2.5 | 2.5 |

§ Control diet was formulated to contain 12% protein, 10% fat, 78% carbohydrate.
§§ PEM diet was formulated to contain 2% protein, 10% fat, 88% carbohydrate.
* AIN-93M mineral mix (206).
¶ AIN-93M modified mineral mix: calcium and phosphorus deleted, potassium citrate·$H_2O$ increased from 28 to 226.55g/kg, sucrose increased from 209.806 to 618.256 g/kg mineral mix.
† AIN-93M vitamin mix (206).

a ventral midline incision. A surgical silk thread was passed under both arteries to allow gentle lifting of the artery. Core body temperature was monitored with a rectal probe and maintained at 37 ± 0.5°C with a homeothermic blanket (Harvard Apparatus Canada, Saint-Laurent, QC, Canada). Tympanic temperature was monitored intraischemically by inserting a fine temperature probe (Barnant Type T Digi-Sense Thermometer, Barrington, IL, USA) through the tympanic membrane. Tympanic temperature was maintained at 36.5 ± 0.2°C by a blanket (Global Medical Products Inc., Burlington, ON, Canada) circulating with warm water wrapped around the gerbil's head. When brain temperature was stable at 36.5°C, two 8 x 1.5mm micro-aneurysm clips (World Precision Instruments, Inc., Sarasota, FL, USA) were applied to both common carotid arteries for five minutes to block the blood flow. The blockage was confirmed visually. After removal of the clips, carotid artery reflow was again verified visually and the incision was closed. Sham surgery procedures were similar; arteries were isolated from other tissue by silk thread, but not occluded, and then released. Animals were all placed under a warm lamp for 2 hr to recover. As they recovered, they could choose to stay under the lamp or not. Animals were then returned to the shoebox cage to be monitored for postischemia activity.

### 3.1.3 Behavioral Monitoring

The model of global ischemia relies on our gerbil supply consistently lacking a complete circle of Willis. However, a recent study by Laidley and Corbett (2005) shows that a significant proportion of gerbils from Charles River no longer have an incomplete circle of Willis, causing variability in neuronal damage after ischemia. Since complete ischemia causes persistent hyperactivity apparent for even three days postischemia (196, 199), one way to screen out animals with undesirable brain vasculature is to monitor

activity continuously post-ischemia. Therefore we monitored activity for 20hr following surgery using the Opto-M3 Activity Meter (Columbus Instruments, Columbus, OH, USA).

This instrument supports a modular multi-channel activity monitoring and a variety of optical beam sensors. The sensors are mounted around a standard cage by bolting the ends of the sensors to four corner brackets. Each sensor pair includes a detector and emitter. Two sensor pairs are required for each monitoring station, so that 2-dimensional monitoring is accomplished as infrared light beams (wavelength = 875 nm) pass through the cage. There are 16 light beams, spaced 1 inch apart and each beam is 0.32cm in diameter. Animal motor activity was measured by counting the interruptions indicated by the sensors. The accumulated two-dimensional total counts were recorded every 15 minutes with computer software (The CI Multi-Device Software, Columbus Instruments, OH, USA). Activity monitoring started 2 hr after the surgery and lasted for 20 hr (for 24hr or 72hr survival animals). Gerbils were then either humanely killed or returned to the animal care room. Gerbils taken to a 6hr survival time were monitored for 4 hrs only.

Criteria for complete ischemia were established as: 1) Activity had to be greater than 3 standard deviation (SD) above the mean activity of sham animals. The mean + 3SD was chosen statistically as it would encompass greater than 99% of the sham population (207). Mean (± SD) activity of sham animals in our laboratory is 190 ± 155 counts/20hr (n=30, combined data from experiments of J.Ji and M.Harmon). Both PEM and CON shams were included in deriving this value because there was no significant effect of diet on activity by a two-factor ANOVA ($p=0.897$) and there was no diet-ischemia interaction ($p=0.782$). 2) This hyperactivity had to be sustained throughout the 20hr period because it has been demonstrated that neuroprotective treatment reduces the period of hyperactivity

(198). Although no fixed tissue was available from my study for histological assessment, my laboratory co-worker (Monique Harmon), using an identical experimental design, assessed hippocampal damage histologically and validated that our activity screening procedure correctly identifies 84.6% of animals with respect to complete forebrain ischemia.

### 3.1.4 Serum Cortisol and Serum Glucose

Since PEM may decrease serum glucose (21-23) and increase serum cortisol (24), and both these factors have been reported to influence stroke outcome (25, 26), blood samples were collected at 6hr following ischemia to measure these two physiological variables. Blood samples were centrifuged (Eppendorf Centrifuge 5417C, Westbury, NY, USA) at 1500 x g for 10 minutes after being clotted at room temperature. Serum samples were transferred to 500µl microcentrifuge tubes and stored at -80°C. Blood samples that were hemolyzed as assessed by visual inspection were excluded. Serum glucose and cortisol were analyzed by Prairie Diagnostic Services, College of Veterinary Medicine, University of Saskatchewan. Serum glucose was analyzed by the hexokinase method spectrophotometrically at 505 nm (Roche/Hitachi 704/911/912, Roche Diagnostics Corporation, IN, USA). Serum cortisol was measured by radioimmunoassay designed to measure total cortisol (Coat-A-Count Cortisol Kit, Diagnostic Products Corporation, LA, CA, USA).

### 3.1.5 NFκB Activation

#### 3.1.5.1 Preparation of Hippocampal Nuclear Extracts

Animals were perfused with heparinized saline by intracardiac injection at 6hr, 24hr

and 72hr postischemia. Brain hippocampus was dissected on ice. The nuclear extraction method was modified from Xu et al.(208). Approximately 0.05g of hippocampal tissue for each animal was homogenized in 0.5ml 0.1% phosphate buffered saline using a Brinkmann Polytron Model PT10-35 (Rexdale, ON, Canada) buffer for 5 strokes, with each stroke lasting 5seconds. Then each sample was centrifuged (Eppendorf Centrifuge 5403, Westbury, NY, USA) at 600 x g for 10 min and the pellet was resuspended in 0.5ml buffer A at pH 7.9 (10 mmol/L HEPES buffer,10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.1 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulphonyfluoride). The mixture was incubated on ice for 10 min and centrifuged again at 600 x g for 10 min. The pellet was again resuspended in 0.3 ml buffer A to which 0.05% Nonidet P-40 was added. This was homogenized again for 5 strokes at 5seconds per stroke to lyse the nuclei. The sample was then centrifuged again at 600 x g for 10 min and resuspended in 0.5 ml buffer C at pH 7.9 (5 mmol/L HEPES, 25% glycerol, 1.5 mmol/L $MgCl_2$, 0.4 mmol/L EDTA, 0.3 mol/L NaCl). Finally, it was mixed well and incubated on ice for 30min before being centrifuged at 14000 x g for 30 min. The nuclear extract (the supernatant) was then aliquoted to microcentrifuge tubes and stored at -80°C.

### 3.1.5.2 Protein Analysis

Total protein concentration of hippocampal nuclear extracts was determined by the bicinchoninic acid (BCA) assay (209). First, the stock protein standard was prepared using bovine serum albumin and deionized distilled water (DD-$H_2O$). Working protein standards were then diluted in DD-$H_2O$ to the following concentrations: 0, 0.0625, 0.125, 0.25, 0.5, and 1 mg/ml. Standards (to make a standard curve), blanks and unknowns were run in triplicates. Cupric sulfate pentahydrate (BDH Chemicals, Toronto, Canada)

solution (4%) was prepared. The working BCA solution contained 1 part cupric sulfate solution and 5 parts BCA. One ml BCA working solution was added to each 100 µl standard, blank or unknown sample. The final dilution of the brain nuclear extracts for the BCA assay was 1:200. The mixture was incubated at 37°C for 30min and allowed to cool down to room temperature. The concentration was measured using a spectrophotometer at 560 nm (Shimadzu, UV-265, UV-visible Recording Spectrophotometer, Columbia, MD, USA). The protein concentration of the sample was calculated from the standard curve by linear regression analysis.

**3.1.5.3 EMSA Analysis and Semiquantification**

NFκB activation was analyzed by EMSA using a gel shift NFκB family kit (Active Motif, Carlsbad, CA, USA). Radioactive labeling of oligonucleotide probe was achieved by adding 2 µl unlabeled wild-type oligonucleotide probe which contained a consensus sequence 5′-GGGGTATTTCC-3′, 5 µl of 5X T4 Kinase buffer, 4 µl (40 µCi) [$\gamma$-$^{32}$P]-ATP, 1 µl of T4 polynucleotide kinase (Invitrogen, Carlsbad, CA, USA), and 8 µl DD-$H_2O$ to a final volume of 20 µl. The probe was incubated at 37°C for 30 min and 5 µl of 1% SDS/100mM EDTA solution was added to stop the kinase reaction. Radioactivity of the labeled probe was determined by measuring 1µl of purified probe in a scintillation counter (Beckman LS6500 multi-purpose scintillation counter, Fullerton, CA, USA). The activity of the probes had to be greater than $1x10^5$ c.p.m/µl.

The extract premix was prepared by adding 5 µl DD-$H_2O$, 4 µl buffer B (Active Motif, Carlsbad, CA, USA), 2 µl stabilizing solution D (Active Motif, Carlsbad, CA, USA), and 5 µl nuclear extracts on ice with gentle mixing by pipetting. The mixture was incubated for 20 min at 4°C. While incubating, probe premix was prepared by adding 2

µl DD-$H_2O$, 2 µl buffer C, 1 µl buffer D and 1µl labeled oligonucleotide probe. The probe premix was then added to pre-incubated extract premix, mixed well and incubated for 20 min at 4°C.

For EMSA detection, the entire content of premixed probe (6µl) and premixed extract (16 µl) were mixed and loaded onto a 5% polyacrylamide (acrylamide:bisacrylamide, 38:2) gel and pre-cooled to 4°C in Tris-Glycine electrophoresis buffer containing 24.8 mM Tris, 190 mM glycine and 1 mM EDTA. Gel components included 5% polyacrylamide, 20% buffer containing 124 mM Tris, 950 mM glycine, 5 mM EDTA, 10% ammonium persulfate, and 1% TEMED (N,N,N',N'-Tetramethylethylenediamine) (pH ~8.3). Sixteen cm plates with 10 wells on each gel were used in the electrophoresis apparatus EC105 (EC Apparatus Corporation, Holbrook, NY, USA). The gels were run at 100V for 1 hr or until the loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) reached the bottom third of the plates. The gels were then dried by a gel dryer (Labconco Gel Dryer, Kansas City, MO, USA), exposed to Kodak X-OMAT film (Eastman Kodak Company, Rochester, NY, USA), and stored at -80°C for 24 hr.

Bands corresponding to the binding reaction between activated NFκB and labeled oligonucleotide probe were detected by autoradiography using a densitometry system (Alphalmager system, Alpha Innotech, San Leandro, CA, USA). The results were expressed as a ratio which was calculated as the density of the sample from an experimental group per µg protein divided by the density of a sample from the reference group per µg protein. Each gel contained a sample from an animal on control diet without exposure to any type of surgery, which was designated as the reference group. This ratio was used to decrease the variability between gels, radioactivity of labeled probe and film

development. All samples were analyzed using the same probe, and all runs were completed over a 2-day period. Six gels were run, and every gel contained 10 samples. In order to keep these variables controlled, the following number of samples were analyzed for all three timepoints (6hr, 24hr, 72hr): PEM-Isch (n=6); CON-Isch (n=6); PEM-Sham (n=2); CON-Sham (n=2).

### 3.1.6 Statistical Analysis

All statistical analyses were conducted using SPSS 12.0 for windows (SPSS Inc., Chicago, IL, USA). Body weight and feed intake pre-ischemia were analyzed by unpaired Student's *t*-test. Activity data for assessing complete ischemia were analyzed to ensure there were no diet effects or diet-ischemia interactions using two-factor ANOVA. Postischemia body weights and biochemical data were analyzed by two-factor ANOVA. Correlation of NFκB activation and activity was analyzed by Pearson's Correlation Coefficient. Differences were considered statistically significant at $p<0.05$. All data were presented as mean ± SEM.

## 3.2 Experiment 2

Since increased hippocampal NFκB activation was observed in PEM-Sham animals in Experiment 1, a second experiment was designed to investigate whether PEM increases NFκB activation in the hippocampus independent of sham surgery. Male Mongolian gerbils of the identical strain and age as used in Experiment 1 were randomly assigned to either CON diet or PEM (n=6 for each group) for 28 days but did not receive any surgical intervention. Tissue perfusion was at a timepoint equivalent to 6 hr

postischemia. Nuclear extracts were prepared from hippocampus and EMSAs were performed for NFκB activation as described previously.

# CAPTER 4

# RESULTS

## 4.1 Experiment 1

### 4.1.1 Body Weight and Feed Intake

Initial and final body weights, 28-day body weight change, and total feed intake are presented in Table 4.1. The initial body weight was not significantly different between CON and PEM groups. After the 28-day period of feeding, the control group gained on average 4.9g body weight and the PEM group lost on average 7.4 g. The differences in final body weight and body weight change between the groups are significant ($p<0.001$). Average total 28-day feed intake per animal per cage is significantly different between the CON and PEM groups ($p<0.001$).

Table 4.1 Body weight and feed intake prior to ischemia

| Parameter | CON | PEM |
|---|---|---|
| Initial Body Weight (g) $^{\pi}$ | 63.4 $\pm$ 0.5 | 62.2 $\pm$ 0.6 |
| Final Body Weight (g) $^{\pi}$ | 68.3 $\pm$ 0.6 | 54.7 $\pm$ 0.8* |
| 28-Day Body Weight Change (g) $^{\pi}$ | + 4.9 $\pm$ 0.5 | - 7.4 $\pm$ 0.7* |
| Total 28-Day Feed Intake (g) $^{\lambda}$ | 109.6 $\pm$ 1.4 | 93.7 $\pm$ 1.8* |

Results in the table are shown as mean ± SEM.

* Indicates significantly different from control group by unpaired T-test. CON or PEM designates animals on control diet or exposed to PEM, respectively.

$^{\pi}$ n = 36

$^{\lambda}$ n = 12 cages (3 animals/cage); presented as average intake/animal/cage.

Following ischemia, there was a significant effect of diet ($p<0.001$) on body

weight but no significant effect of ischemia (p=0.260, 0.379, 0.124, at 6hr, 24hr, 72hr respectively) or interaction between surgery and diet at any timepoint (p=0.373, p=0.806, p=0.635 at 6hr, 24hr, 72hr respectively) (Table 4.2). All experimental groups had weight loss during the 72 hr period following surgery except the CON-Sham group. Data on feed intake are shown in Figure 4.1 for animals taken to 24hr and 72hr timepoints postischemia. These data are shown descriptively because there was an insufficient sample size to undertake statistical analysis. Feed intake was decreased at 24hr postischemia and returned to preischemic levels by 72 hr.

Table 4.2 Pattern of body weight post-ischemia

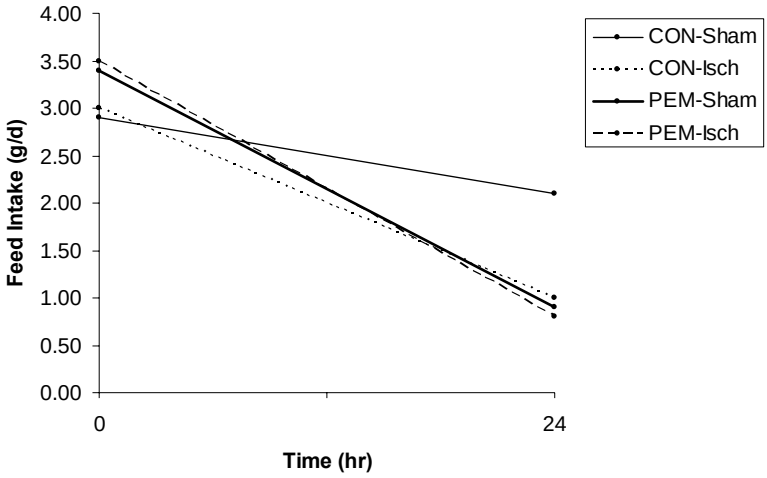
| Timepoint | CON-Sham | CON-Isch | PEM-Sham | PEM-Isch |
|---|---|---|---|---|
| 6hr | 66.4 ± 0.8 | 66.1 ±1.0 | 56.3 ± 1.2* | 54.1 ± 0.8* |
| 24hr | 65.9 ± 0.7 | 64.8 ± 1.8 | 55.5 ± 1.8* | 53.5 ± 1.2* |
| 72hr | 66.2 ± 1.2 | 62.6 ± 1.6 | 51.4 ± 1.6* | 49.5 ± 1.4* |

Results in the table are shown as mean ± SEM, n=6 for each ischemic group, n=3 for each sham group. *PEM significantly decreased body weight at all timepoints (2-factor ANOVA, P<0.001).

### 4.1.2 Serum Glucose

Serum glucose concentration at 6 hr postischemia is shown in Figure 4.2. There was no significant effect of diet (p=0.242) or ischemia (p=0.359) on serum glucose and no interaction between diet and ischemia (p=0.844). Table 4.3 shows data for serum glucose concentration when data for animals with incomplete ischemia are removed.

A




B

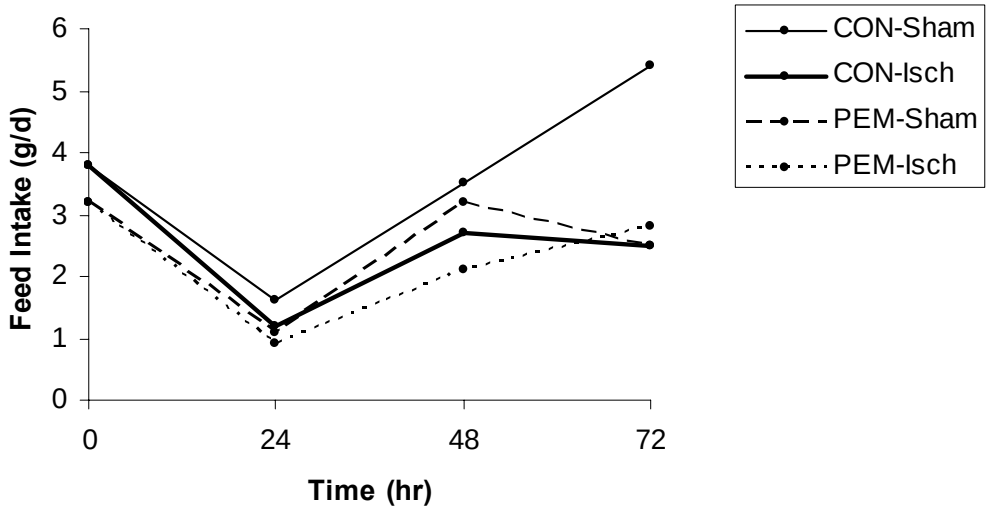



Figure 4.1 Pattern of postischemic feed intake. Average feed intake/day was calculated as total feed intake/day/number of animals in the cage. n =2 cages (3 animals /cage) for each ischemia group, n=1 cage (3 animals /cage) for each sham group. Animals taken to a 24hr endpoint are shown in A and 72hr endpoint data are shown in B. CON-Sham: sham animals with control diet; CON-Isch: ischemic animals with control diet; PEM-Sham: sham animals with PEM; PEM-Isch: ischemic animals with PEM.

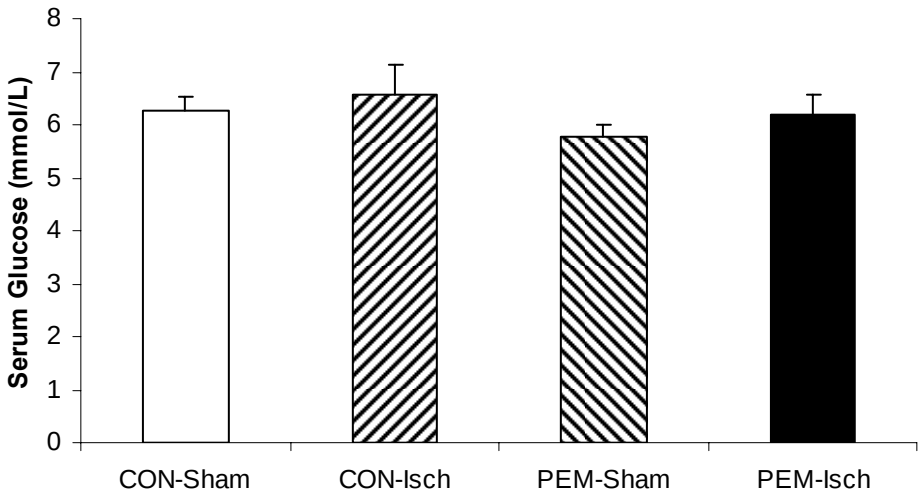



Figure 4.2 The effect of PEM and ischemia on serum glucose concentrations at 6hr postischemia. Results are presented as mean ± SEM; n=6. No significant effects of treatments were found by a two-factor ANOVA ($p>0.05$). CON-Sham: sham animals with control diet; CON-Isch: ischemic animals with control diet; PEM-Sham: sham animals with PEM; PEM-Isch: ischemic animals with PEM.

Table 4.3 Individual serum glucose concentrations at 6 hr postsurgery for gerbils with sham surgery or complete forebrain ischemia.

| Serum Glucose (mmol/L) | | | |
|---|---|---|---|
| CON-Sham | CON-Isch | PEM-Sham | PEM-Isch |
| 7.2 | 5.3 | 5.5 | 5.5 |
| 7.4 | 4.9 | 5.4 | 5.6 |
| 6 | | 6.7 | 6.7 |
| 6.5 | | 5.4 | 6.3 |
| 5.8 | | 5 | |
| 4.8 | | 6 | |

### 4.1.3 Serum Cortisol

Serum cortisol concentration at 6hr postischemia is shown in Figure 4.3. There was no significant effect of diet (p=0.878) or ischemia (p=0.702) on serum cortisol and no interaction was found between ischemia and diet (p=0.832). Table 4.4 shows data for serum cortisol concentration when data for animals with incomplete ischemia are removed.

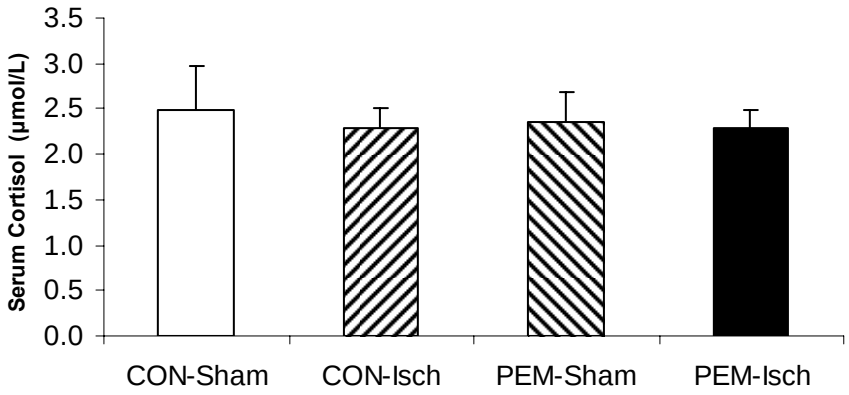



Figure 4.3 The effect of PEM and ischemia on serum cortisol concentration at 6hr postischemia. Results are mean ± SEM; n=6. No significant effects of treatments were found by a two-factor ANOVA (p>0.05). CON-Sham: sham animals with control diet; CON-Isch: ischemic animals with control diet; PEM-Sham: sham animals with PEM; PEM-Isch: ischemic animals with PEM.

### 4.1.4 Activity Monitoring

Activity criteria for complete ischemia were set at >3SD above mean activity of sham animals (n=30) as described in the Methods section. This was calculated as 654 mean counts/20 hr. In addition, hyperactivity had to be present for the entire monitoring period. 45.8% (11/24) of occluded animals passed this criteria.

Table 4.4 Individual serum cortisol concentrations at 6 hr postsurgery for gerbils with sham surgery or complete forebrain ischemia.

| Serum Cortisol (μmol/L) | | | |
|---|---|---|---|
| CON-Sham | CON-Isch | PEM-Sham | PEM-Isch |
| 1.1 | 1.6 | 3.2 | 5.5 |
| 4.6 | 3.0 | 3.7 | 5.6 |
| 4.9 | | 3.2 | 6.7 |
| 1.3 | | 1.1 | 6.3 |
| 3.6 | | 4.3 | |
| 4.1 | | 3.0 | |

Figure 4.4 shows representative major patterns of activity obtained when activity was monitored over 20hr as an indicator of complete ischemia. Sham animals showed very low activity over the entire period. The pattern of activity seen in animals subjected to ischemia can be divided into three subgroups. 1) A group showing persistent hyperactivity which indicates complete ischemia. 2) A second group showing low activity representative of incomplete ischemia. 3) The third subgroup had hyperactivity which was not persistant over the entire time of monitoring, also representative of incomplete ischemia.

Table 4.5 shows the activity data obtained from individual animals in this experiment. The data from animal killed at 6 hr could not be analyzed because 4 hr is not a sufficiently long period to assess persistent hyperactivity.

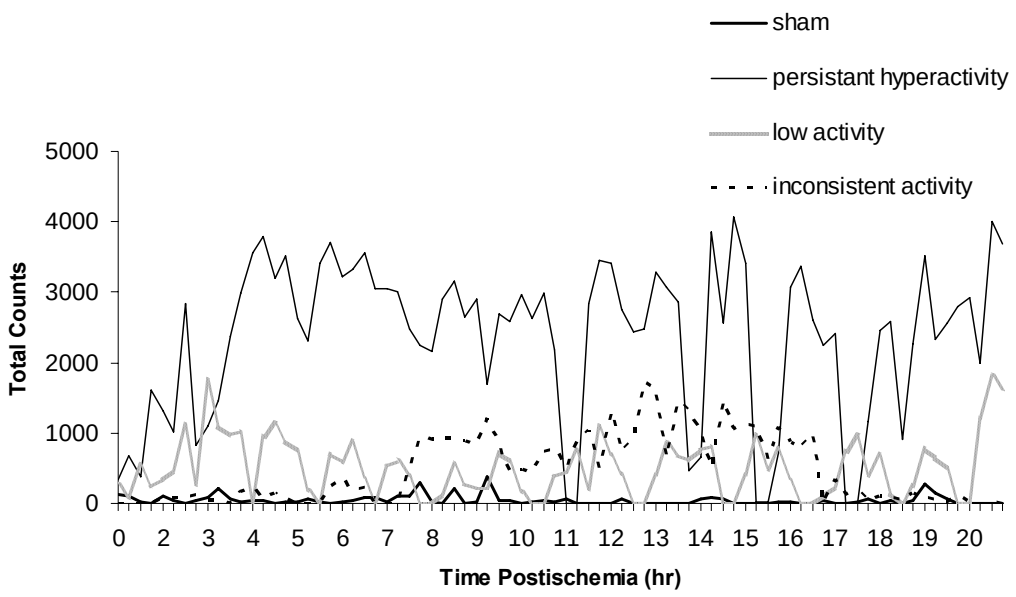



Figure 4.4 Pattern of activity postischemia.

Table 4.5 Activity monitoring on individual animals to assess complete ischemia

| Activity (Mean Counts/20hr) | | | |
|---|---|---|---|
| 24hr CON-Isch | 24hr PEM-Isch | 72hr CON-Isch | 72hr PEM-Isch |
| 72 | 1015* | 170 | 46 |
| 2002* | 224 | 2348* | 475 |
| 1961* | 1000* | 179 | 1306* |
| 31 | 2974* | 123 | 502 |
| 234 | 526 | 904* | 2056* |
| 107 | 999* | 179 | 1367* |

* indicates the animal passed criteria >mean + 3SD for sham animals (=654 mean counts/20 hr) and the activity was persistent over 20hr.

### 4.1.5 NFkB Activation Analysis

#### 4.1.5.1 Protein Analysis of hippocampal Nuclear Extracts

Total protein concentrations of nuclear extracts are shown in Table 4.6. There was no significant effect of PEM (p=0.210, 0.108, 0.972 at 6hr, 24hr, and 72hr respectively) on protein concentration. Ischemia significantly increased protein concentration at 24 hr (p=0.013) but not at 6 hr (p=0.065), and there was no effect at 72 hr (p=0.854). There was no interaction between diet and ischemia at any timepoint.

Table 4.6 Protein concentration of hippocampal nuclear extracts at different timepoints post-ischemia (mg/ml)

| | CON-Sham | CON-Isch | PEM-Sham | PEM-Isch |
|---|---|---|---|---|
| 6 hr | 3.2 ± 0.4 | 2.0 ± 0.3 | 2.2 ± 0.4 | 2.0 ± 0.3 |
| 24 hr | 1.7 ± 0.2 | 2.5 ± 0.2* | 2.3 ± 0.3 | 2.6 ±0.1* |
| 72 hr | 2.1 ± 0.4 | 2.2 ± 0.2 | 2.3 ± 0.3 | 2.0 ± 0.3 |

Protein concentrations in the table are shown as mean ± SEM. n=3 for each sham group; n=6 for all ischemic groups except n=5 for 24 hr CON-Isch group. Pooled mean ± SEM for ischemic groups are 2.0 ± 0.2, 2.6 ± 0.1, and 2.1 ± 0.2 mg/ml at 6 hr, 24 hr, and 72 hr respectively; pooled mean ± SEM for sham groups are 2.7 ± 0.3, 2.0 ± 0.2, and 2.2 ± 0.2 mg/ml at 6 hr, 24 hr, and 72 hr respectively. * Protein concentration was increased by ischemia (p=0.013).

#### 4.1.5.2 NFkB Activation as Assessed by EMSA

Figure 4.5 shows representative autoradiograms of NFκB activation in hippocampal nuclear extracts measured by electrophoresis mobility shift assay (EMSA). Generally, activation of NFκB appeared to be more pronounced as a result of PEM than from global ischemia.

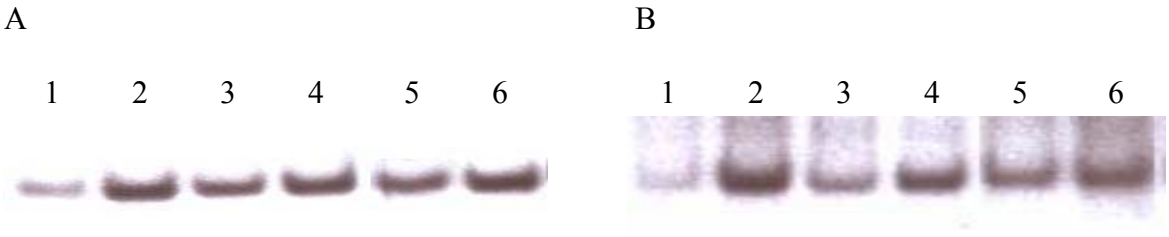



Figure 4.5 Representative autoradiograms of NFκB activation in hippocampal nuclear extracts measured by EMSA. A, Effect of ischemia on NFκB activation at various times postischemia. Times postischemia are 6 hr, lanes 1 and 2, 24 hr, lanes 3 and 4, and 72 hr, lanes 5 and 6; Lane 1 = CON-Isch; Lane 2 = PEM-Isch; Lane 3 = CON-Isch; Lane 4 = PEM-Isch; Lane 5 = CON-Isch; Lane 6 = PEM-Isch. B, Effect of PEM on NFκB activation. Times postsurgery are 6hr, lanes 1-2, 24hr, lanes 3-6; Lane 1 = CON-Sham; Lane 2 = PEM-Sham; Lane 3 = CON-Sham; Lane 4 = PEM-Sham; Lane 5 = CON-Isch; Lane 6 = PEM-Isch.

#### 4.1.4.3 Semi-quantification of EMSA Results

At 6 hr postsurgery, PEM significantly increased NFκB activation ($p=0.014$) (Figure 4.6). Ischemia didn't significantly affect NFκB activation ($p=0.169$) and there was no interaction between diet and ischemia ($p=0.270$). Because of the pronounced effects of PEM on NFκB activation independent of ischemia and the small sample size, those animals with incomplete ischemia were included in the analysis.

At 24hr postsurgery, there was a trend for PEM to increase NFκB activation ($p=0.097$) (Figure 4.7). There was no significant effect of ischemia ($p=0.644$) or interaction between diet and ischemia ($p=0.360$). Table 4.7 shows data for NFκB activation when data for animals with incomplete ischemia are removed.

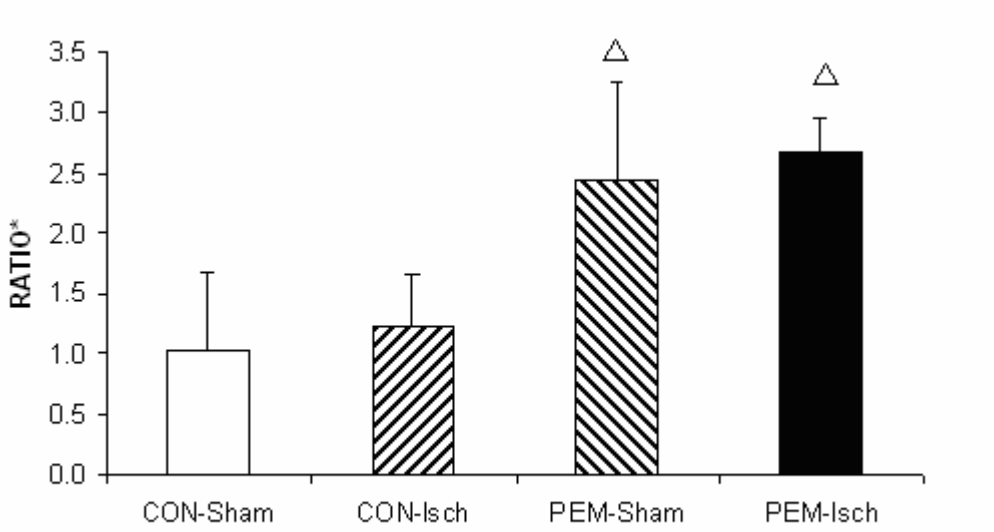



Figure 4.6 The effect of PEM and ischemia on NFκB activation in hippocampus at 6 hr postsurgery. Results are presented as mean ± SEM; n=6 for each ischemic group and n=2 for each sham group. $^{\Delta}$ There is a significant effect of diet by a two-factor ANOVA ($p=0.014$). * Arbitrary density unit of the experimental sample/μg protein divided by the arbitrary density unit of the reference sample/μg protein.

Table 4.7 Individual hippocampal NFκB activation ratios at 24 hr postsurgery for gerbils with sham surgery or complete forebrain ischemia.

| **NFκB activation (Ratio)*** | | | |
|---|---|---|---|
| CON-Sham | CON-Isch | PEM-Sham | PEM-Isch |
| 0.71 | 2.31 | 2 | 3.89 |
| 1.3 | 1.95 | 1.33 | 3.28 |
| | | | 1.35 |
| | | | 1.51 |

* Arbitrary density unit of the experimental sample/μg protein divided by the arbitrary density unit of the reference sample/μg protein.

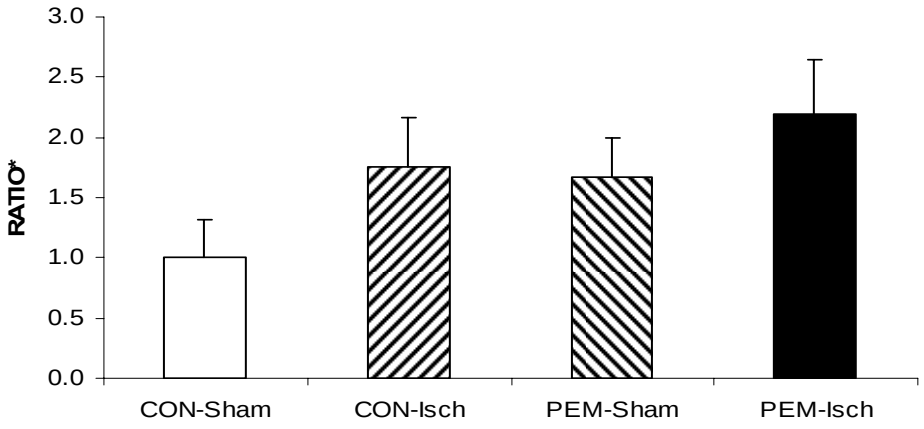



Figure 4.7 The effect of PEM and ischemia on NFκB activation in hippocampus at 24 hr postsurgery. Results are presented as mean ± SEM; n=6 for PEM-Isch group, n=5 for CON-Isch group, n=2 for each sham group. There was a trend for PEM to increase NFκB activation by a two-factor ANOVA (p=0.097). * Arbitrary density unit of the experimental sample/μg protein divided by the arbitrary density unit of the reference sample/μg protein.

At 72 hr postsurgery, the increased NFκB activation caused by PEM appeared to be absent by a two-factor ANOVA analysis (Figure 4.8) (diet, p=0.189; ischemia, p=0.762; interaction between diet and ischemia, p=0.983). However, these data are technically flawed because too much radioactivity was added to the probe, and the films were saturated, resulting in loss of sensitivity to detect differences in NFκB activation.

### 4.1.6 Correlation of NFκB activation and Postischemia Activity

Correlation data between NFκB activation and postischemia activity from the animals taken to a 24 hr postischemia endpoint are shown in Figure 4.9. There was no

significant correlation between NFκB activation and activity level in ischemic animals fed control diet as detected by a Pearson's Correlation Coefficient ($p=0.132$, $R^2=0.764$, $n=5$). The correlation was also not significant in ischemic animals with PEM ($p=0.790$, $R^2=-0.141$, $n=6$). Similar analysis could not be performed for the other two timepoints. At 6hr, accurate activity data could not be collected to access if hyperactivity was persistent. At 72 hr, the NFκB activation data were flawed due to saturation of the film.

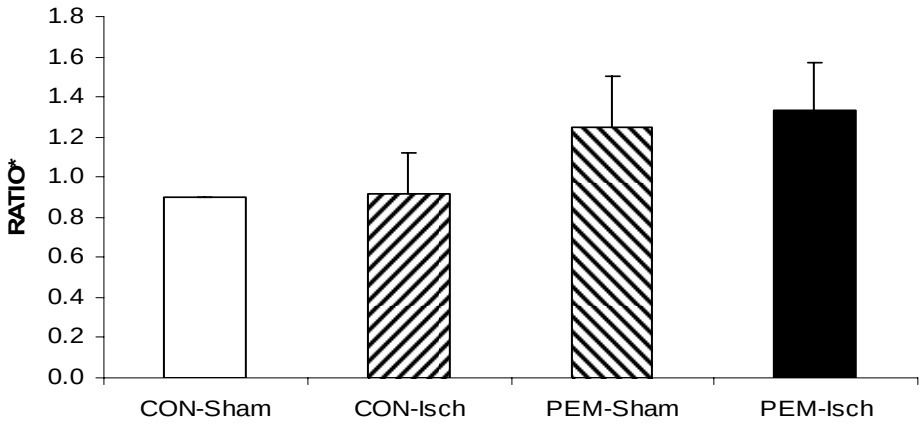



Figure 4.8 The effect of PEM and ischemia on NFκB activation in hippocampus at 72 hr postsurgery. Results are shown as mean ± SEM; n=6 for each ischemic group and n=2 for each sham group. There were no significant differences shown by a two-factor ANOVA ($p>0.05$). * Arbitrary density unit of the experimental sample/μg protein divided by the arbitrary density unit of the reference sample/μg protein.

## 4.2 Experiment 2

### 4.2.1 Body Weight and Feed Intake

The data on body weight and feed intake showed the same pattern as in the first experiment. The initial body weight was not significantly different between CON group

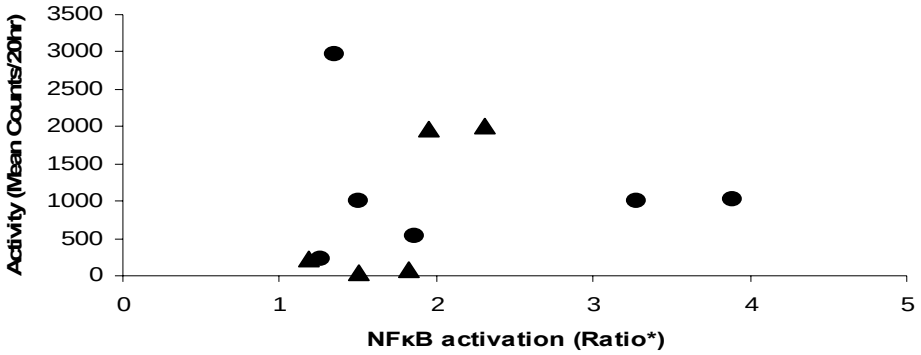



Figure 4.9 Correlation of NFκB activation and postischemia activity level at 24 hr postsurgery. ▲indicates the ischemic animals on control diet (n=5); ● indicates the ischemic animals with PEM (n=6). * Arbitrary density unit of the experimental sample/μg protein divided by the arbitrary density unit of the reference sample/μg protein.

and PEM group (p=0.809) (Table 4.8). After the 28-day period of feeding, the CON group gained on average 4.3g body weight and the PEM group lost 7.1g. The difference in 28-day body weight and body weight change is significant (both $p<0.001$). Total 28-day feed intake is also significantly different between CON and PEM animals ($p<0.001$).

Table 4.8 Body weight and feed intake

| | CON | PEM |
|---|---|---|
| Initial Body Weight (g) $^{\pi}$ | 62.8 ± 0.6 | 63.4 ± 0.4 |
| Day28 Body Weight (g) $^{\pi}$ | 67.1 ± 0.9 | 56.4 ± 0.7* |
| 28-Day Body Weight Change (g) $^{\pi}$ | + 4.3 ± 0.9 | - 7.1 ± 0.7* |
| 28-Day Feed Intake (g) $^{\lambda}$ | 105.1 ± 4.0 | 91.7 ± 3.1* |

Results in the table indicate mean ± SEM; * Indicates significantly different from control group by unpaired T-test. $^{\pi}$ n = 6, $^{\lambda}$ n = 2 cages (3 animals/cage); presented as average intake/animal/cage.

### 4.2.2 NFκB Activation Analysis

Figure 4.10 shows that hippocampal NFκB activation was increased in PEM animals compared to CON animals even in the absence of sham surgery. Unlike the previous EMSA results which were presented as a ratio (relative to data from a reference animal), these data are shown as actual density per μg protein. The reason is that these data come from the reference animals and they were thus run on separate gels. PEM consistently increased NFκB activation when compared to the control animal run on the same gel. The variability is much higher than in Experiment 1 because of the inability to control for variability among gels, and thus the effect of PEM is not significant ($p=0.320$; $n=4$) by unpaired t-test.

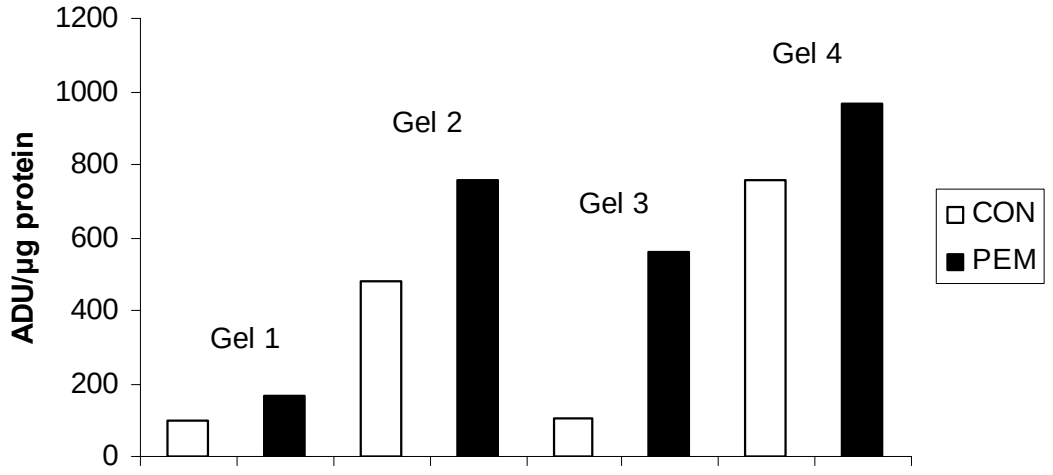



Figure 4.10 The influence of PEM on hippocampal NFκB activation in the absence of sham surgery. The results are given as density units per μg protein. Each pair of values presents the results from one gel. ADU, Arbitrary density unit.

# CHAPTER 5

# DISCUSSION

It has been previously shown in our laboratory that gerbils fed with 2% protein diet for 28 days have decreased feed intake, significant weight loss, decreased liver GSH, increased liver lipid concentration (17) and decreased serum albumin concentration (210), indicating that a moderate PEM was achieved. Consistently, using the same model in the current study, gerbils fed 2% protein had the same magnitude of decrease in feed intake and weight loss. Gerbils appeared normal with no signs of sparse hair, edema, diarrhea or other signs of severe protein deficiency. Our model is distinguished from two other models of PEM. One is a milder protein deficiency induced by feeding 7.5% protein in which voluntary feed intake is not decreased (211, 212). The second is a severe PEM in which rats are fed a protein-free diet for two weeks, causing severe protein deficiency symptoms (213). Increased liver lipid in our model is consistent with a diet that supplies a relatively higher proportion of energy from carbohydrate than protein, causing decreased synthesis of the apolipoprotein required for transport of triglycerides out of the liver (214). Serum albumin is the protein in highest concentration in human blood and key to the regulation of the osmolality of blood. Due to its half-life of eighteen to twenty days, serum albumin is not necessarily decreased in early PEM (215). However, as severe protein depletion worsens, albumin and other serum protein decrease, resulting in reduced intravascular oncotic pressure and edema. The combination of decreased feed intake and body weight shown in this study combined with previous findings of increased liver lipid and decreased serum albumin indicate that our model is a combination of both protein and energy deficiency, which also can be characterized as marasmic kwashiorkor.

PEM resulted in a loss of body weight at all times sampled during the post-ischemic period, and this did not differ between the PEM sham and PEM ischemic groups. This was seen in a previous study using the same model of global ischemia (146). The ischemic group fed control diet also lost weight following surgery. Because of the small sample size, post-ischemic feed intake was shown only descriptively. Feed intake appeared to decrease after surgery in all groups and return to pre-ischemic level by three days. Post-ischemic feed intake and weight loss in rodent surgical models of stroke are often not reported but are very important as they can exert effects on brain damage independent of the experimental factors being studied, and thus are potential confounding variables.

Our laboratory has previously shown that PEM impairs functional outcome up to 10 days following global ischemia as measured by habituation in the open field. A subset of these animals also showed marked reactive gliosis that correlated with the functional impairment (17). Reactive gliosis is characterized by the hypertrophy of astrocytes and proliferation of both astrocytes and microglial cells around the injured region (216). These findings suggest that the relationship between undernutrition immediately after stroke and reduced survival, functional ability and living circumstances six months later detected by the FOOD Trial Collaboration may be causal (16). The findings also support those of another study demonstrating that stroke patients had a shorter hospital stay when fed within 72 hr of the ischemic insult (144).

The major objective of the current study was to identify a mechanism by which PEM impairs outcome following global ischemia. The reactive gliosis suggested an increased inflammatory response. Inflammatory mechanisms modulate the proliferation of reactive astrocytes (153), and microglia can produce toxic molecules including pro-inflammatory

cytokines and also reactive species including $NO^{\bullet}$, oxygen radicals and arachidonic acid derivatives (154). PEM had been shown to increase the inflammatory response in other studies. The inflammatory mediators, IL-6, C-reactive protein, and soluble receptors of TNF-α, were found to be greater in the plasma of PEM children (18). Since NFκB plays a central role in regulating the inflammatory response following ischemia (132), and protein-malnourished mice show increased activation of NFκB and increased transcription of its downstream genes, IL-1β and TNF-α, in liver when induced by lipopolysaccharide (20), we questioned if the effects of PEM on outcome in global ischemia could be linked to increased NFκB activation.

In this study, PEM caused a marked increase, independent of ischemia, in increasing NFκB activation in hippocampus at 6 hr following global ischemia ($p=0.014$) and a trend at 24 hr ($p=0.097$). This is the first report of PEM influencing NFκB activation in brain. The data collected at 72 hr post-ischemia were technically flawed, so it is not known if there is prolonged activation of the transcription factor. It is important to examine this in future studies because prolonged NFκB activation in PEM following ischemia could regulate secondary damage. Clemens et al (11) found that NFκB activation was increased in CA1 neurons at 72 h following global ischemia in rats. The persistent increase of NFκB activation at 72 h after ischemia may be due to the inability of these neurons to synthesize IκB because of the long-term inhibition of synthesis of many proteins that occurs after global ischemia. Global ischemia may also cause a prolonged inhibition of synthesis of protective factors in this area. Another possibility is that persistent NFκB activation induces production of pro-apoptotic proteins.

The findings that PEM increases NFκB activation after global ischemia suggest that protein-energy deficiency, if present at the time of stroke, will increase the inflammatory

response. Future studies should investigate the pro-inflammatory target genes that are regulated by NFκB to determine whether PEM enhances the inflammatory response following brain ischemia. For example, the cytokine, TNF-α, a mediator of several cellular and molecular mechanisms involved in the development of ischemic damage, directly promotes activation and proliferation of glial cells (217). By examining the TNF-α expression using immunohistochemistry or enzyme-linked immunosorbent assay, it could be determined if the marked reactive gliosis observed following global ischemia in protein-energy malnourished gerbils (17) is linked to activated NFκB with associated upregulation of TNF-α production. Similarly, gene expression of IL-1β, ICAM-1, COX-2 and iNOS are also regulated by NFκB and are good markers to investigate the inflammatory response following ischemia.

Although it was hypothesized that PEM would enhance NFκB activation stimulated by global ischemia, no interaction between PEM and global ischemia was observed in this study. This finding needs to be confirmed with further study because of several limitations of this study. The first was that an insufficient number of sham animals was included in the study. A second limitation is the variability introduced by the inconsistent severity of ischemia related to the presence of unilateral or bilateral posterior communicating arteries in some gerbils. Screening for hyperactivity identified that 54.2% of the study animals with bilateral carotid occlusion were exposed to incomplete ischemia. All occluded gerbils were included in the NFκB analysis because there was an obvious increase in NFκB activation seen across gels for samples from all protein-energy malnourished animals, regardless of ischemic severity. Although the correlation between NFκB activation and hyperactivity monitoring at 24 hr post-ischemia was not statistically significant for either the control or protein-energy malnourished animals, the correlation

was much higher in the control group (P=0.132) than in the PEM animals (P=0.780), supporting the idea that PEM might be an independent predictor of NFκB activation. With a larger sample size, it appears that the correlation between activity and NFκB activation might become significant in the control group only. However, this type of correlation analysis relies on the assumption that the relationship between ischemic severity and persistent hyperactivity is linear. While hyperactivity is a good predictor of ischemic severity (198, 199), there are no data demonstrating that the relationship is linear. It will be necessary to undertake further studies with large sample size and hyperactivity monitoring to screen out gerbils with incomplete ischemia to determine if PEM and ischemia interact or act independently to increase NFκB activation.

We also did not observe the elevated NFκB binding at 6 and 24 hr post-ischemia demonstrated by others in both focal and global ischemia (8-11, 129, 130). Domanska-Janik et al (8) have observed NFκB binding as early as 3 hr and continuing up to 7 days in hippocampus after global ischemia in the gerbil. Clemens and coworkers (10, 11) found that neuronal NFκB immunoreactivity in the hippocampus was induced 6 hr after global ischemia and persisted for three days until apoptotic degeneration occurred in CA1 neurons (10). Importantly, similar observations were obtained by Schneider and coworkers (130) in mice in which prominent p65 immunoreactivity was seen in the apoptotic neurons of the penumbral area 3 days after transient focal ischemia.

There are a number of reasons that might explain why we did not detect an increase in NFκB activation with ischemia. High variability in NFκB activation occurred following global ischemia, and this can be accounted for by the inclusion of animals with incomplete ischemia in which one expects to find attenuated NFκB activation. This is supported by Figure 4.7 which shows increasing but variable NFκB activation in the

ischemic group fed control diet. Furthermore, the unexpected high NFκB activation found in protein-energy malnourished sham animals and the small sample size for both sham groups made it more difficult to detect an increase in NFκB activation with ischemia in animals fed control diet.

Whether NFκB activation is neurodegenerative or neuroprotective remains a controversial question. In studies of cerebral ischemia, there is evidence for both, although there is more evidence indicating that sustained NFκB activation is detrimental, leading to prolonged secondary damage (218). This is supported by a large amount of research that revealed persistent NFκB activation following ischemia associated with increased cytokines (110, 111, 114) and iNOS (122), which promote neuronal death. However, it appears that NFκB is a signal transducer that has dual effects on the immune response (218). As previously reported (8, 219), we detected constitutive binding activity of NFκB in hippocampus of sham controls. Depending on the intensity and duration of the stimuli, low level and transient NFκB activation could be important and necessary as a key regulator for the innate immune response. This is supported by data showing that neuronal damage can be increased under conditions when NFκB activation is impaired. In hippocampus of p50 knockout mice, NFκB DNA-binding activity is decreased and neuronal damage has been reported to be increased after the administration of the excitotoxin kainate (220). However, this evidence is in apparent contrast to that of Schneider et al (130) in which p50 knockout mice had significantly reduced neural damage in response to cerebral ischemia. Future studies with longer survival times are needed to reveal the role of NFκB activation in ischemia. Increased NFκB activation in hippocampus from protein-energy malnourished animals could be an explanation for an increased inflammatory response leading to the reactive gliosis previously reported in our

laboratory (17). However, the current results do not explain the impaired functional outcome demonstrated in the protein-energy malnourished gerbils following global ischemia because enhanced NFκB activation is also present in protein-energy deficient gerbils exposed to sham surgery. The latter animals showed normal habituation patterns in the open field (17).

The results of this study have been significantly hampered by the high proportion of animals detected by activity monitoring to have a partial or complete circle of Willis, which is known to cause high variability in hippocampal CA1 damage (204). As we described before, using our criteria of persistent hyperactivity over 20 hr as an indirect indicator of ischemic severity, 54.2% of animals exposed to bilateral carotid artery occlusion in this study showed incomplete ischemia. This number compares quite well with the 61.4% reported by Laidley and coworkers when they used direct assessment of cerebral vasculature to identify bilateral or significant unilateral posterior communicating arteries in gerbils from Charles River (204). Our laboratory activity screening method has recently been shown by a co-worker in the laboratory to classify approximately ~85% of gerbils correctly when assessed against histological evaluation of hippocampal CA1 neuron death (Monique Harmon, unpublished communication). Using the same experimental model, combined data from both experiments showed that PEM did not alter the hyperactivity ($p=0.897$) or interact with ischemia ($p=0.782$). This screening method is therefore useful for studying PEM and impaired outcome. However, it is not suitable for investigating treatments that improve ischemic outcome, since neuroprotective agents interfere with activity (198) and thus would confound the screening tool. Although this screening could be used to reduce inter-animal variability to an acceptable level, a more direct method with greater accuracy suggested by Laidley et

al (204), is to use laser Doppler flowmetry to ensure blood flow is reduced to an acceptable level in the brain region of interest. Regardless of the method of screening, however, the need to screen out such a high proportion of animals will lead to unacceptably high cost and time required to complete an experiment. Unless a supply of gerbils bred to consistently lack posterior communicating arteries becomes commercially available, it is likely that the gerbil model of global ischemia will be abandoned by stroke researchers and replaced by either the 2-vessel occlusion or 4-vessel occlusion models of global ischemia in the rat.

After demonstrating that NFκB activation was also increased in hippocampus from protein-energy deficient gerbils exposed only to sham surgery, Experiment 2 was performed to determine if this was also the case in the absence of surgery. Again, we found greater NFκB activation in hippocampus with PEM. These findings add further weight to those of Experiment 1, in which the sample size for sham animals was small. While unrelated to the subject of this thesis, this finding has important implications since PEM is still a global problem. In 2000, the WHO Global Database showed that the prevalence of PEM in children under five years of age in developing countries world-wide was 32.5 % (221). In adults, PEM may occur secondary to many diseases (222). Our observation suggests that increased NFκB activation and inflammation may be a general feature of PEM. It will be important to investigate the effect of PEM on NFκB activation in other tissues, such as liver, lung and kidney, so that the impact of PEM on the inflammatory response of the whole body can be revealed. Alternatively, the increased NFκB activation in PEM could represent a response to pre-surgery stress or anaesthesia. A survival time longer than 24 hr is needed to confirm whether PEM causes a sustained increase in NFκB activation or a short-term response to stress.

The study results are also limited by the technical difficulty of running EMSA. While an EMSA provides good evidence for NFκB binding to DNA, semi-quantifying accurately with densitometry across different gels is difficult. Which dimers of NFκB are translocated has not been determined in this study which used gel shift methodology. This can be determined in future with supershift assays which employ the same techniques but with the addition of antibodies against the subunits of NFκB. A DNA-binding ELISA could also provide a more quantitative approach to measuring NFκB activation and specific subunit translocation. Future studies should also investigate whether PEM decreases the cytosolic inhibitor, IκB, which allows the increased NFκB translocation into the nucleus. Phosphorylation and degradation of IκBα has been shown to be involved in NFκB activation and deactivation in ischemia (104, 135). IκBα can also be measured by DNA-binding ELISA.

This study has not addressed the mechanisms by which PEM affects hippocampal NFκB activation. As described, PEM may affect the inflammatory response (18, 19, 150) and oxidative stress (17, 223), two key regulators of NFκB activation. There is a wide range of candidate molecules that could be responsible for the increase in NFκB activation. For example, as an inflammatory mediator, TNF-α is one of the stimuli that could be directly triggering NFκB activation since TNF-α levels have been found to be higher in plasma from protein-energy malnourished children (18). Thus, PEM may trigger NFκB activation through increasing TNF-α expression.

PEM may also affect NFκB activation by influencing cell redox status, possibly by decreased GSH concentration in the brain. Cysteine is the limiting amino acid for GSH synthesis (2). In PEM, decreased dietary cysteine leads to limited GSH synthesis (146). Decreased GSH has been demonstrated in the liver of protein-energy malnourished

gerbils (17) and in liver and lung of protein-energy deficient rats (158) and protein-deficient mice (157). When substrate is limited, GSH may also be used to supply cysteine for critical proteins, resulting in a more vulnerable antioxidant defense system (224). The response of brain is less clear. Bobyn et al (159) have shown decreased GSH concentration in neocortex and thalamus of rats with acute, severe dietary sulfur amino acid deficiency. However, brain GSH appeared to be well regulated and unaffected by a low protein diet in mice and rats (211, 225, 226). Although it was hypothesized that PEM would exacerbate a global ischemia-induced depletion of GSH due to decreased substrate for synthesis in the face of increased demand, brain GSH was not decreased by PEM at 12 hr following global ischemia in previous studies in our laboratory (17). However, this will need to be re-evaluated in a more consistent model of global ischemia since GSH was not depleted by global ischemia in animals fed control diet, suggesting that oxidative stress was mild. This is in contrast to most reports in global ischemia models in which brain GSH is markedly depleted (62, 184, 227, 228) and is likely related to varying degrees of ischemic severity in our failing global ischemia model.

It is also possible that PEM increases NFκB activation through an increase in oxidative stress that is independent of a change in GSH concentration.
This is supported by the study of Bobyn et al (17) which reported PEM- and ischemia-induced decreases in hippocampal protein thiol levels and a trend ($p=0.07$) for GSH reductase activity in hippocampus to decrease with ischemia only when the animals were also protein-energy malnourished. These serve as markers of oxidative stress because of the susceptibility of thiols groups to oxidation (160).

Other studies also support an increase in oxidative stress with protein deficiency. Increased protein carbonyls in hippocampus, as a marker of oxidative damage to proteins,

has been reported in protein malnourished rats, as has increased activity of the antioxidant enzyme, SOD (223). The latter can lead to the production of hydrogen peroxide, which can react with iron to generate hydroxyl radicals, which are believed to be the most toxic oxygen molecules in vivo (223). In patients with kwashiorkor, the plasma total antioxidant status has been reported to be reduced to less than 50% of that of healthy controls, whereas erythrocyte GSH concentration was decreased and plasma nitrite and nitrate were increased (229). This evidence suggests that PEM may affect cell redox status which could regulate NFκB activation and modify stroke outcome.

This study was also designed to rule out the possibility that PEM influences the outcome from global ischemia by altering serum glucose or GCs. These physiological variables are known to affect ischemic brain injury. Hyperglycemia has been reported in several brain ischemia models and is correlated with poor prognosis in ischemic stroke because elevated glucose levels lead to elevated intra-ischemic lactic acid levels and lactic acidosis, thus exacerbating brain damage (164). PEM has been shown in several studies to decrease serum glucose (21, 23), while insulin has been reported decreased to promote glucose homeostasis (24). However, in the model of malnutrition used in this study, serum glucose was not affected by PEM or ischemia, at least at 6 hr post-ischemia. Although only one timepoint has been measured so far, there is no evidence at present that PEM alters ischemic brain injury by influencing glucose homeostasis. This question could be addressed better in the rat models of global ischemia in which the animal's larger blood volume would allow blood sampling at several timepoints surrounding ischemia.

High GCs have been associated with cognitive dysfunction and severity of hemiparesis after stroke, and may predict poor stroke outcome (172-174). A high level of

GCs is often reported in response to acute deficits of protein and energy (24) although this varies with severity and age of onset of the deficiency. Blood cortisol in acutely protein-energy malnourished children (169) and serum corticosterone concentration in acutely protein-energy malnourished young adult rodents (171) have been reported to range from 1.3 to 6-fold that found in matched healthy subjects. As well as influencing ischemic brain injury, GC levels were of interest because of their ability to decrease NFκB activation (155). In the current study, we found no effect of PEM on serum cortisol concentration. However, the serum cortisol data may be flawed as the levels were variable and unphysiologically high. This is likely due to acute pre-anesthesia stress. As stress-induced inflation of basal levels has been shown to mask an effect caused by PEM (230)**,** these results should be confirmed in another study to confirm that PEM is not mediating its effects on ischemic outcome through increased circulating GCs. Shipp and Woodward (230) have published a protocol for anesthesia induction and acclimatization of animals to transport and environment that minimizes this problem.

In conclusion, the results of this study demonstrated that PEM increases hippocampal NFκB activation following global ischemia. Whether the increased NFκB activation causes an increase in its proinflammatory target genes is important to investigate in future studies, as the findings suggest that patients who are protein-energy compromised at the time of presentation for stroke will have an increased inflammatory response. Whether PEM increases NFκB activation by increasing oxidative stress should also be studied in an alternate model of global ischemia in which neuronal damage is more consistent. These factors are essential for determining the death or survival of neurons following ischemia. The data suggest that neither serum cortisol nor serum glucose influenced ischemic severity in the protein-energy malnourished gerbils, as neither parameter was

altered at 6 hr following global ischemia. Since PEM enhanced hippocampal NFκB activation in sham surgical controls and in the absence of surgery as well, the findings may also have other important implications for the inflammatory response of the many individuals affected globally by malnutrition.

Future Directions:

1. The results of this study have been significantly hampered by the high proportion of gerbils having a partial or complete circle of Willis. This is known to cause high variability in hippocampal CA1 damage following bilateral carotid artery occlusion. Therefore, an alternate global ischemia model, such as the rat 2-vessel or 4-vessel occlusion model, should be used for future studies.
2. It is important to further investigate which specific NFκB dimer is activated in hippocampus by PEM. This can be done by using antibodies against p50 and p65 in a supershift assay or DNA-binding ELISA.
3. It is also important to investigate whether the increased NFκB activation observed with PEM causes an increase in its pro-inflammatory target genes such as TNF-α and IL-1β. This can be measured by RT-PCR, ELISA, and immunohistochemistry.
4. Future studies should also investigate whether PEM decreases the cytosolic inhibitor, IκB, since phosphorylation and degradation of IκB allows NFκB translocation into the nucleus. IκB can be measured by ELISA.
5. Whether PEM increases NFκB activation in other tissues, such as liver, kidney, heart and lung, needs to be determined as well so that the impact of PEM on the inflammatory response of the whole body can be revealed.

References:

1. Statistics & Background Information - Stroke Statistics. Heart and Stroke Foundation of Canada; 2002.
2. Juurlink BHJ, Paterson PG. Review of oxidative stress in brain and spinal cord injury. J Spinal Cord Med. 1998;21:309-34.
3. Dirnagl U, Iadecola C, Moskowitz MA. Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci. 1999;22:391-7.
4. Juurlink BHJ, Sweeney MI. Mechanisms that result in damage during and following cerebral ischemia. Neurosci Biobehav Rev. 1997;21:121-8.
5. Han HS, Yenari MA. Cellular targets of brain inflammation in stroke. Curr Opin Investig Drugs. 2003;4:522-9.
6. Lee J, Burckart GJ. Nuclear factor kappa B: important transcription factor and therapeutic target. J Clin Pharmacol. 1999;38:981-3.
7. Cechetto DF. Role of nuclear factor kappa B in neuropathological mechanisms. Prog Brain Res. 2002;132:391-404.
8. Domanska-Janik K, Bronisz-Kowalczyk A, Zajac H, Zablocka B. Interrelations between nuclear-factor kappa B activation, glial response and neuronal apoptosis in gerbil hippocampus after ischemia. Acta Neurobiol Exp. 2001;61:45-51.
9. Salminen A, Liu PK, Hsu CY. Alteration of transcription factor binding activities in the ischemic rat brain. Biochem Biophys Res Commun. 1995;212:939-44.
10. Clemens JA, Stephenson DT, Dixon EP, Smalstig EB, Mincy RE, Rash KS, Little SP. Global cerebral ischemia activates nuclear factor-kappa B prior to evidence of DNA fragmentation. Brain Res Mol Brain Res. 1997a;48:187-96.
11. Clemens JA, Stephenson DT, Smalstig EB, Dixon EP, Little SP. Global ischemia activates nuclear factor-kappa B in forebrain neurons of rats. Stroke. 1997b;28:1073-80.
12. Davalos A, Ricart W, Gonzalez-Huix F, Soler S, Marrugat JM, A., Suner R, Genis D. Effect of malnutrition after acute stroke on clinical outcome. Stroke. 1996;27:1028-32.
13. Axelsson K, Asplund K, Norberg A, Alafuzoff I. Nutritional status in patients with acute stroke. Acta Med Scand. 1988;224:217-24.
14. Gariballa SE, Parker SG, Taub N, Castleden M. Nutritional status of hospitalized acute stroke patients. Br J Nutr. 1998a;79:481-7.
15. Finestone HM, Greene-Finestone LS, Wilson ES, Teasell RW. Malnutrition in stroke patients on the rehabilitation service and at follow-up: prevalence and predictors. Arch Phys Med Rehabil. 1995;76:310-6.
16. Poor nutritional status on admission predicts poor outcomes after stroke: observational data from the FOOD trial. Stroke. 2003;34:1450-6.
17. Bobyn PJ, Corbett D, Saucier DM, Noyan-Ashraf MH, Juurlink BHJ, Paterson PG. Protein-energy malnutrition impairs functional outcome in global ischemia. Exp Neurol. 2005;196:308-15.
18. Sauerwein RW, Mulder JA, Mulder L, Lowe B, Peshu N, Demacker PN, van der Meer JW, Marsh K. Inflammatory mediators in children with protein-energy malnutrition. Am J Clin Nutr. 1997;65:1534-9.
19. Mayatepek E, Becker K, Gana L, Hoffmann GF, Leichsenring M. Leukotrienes in the pathophysiology of kwashiorkor. Lancet. 1993;342:958-60.
20. Li J, Quan N, Bray TM. Supplementation of N-acetylcysteine normalizes lipopolysaccharide-induced nuclear factor kappaB activation and proinflammatory

cytokine production during early rehabilitation of protein malnourished mice. J Nutr Biochem. 2002;132:3286-92.
21. Das BK, Jayanthy R, Agarwal JK, Mishra OP, Bhatt RP. Blood sugar and serum insulin response in protein energy malnutrition. J Trop Pediatr. 1998;44:139-41.
22. Manary M. Protein-Energy Malnutrition: There Is Still Work to Do. J Pediatric Gastroenterol Nutr. 2001;32:516-8.
23. Solomon T, Felix JM, Samuel M. Hypoglycemia in pediatric admissions in Mozambique. Lancet. 1994;343:149-50.
24. Woodward B. Depressed adaptive immune competence in acute protein-energy malnutrition: A regulated pathophysiology controlled by endocrine hormones and cytokines Toronto: TSAR Health; 2004.
25. Dinkel K, Ogle WO, Sapolsky RM. Glucocorticoids and central nervous system inflammation. J Neurovirol. 2002;8:513-28.
26. Schurr A. Glucose and the ischemic brain: a sour grape or a sweet treat? Curr Opin Clin Nutr Metab Care. 2001;4:287-92.
27. Hayashi R, Wada H, Ito K, Adcock IM. Effects of glucocorticoids on gene transcription. Eur J Pharmacol. 2004;500:51-62.
28. Gariballa SE. Nutritional factors in stroke. Br J Nutr. 2000;84:5-17.
29. Poungvarin N. Stroke in the developing world. Lancet. 1998;352 SIII 19-22.
30. Zhang LF, Yang J, Hong Z, Yuan GG, Zhou BF, Zhao LC, Huang YN, Chen J, Wu YF. Proportion of different subtypes of stroke in China. Stroke. 2003;34:2091-6.
31. Iadecole C. Cerebral Ischemia: Molecular and Cellular Pathophysiology; 1999.
32. Finestone HM, Greene-Finestone LS, Wilson ES, Teasell RW. Prolonged length of stay and reduced functional improvement rate in malnourished stroke rehabilitation patients. Arch Phys Med Rehabil. 1996;77:340-5.
33. Ginsberg MD. Expression, Neuroprotection: The 2002 Thomas Willis Lecture. Adventures in the Pathophysiology of Brain Ischemia: Penumbra, Gene. Stroke. 2003;34:214-23.
34. Siesjo BK. Acidosis and ischemic brain damage. Neurochem Pathol. 1988a;9:31-88.
35. Symon L. The relationship between CBF, evoked potentials and the clinical features in cerebral ischemia. Acta Neurol Scand. 1980;78:175-90.
36. Siesjo B. Mechanisms of ischemic brain damage. Crit Care Med. 1988b;16:954-63.
37. Kermer P, Klocker N, Bahr M. Neuronal death after brain injury. Models, mechanisms, and therapeutic strategies in vivo. Cell Tissue Res. 1999;298:383-95.
38. Kristian T, Ouyang Y, Siesjo BK. Calcium-induced neuronal cell death in vivo and in vitro: are the pathophysiologic mechanisms different? Adv Neurol. 1996;71:107-13.
39. Ding Y, Clark JC. Cerebrovascular injury in stroke. Neurol Res. 2006;28:3-10.
40. Gunter TE, Pfeiffer DR. Mechanisms by which mitochondria transport calcium. Am J Physiol. 1990;258:C755-86.
41. Zheng Z, Yenari MA. Post-ischemic inflammation: molecular mechanisms and therapeutic implications. Neurol Res. 2004;26:884-92.
42. Price GM, Halliday D, Pacy PJ, Quevedo MR, Millward DJ. Nitrogen homeostasis in man: influence of protein intake on the amplitude of diurnal cycling of body nitrogen. Clin Sci. 1994;86:91-102.

43. Barone FC, Feuerstein GZ. Inflammatory mediators and stroke: new opportunities for novel therapeutics. J Cereb Blood Flow Metab. 1999;19:819-34.
44. Cherubini A, Ruggiero C, Polidori MC, Mecocci P. Potential markers of oxidative stress in stroke. Free Radic Biol Med. 2005;39:841-52.
45. Margaill I, Plotkine M, Lerouet D. Antioxidant strategies in the treatment of stroke. Free Radic Biol Med. 2005;39:429-33.
46. Patel M, Day BJ, Crapo JD, Fridovich I, McNamara JO. Requirement for superoxide in excitotoxic cell death. Neuron. 1996;16:345-55.
47. Terada LS, Willingham IR, Rosandich ME, Leff JA, Kindt GW, Repine JE. Generation of superoxide anion by brain endothelial cell xanthine oxidase. J Cell Physiol. 1991;148:191-96.
48. Moro MA, Almeida A, Bolanos JP, Lizasoain I. Mitochondrial respiratory chain and free radical generation in stroke. Free Radic Biol Med 2005;39:1291-304.
49. Bredt DS, Snyder SH. Nitric oxide: a physiologic messenger molecule. Annu Rev Biochem. 1994;63:175-95.
50. Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol Rev. 1991;43:109-42.
51. Forster C, Clark HB, Ross ME, Iadecola C. Inducible nitric oxide synthase expression in human cerebral infarcts. Acta Neuropathol. 1999;97:215-20.
52. Iadecola C, Zhang F, Xu S, Casey R, Ross ME. Inducible nitric oxide synthase gene expression in brain following cerebral ischemia. J Cereb Blood Flow Metab. 1995;15:378-84.
53. Iadecola C, Zhang F, Casey R, Clark HB, Ross ME. Inducible nitric oxide synthase gene expression in vascular cells after transient focal cerebral ischemia. Stroke. 1996;27:7373-80.
54. Serteser M, Ozben T, Gumuslu S, Balkan S, Balkan E. Lipid peroxidation in rat brain during focal cerebral ischemia: prevention of malondialdehyde and lipid conjugated diene production by a novel antiepileptic, lamotrigine. Neurotoxic. 2002;23:111-19.
55. Juurlink BHJ. Management of oxidative stress in the CNS: the many roles of glutathione. Neurotox Res. 1999;1:119-40.
56. Warner DS, Sheng H, Batinic-Haberle I. Oxidants, antioxidants and the ischemic brain. J Exp Biol. 2004;207:3221-31.
57. Khan M, Sekhon B, Jatana M, Giri S, Gilg AG, Sekhon C, Singh I, Singh AK. Administration of N-acetlycysteine after focal cerebral ischemia protects brain and reduces inflammation in a rat model of experimental stroke. J Neurosci Res. 2004;76:519-27.
58. Uemura Y, Miller JM, Matson WR, Beal MF. Neurochmical analysis of focal ischemia in rats. Stroke. 1991;22:1548-53.
59. Fabian RH, DeWitt DS, Kent TA. In vivo detection of superoxide anion production by the brain using a cytochrome c electrode. J Cereb Blood Flow Metab. 1995;15:242-7.
60. Lancelot E, Callebert J, Revaud ML, Boulu RG, Plotkine M. Detection of hydroxyl radicals in rat striatum during transient focal cerebral ischemia: possible implication in tissue damage. Neurosci Lett. 1995;197:85-8.
61. Kato N, Yanaka K, Nagase S, Hirayama A, Nose T. The antioxidant EPC-K1 ameliorates brain injury by inhibiting lipid peroxidation in a rat model of transient focal ischaemia. Acta Neurochir. 2003;145:489-93.

62. Candelario-Jalil E, Mhadu NH, Al-Dalain SM, Martinez G, Leon OS. Time course of oxidant damage in different brain regions following transient cerebral ischemia in gerbil. Neurosci Res. 2001;41:233-41.
63. Alexandrova M, Bochev P, Markova V, Bechev B, Popova M, Danovska M, Simeonova V. Dynamics of free radical processes in acute ischemic stroke: influence on neurological status and outcome. J Clin Neurosci. 2004;11:501-6.
64. Sharpe PC, Mulholland T, Trinick T. Ascorbate and malondialdehyde in stroke patients. Ir J Med Sci. 1994;163:488-91.
65. Kochanek PM, Hallenbeck JM. Polymorphonuclear leukocytes and monocytes/macrophages in the pathogenesis of cerebral ischemia and stroke. Stroke. 1992;23:1367-79.
66. Garcia JH, Liu KF, Yoshida Y, Lian J, Chen S, del Zoppo GJ. Influx of leukocytes and platelets in an evolving brain infarct (Wistar rat). Am J Pathol. 1994;144:188-99.
67. Clark RK, Lee EV, Fish CJ, White RF, Price WJ, Jonak ZL, Feuerstein GZ, Barone FC. Development of tissue damage, inflammation and resolution following stroke: an immunohistochemical and quantitative planimetric study. Brain Res Bull. 1993;31:565-72.
68. Akopov SE, Simonian NA, Grigorian GS. Dynamics of polymorphonuclear leukocyte accumulation in acute cerebral infarction and their correlation with brain tissue damage. Stroke. 1996;27:1739-43.
69. Heinel LA, Rubin S, Rosenwasser RH, Vasthare US, Tuma RF. Leukocyte involvement in cerebral infarct generation after ischemia and reperfusion. Brain Res Bull. 1994;34:137-41.
70. Matsuo Y, Onodera H, Shiga Y, Nakamura M, Ninomiya M, Kihara T, Kogure K. Correlation between myeloperoxidase-quantified neutrophil accumulation and ischemic brain injury in the rat. Effects of neutrophil depletion. Stroke. 1994;25:1469-75.
71. Connolly ES, Jr; Winfree CJ, Springer TA, Naka Y, Liao H, Yan SD, Stern DM, Solomon RA, Gutierrez-Ramos JC, Pinsky DJ. Cerebral protection in homozygous null ICAM-1 mice after middle cerebral artery occlusion. Role of neutrophil adhesion in the pathogenesis of stroke. J Clin Invest. 1996;97:209-16.
72. Hartl R, Schurer L, Schmid-Schonbein GW, del Zoppo GJ. Experimental antileukocyte interventions in cerebral ischemia. J Cereb Blood Flow Metab. 1996;16:1108-19.
73. Walder CE, Green SP, Darbonne WC, Mathias J, Rae J, Dinauer MC, Curnutte JT, Thomas GR. Ischemic stroke injury is reduced in mice lacking a functional NADPH oxidase. Stroke. 1997;28:2252-58.
74. Hansson E, Ronnback L. Glial neuronal signaling in the central nervous system. FASEB J. 2003;17:341-48.
75. Hanisch UK. Microglia as a source and target of cytokines. Glia. 2002;40:140-55.
76. Tikka T, Fiebich BL, Goldsteins G, Keinanen R, Koistinaho J. Minocycline, a tetracycline derivative, is neuroprotective against excitotoxicity by inhibiting activation and proliferation of microglia. J Neurosci. 2001;21:2580-88.
77. Lehrmann E, Christensen T, Zimmer J, Diemer NH, Finsen B. Microglial and macrophage reactions mark progressive changes and define the penumbra in the rat neocortex and striatum after transient middle cerebral artery occlusion. J Comp Neurol. 1997;386:461-76.

78. Yrjanheikki J, Tikka T, Keinanen R, Goldsteins G, Chan PH, Koistinaho J. A tetracycline derivative, minocycline, reduces inflammation and protects against focal cerebral ischemia with a wide therapeutic window. Proc Natl Acad Sci USA. 1999;96:13496-500.
79. Korematsu K, Goto S, Nagahiro S, Ushio Y. Microglial response to transient focal cerebral ischemia: an immunocytochemical study on the rat cerebral cortex using anti-phosphotyrosine antibody. J Cereb Blood Flow Metab. 1994;14:825-30.
80. Mabuchi T, Kitagawa K, Ohtsuki T, Kuwabara K, Yagita Y, Yanagihara T, Hori M, Matsumoto M. Contribution of microglia/macrophages to expansion of infarction and response of oligodendrocytes after focal cerebral ischemia in rats. Stroke. 2000;31:1735-43.
81. Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. Trends Neurosci. 1996;19:312-18.
82. Bhat RV, DiRocco R, Marcy VR, Flood DG, Zhu Y, Dobrzanski P, Siman R, Scott R, Contreras PC, Miller M. Increased expression of IL-1beta converting enzyme in hippocampus after ischemia: selective localization in microglia. J Neurosci. 1996;16:4146-54.
83. Gottschall PE, Yu X, Bing B. Increased production of gelatinase B (matrix metalloproteinase-9) and interleukin-6 by activated rat microglia in culture. J Neurosci Res. 1995;42:335-42.
84. Nakashima MN, Yamashita K, Kataoka Y, Yamashita YS, Niwa M. Time course of nitric oxide synthase activity in neuronal, glial, and endothelial cells of rat striatum following focal cerebral ischemia. Cell Mol Neurobiol. 1995;15:341-9.
85. Sairanen T, Ristimaki A, Karjalainen-Lindsberg ML, Paetau A, Kaste M, Lindsberg PJ. Cyclooxygenase-2 is induced globally in infarcted human brain. Ann Neurol. 1998;43:738-47.
86. Lehrmann E, Kiefer R, Christensen T, Toyka KV, Zimmer J, Diemer NH, Hartung HP, Finsen B. Microglia and macrophages are major sources of locally produced transforming growth factor-beta1 after transient middle cerebral artery occlusion in rats. Glia. 1998;24:437-48.
87. Mesples B, Plaisant F, Gressens P. Effects of interleukin-10 on neonatal excitotoxic brain lesions in mice. Brain Res Dev Brain Res. 2003;141:25-32.
88. Urabe T, Hattori N, Yoshikawa M, Yoshino H, Uchida K, Mizuno Y. Colocalization of Bcl-2 and 4-hydroxynonenal modified proteins in microglial cells and neurons of rat brain following transient focal ischemia. Neurosci Lett. 1998;247:159-62.
89. Zeev-Brann AB, Lazarov-Spiegler O, Brenner T, Schwartz M. Differential effects of central and peripheral nerves on macrophages and microglia. Glia. 1998;23:181-90.
90. Faber-Elman A, Solomon A, Abraham JA, Marikovsky M, Schwartz M. Involvement of wound-associated factors in rat brain astrocyte migratory response to axonal injury: in vitro simulation. J Clin Invest. 1996;97:162-71.
91. Ayata C, Ropper AH. Ischaemic brain oedema. J Clin Neurosci. 2002;9:113-24.
92. Yang GY, Betz AL. Reperfusion-induced injury to the blood-brain barrier after middle cerebral artery occlusion in rats. Stroke. 1994;25:1658-64.
93. Davalos A, Toni D, Iweins F, Lesaffre E, Bastianello S, Castillo J. Neurological deterioration in acute ischemic stroke: potential predictors and associated factors in the European cooperative acute stroke study (ECASS) I. Stroke. 1999;30:2631-36.
94. Nagayama M, Niwa K, Nagayama T, Ross ME, Iadecola C. The cyclooxygenase-2 inhibitor NS-398 ameliorates ischemic brain injury in wild-type mice but not in mice

with deletion of the inducible nitric oxide synthase gene. J Cereb Blood Flow Metab. 1999;19:1213-19.
95. Murphy PG, Borthwick LS, Johnston RS, Kuchel G, Richardson PM. Nature of the retrograde signal from injured nerves that induces interleukin-6 mRNA in neurons. J Neurosci. 1999;19:3791-800.
96. Tchelingerian JL, Le Saux F, Jacque C. Identification and topography of neuronal cell populations expressing TNF alpha and IL-1 alpha in response to hippocampal lesion. J Neurosci Res. 1996;43:99-106.
97. Yang L, Blumbergs PC, Jones NR, Manavis J, Sarvestani GT, Ghabriel MN. Early expression and cellular localization of proinflammatory cytokines interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in human traumatic spinal cord injury. Spine. 2004;29:966-71.
98. Hirabayashi H, Takizawa S, Fukuyama N, Nakazawa H, Shinohara Y. Nitrotyrosine generation via inducible nitric oxide synthase in vascular wall in focal ischemia-reperfusion. Brain Res. 2000;852:319-25.
99. Han HS, Qiao Y, Karabiyikoglu M, Giffard RG, Yenari MA. Influence of mild hypothermia on inducible nitric oxide synthase expression and reactive nitrogen production in experimental stroke and inflammation. J Neurosci. 2002;22:3921-8.
100. Sen R, Baltimore D. Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. Cell. 1986;47:921-28.
101. Ghosh S, May MJ, Kopp EB. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol. 1998;16:225-60.
102. Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. Annu Rev Immunol. 1994;12:141-79.
103. Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol. 2000;18:621-63.
104. Shen W-H, Zhang C-Y, Zhang G-Y. Modulation of IκB kinase autophosphorylation and activity following brain ischemia. Acta Pharmacol Sin. 2003;24:311-15.
105. Shen W, Zhang C, Zhang G. Nuclear factor kappa B activation is mediated by NMDA and non-NMDA receptor and L-type voltage-gated $Ca^{2+}$ channel following severe global ischemia in rat hippocampus. Brain Res. 2002;933:23-30.
106. DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M. A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. Nature. 1997;388:548-54.
107. Mercurio F, Zhu H, Murray BW, Shevchenko A, Bennett BL, Li J, Young DB, Barbosa M, Mann M, Manning A, Rao A. IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. Science. 1997;278:860-66.
108. Zandi E, Rothwarf DM, Delhase M, Hayakawa M, Karin M. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. Cell. 1997;91:243-52.
109. Siebenlist U, Franzoso G, Brown K. Structure, regulation and function of NF-kappa B. Annu Rev Cell Biol. 1994;10:405-55.
110. Shakhov AN, Collart MA, Vassalli P, Nedospasov SA, Jongeneel CV. Kappa B-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor alpha gene in primary macrophages. J Exp Med. 1990;171:35-47.

111. Hiscott J, Marois J, Garoufalis J, D'Addario M, Roulston A, Kwan I, Pepin N, Lacoste J, Nguyen H, Bensi G. Characterization of a functional NF-kappa B site in the human interleukin 1 beta promoter: evidence for a positive autoregulatory loop. Mol Cell Biol. 1993;13:6231-40.
112. Kunsch C, Rosen CA. NF-kappa B subunit-specific regulation of the interleukin-8 promoter. Mol Cell Biol. 1993;13:6137-46.
113. Lai JH, Horvath G, Subleski J, Bruder J, Ghosh P, Tan TH. RelA is a potent transcriptional activator of the CD28 response element within the interleukin 2 promoter Mol Cell Biol. 1995;15:4260-71.
114. Libermann TA, Baltimore D. Activation of interleukin-6 gene expression through the NF-kappa B transcription factor. Mol Cell Biol. 1990;10:2327-34.
115. Mori N, Prager D. Transactivation of the interleukin-1alpha promoter by human T-cell leukemia virus type I and type II Tax proteins. Blood. 1996;87:3410-17.
116. Murphy TL, Cleveland MG, Kulesza P, Magram J, Murphy KM. Regulation of interleukin 12 p40 expression through an NF-kappa B half-site. Mol Cell Biol. 1995;15:5258-67.
117. Hong SL, Wang JB, Liu YH, Si JY, Xu XM, Guo XC, Zeng Y. Identification and assessment of multiple human papillomavirus types in condyloma acuminata lesions from patients with genital warts in Beijing area. Zhongguo Yi Yue Ke Xue Yuan Xue Bao. 2002;24:397-400.
118. Smith MF, Jr; Eidlen D, Arend WP, Gutierrez-Hartmann A. LPS-induced expression of the human IL-1 receptor antagonist gene is controlled by multiple interacting promoter elements. J Immunol. 1994;153:3584-93.
119. Xu LG, Wu M, Hu J, Zhai Z, Shu HB. Identification of downstream genes up-regulated by the tumor necrosis factor family member TALL-1. J Leukoc Biol. 2002;72:410-6.
120. Kaltschmidt B, Linker RA, Deng J, Kaltschmidt C. Cyclooxygenase-2 is a neuronal target gene of NF-kappaB. BMC Mol Biol. 2002;3:16-28.
121. Yamamoto K, Arakawa T, Ueda N, Yamamoto S. Transcriptional roles of nuclear factor kappa B and nuclear factor-interleukin-6 in the tumor necrosis factor alpha-dependent induction of cyclooxygenase-2 in MC3T3-E1 cells. J Biol Chem. 1995;270:31315-20.
122. Morris KR, Lutz RD, Choi HS, Kamitani T, Chmura K, Chan ED. Role of the NF-kappaB signaling pathway and kappaB cis-regulatory elements on the IRF-1 and iNOS promoter regions in mycobacterial lipoarabinomannan induction of nitric oxide. Infect Immun. 2003;71:1442-52.
123. van de Stolpe A, Caldenhoven E, Stade BG, Koenderman L, Raaijmakers JA, Johnson JP, van der Saag PT. 12-O-tetradecanoylphorbol-13-acetate- and tumor necrosis factor alpha-mediated induction of intercellular adhesion molecule-1 is inhibited by dexamethasone. Functional analysis of the human intercellular adhesion molecular-1 promoter. J Biol Chem. 1994;269:6185-92.
124. Iademarco MF, McQuillan JJ, Rosen GD, Dean DC. Characterization of the promoter for vascular cell adhesion molecule-1 (VCAM-1). J Biol Chem. 1992;267:16323-9.
125. Schindler U, Baichwal VR. Three NF-kappa B binding sites in the human E-selectin gene required for maximal tumor necrosis factor alpha-induced expression. Mol Cell Biol. 1994;14:5820-31.

126. Das KC, Lewis-Molock Y, White CW. Thiol modulation of TNF alpha and IL-1 induced MnSOD gene expression and activation of NF-kappa B. Mol Cell Biochem. 1995;148:45-57.
127. Terai K, Matsuo A, McGeer EG, McGeer PL. Enhancement of immunoreactivity for NF-kappa B in human cerebral infarctions. Brain Res. 1996;739:343-9.
128. Schmidt KN, Amsad P, Cerutti P, Baeuerle PA. Identification of hydrogen peroxide as the relevant messenger in the activation pathway of the transciption factor NFκB. Adv Exp Med Biol. 1996;387:63-8.
129. Stephenson D, Yin T, Smalstig EB, Hsu MA, Panetta J, Little S, Clemens J. Transcription factor nuclear factor-kappa B is activated in neurons after focal cerebral ischemia. J Cereb Blood Flow Metab. 2000;20:592-603.
130. Schneider A, Martin-Villalba A, Weih F, Vogel J, Wirth T, Schwaninger M. NF-kappaB is activated and promotes cell death in focal cerebral ischemia. Nat Med. 1999;5:554-59.
131. Duckworth EAM, Butler T, Collier L, Collier S, Pennypacker KR. NF-κB protects neurons from ischemic injury after middle cerebral artery occlusion in mice. Brain Res. 2006;1088:167-75.
132. Christman JW, Blackwell TS, Juurlink BHJ. Redox regulation of nuclear factor kappa B: therapeutic potential for attenuating inflammatory responses. Brain Pathol. 2000;10:153-62.
133. Flohe L, Brigelius-Flohe R, Saliou C, Traber MG, Packer L. Redox regulation of NF-kappa B activation. Free Radic Biol Med. 1997;22:11-26.
134. Carroll JE, Howard EF, Hess DC, Wakade CG, Chen Q, Cheng C. Nuclear factor-kappa B activation during cerebral reperfusion: effect of attenuation with N-acetylcysteine treatment. Brain Res Mol Brain Res. 1998;56:186-91.
135. Shen WH, Zhang CY, Zhang GY. Antioxidants attenuate reperfusion injury after global brain ischemia through inhibiting nuclear factor-kappa B activity in rats. Acta Pharmacol Sin. 2003;24:1125-30.
136. Gariballa SE, Sinclair AJ. Assessment and treatment of nutritional status in stroke patients. Postgrad Med J. 1998;74:395-9.
137. Abbasi AA, Rudman D. Undernutrition in the nursing home: prevalence, consequences, causes and prevention. Nutr Rev. 1994;52:113-22.
138. Lipschitz DA. Malnutrition in the elderly. Semin Dermatol. 1991;10:273-81.
139. Gropper SS, Smith JL, Groff JL. Advanced nutrition and human metabolism. 4th ed ed. Belmont, CA Thomson Wadsworth; 2005.
140. Corish CA, Kennedy NP. Protein-energy undernutrition in hospital in-patients. Br J Nutr. 2000;83:575-91.
141. Choi-Kwon S, Yang YH, Kim EK, Jeon MY, Kim JS. Nutritional status in acute stroke: undernutrition versus overnutrition in different stroke subtypes. Acta Neurol Scand. 1998;98:187-92.
142. Gariballa SE, Parker SG, Taub N, Castleden CM. Influence of nutritional status on clinical outcome after acute stroke. Am J Clin Nutr. 1998b;68:275-81.
143. Martineau J, Bauer JD, Isenring E, Cohen S. Malnutrition determined by the patient-generated subjective global assessment is associated with poor outcomes in acute stroke patients Clin Nutr. 2005;24:1073-77.
144. Nyswonger GD, Helmchen RH. Early enteral nutrition and length of stay in stroke patients. J Neurosci Nurs. 1992;24:220-3.

145. Aptaker RL, Roth EJ, Reichhardt G, Duerden ME, Levy CE. Serum albumin level as a predictor of geriatric stroke rehabilitation outcome. Arch Phys Med Rehabil. 1994;75:80-4.
146. Bobyn PJ. Nutritional influence on oxidative stress in global ischemia. Saskatoon: University of Saskatchewan; 2003.
147. Gariballa SE. Malnutrition in hospitalized elderly patients: when does it matter? ClinNutr. 2001;20:487-91.
148. Lesourd B, Mazari L. Nutrition and immunity in the elderly. Proc Nutr Soc. 1999;58:685-95.
149. Mazari L, Lesourd BM. Nutritional influences on immune response in healthy aged persons. Mech Ageing Dev. 1998;104:25-40.
150. Woodward B. The effect of protein-energy malnutrition on immune competence. Philadelphia: Nestle NUtrition Workshop, Vevey/Lippincott Williams & Wilkins; 2001.
151. Liu J, Bartels M, Lu A, Sharp FR. Microglia/macrophages proliferate in striatum and neocortex but not in hippocampus after brief global ischemia that produces ischemic tolerance in gerbil brain. J Cereb Blood Flow Metab. 2001;21:361-73.
152. Park CK, Ju WK, Hofmann HD, Kirsch M, Ki-Kang J, Chun MH, Lee MY. Differential regulation of ciliary neurotrophic factor and its receptor in the rat hippocampus following transient global ischemia. Brain Res. 2000;861:345-53.
153. Anderson MF, Blomstrand F, Blomstrand C, Eriksson PS, Nilsson M. Astrocytes and Stroke: Networking for Survival? Neurochem Res. 2002;28:293-305.
154. Danton GH, Dietrich WD. Inflammatory Mechanisms after Ischemia and Stroke. J Neuropathol Exp Neurol. 2003;62:127-36.
155. Kaltschmidt B, Widera D, Kaltschmidt C. Signaling via NF-kappaB in the nervous system. Biochim Biophys Acta. 2005;1745:287-99.
156. Bray TM, Taylor CG. Enhancement of tissue glutathione for antioxidant and immune functions in malnutrition. Biochem Pharmacol. 1994;47:2113-23.
157. Li J, Wang H, Stoner GD, Bray TM. Dietary supplementation with cysteine prodrugs selectively restores tissue glutathione levels and redox status in protein-malnourished mice. J Nutr Biochem. 2002;13:625-33.
158. Levy MA, Sikorski B, Bray TM. Selective elevation of glutathione levels in target tissues with L-2-oxothiazolidine-4-carboxylate (OTC) protects against hyperoxia-induced lung damage in protein-energy malnourished rats: implications for a new treatment strategy. J Nutr. 1998;128:671-6.
159. Bobyn PJ, Franklin JL, Wall CM, Thornhill JA, Juurlink BHJ, Paterson PG. The effects of dietary sulfur amino acid deficiency on rat brain glutathione concentration and neural damage in global hemispheric hypoxia-ischemia. Nutr Neurosci. 2002;5:407-16.
160. Tabatabaie T, Floyd RA. Susceptibility of glutathione peroxidase and glutathione reductase to oxidative damage and the protective effect of spin trapping agents. Arch Biochem Biophys. 1994;314:112-9.
161. Myers RE, Yamaguchi S. Nervous system effects of cardiac arrest in monkeys. Preservation of vision. Arch Neurol. 1977;34:65-74.
162. Melamed E. Reactive hyperglycaemia in patients with acute stroke. J Neurosci. 1976;29:267-75.
163. Chalela JA, Merino JG, Warach S. Update on stroke. Curr Opin Neurol. 2004;17:447-51.
164. Samson Y, Bruandet M, Lejeune M, Deltour S, Grimaldi A. Insulin in the treatment of ischemic stroke. Presse Med. 2006;35:696-8.

165. Capes SE, Hunt D, Malmberg K, Pathak P, Gerstein HC. Stress hyperglycemia and prognosis of stroke in nondiabetic and diabetic patients: a systematic overview. Stroke. 2001;32:2426-32.
166. Garg R, Chaudhuri A, Munschauer F, Dandona P. Hyperglycemia, insulin, and acute ischemic stroke: a mechanistic justification for a trial of insulin infusion therapy. Stroke. 2006;37:267-73.
167. Baird TA, Parsons MW, Phanh T, Butcher KS, Desmond PM, Tress BM, Colman PG, Chambers BR, Davis SM. Persistent poststroke hyperglycemia is independently associated with infarct expansion and worse clinical outcome. Stroke. 2003;34:2208-14.
168. Dhindsa S, Tripathy D, Mohanty P, Ghanim H, Syed T, Aljada A, Dandona P. Differential effects of glucose and alcohol on reactive oxygen species generation and intranuclear nuclear factor-kappaB in mononuclear cells. Metabolism. 2004;53:330-4.
169. Smith IF, Latham MC, Azubuike JA, Butler WR, Phillips LS, Pond WG, Enwonwu CO. Blood plasma levels of cortisol, insulin, growth hormone and somatomedin in children with marasmus, kwashiorkor and intermediate forms of protein-energy malnutrition. Proc Soc Exp Biol Med. 1981;167:607-11.
170. Barone KS, O'Brien PC, Stevenson JR. Characterization and mechanisms of thymic atrophy in protein-malnourished mice: role of corticosterone. Cell Immunol. 1993;148:226-33.
171. Hill AD, Naama HA, Gallagher HJ, Shou J, Calvano SE, Daly JM. Glucocorticoids mediate macrophage dysfunction in protein calorie malnutrition. Surgery. 1995;118:130-6.
172. Murros K, Fogelholm R, Kettunen S, Vuorela AL. Serum cortisol and outcome of ischemic brain infarction. J Neurol Sci. 1993;116:12-7.
173. Olsson T. Urinary free cortisol excretion shortly after ischaemic stroke. J Intern Med. 1990;228:177-81.
174. Marklund N, Peltonen M, Nilsson TK, Olsson T. Low and high circulating cortisol levels predict mortality and cognitive dysfunction early after stroke. J Intern Med. 2004;256:15-21.
175. Wiegers GJ, Reul JM. Induction of cytokine receptors by glucocorticoids: functional and pathological significance. Trends Pharmacol Sci. 1998;19:317-21.
176. Liao J, Keiser JA, Scales WE, Kunkel SL, Kluger MJ. Role of corticosterone in TNF and IL-6 production in isolated perfused rat liver. Am J Physiol. 1995;268:R699-706.
177. Alcorn JM, Fierer J, Chojkier M. The acute-phase response protects mice from D-galactosamine sensitization to endotoxin and tumor necrosis factor-alpha. Hepatology. 1992;15:122-9.
178. McEwen BS, Dekloet ER, Rostene W. Adrenal steroid receptors and actions in the nervous system. Physiol Rev. 1986;66:1121-88.
179. Stapleton PP, Barry LK, Freeman TA, Mestre JR, Daly JM. Decreased peritoneal macrophage NF-kappaB translocation to the nucleus in protein energy malnutrition--a role for the glucocorticoid response? Clin Nutr. 2004;23:177-82.
180. Ginsberg MD. The validity of rodent brain-ischemia models is self-evident. Arch Neurol. 1996;53:1065-7.
181. Ginsberg MD, Busto R. Rodent models of cerebral ischemia. Stroke. 1989;20:1627-42.
182. Small DL, Buchan AM. Animal models. Br Med Bull. 2000;56:307-17.

183. Seta KA, Crumrine RC, Whittingham TS, Lust WD, Candless DW. Experimental models of human stroke. Totowa, NJ: The Humana Press, Inc.; 1992.
184. Park EM, Choi JH, Park JS, Han MY, Park YM. Measurement of glutathione oxidation and 8-hydroxy-2'-deoxyguanosine accumulation in the gerbil hippocampus following global ischemia. Brain Res Protoc. 2000;6:25-32.
185. Cervantes M, Gonzalez-Vidal MD, Ruelas R, Escobar A, Morali G. Neuroprotective effects of progesterone on damage elicited by acute global cerebral ischemia in neurons of the caudate nucleus. Arch Med Res. 2002;33:6-14.
186. Hamberg LM, Macfarlane R, Tasdemiroglu E, Boccalini P, Hunter GJ, Belliveau JW, Moskowitz MA, Rosen BR. Measurement of cerebrovascular changes in cats after transient ischemia using dynamic magnetic resonance imaging. Stroke. 1993;24:444-50.
187. Pulsinelli WA, Brierley JB. A new model of bilateral hemispheric ischemia in the unanesthetized rat. Stroke. 1979;10:267-72.
188. Kirino T. Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res. 1982;239:57-69.
189. Smith ML, Auer RN, Siesjo BK. The density and distribution of ischemic brain injury in the rat following 2-10 min of forebrain ischemia. Acta Neuropathol. 1984;64:319-32.
190. Ljunggren B, Schutz H, Siesjo BK. Changes in energy state and acid-base parameters of the rat brain during complete compression ischemia. Brain Res. 1974;73:277-89.
191. Hendrickx HH, Safar P, Miller A. Asphyxia, cardiac arrest and resuscitation in rats. II. Long term behavioral changes. Resuscitation. 1984;12:117-28.
192. Levine S, Payan H. Effects of ischemia and other procedures on the brain and retina of the gerbil (Meriones unguiculatus). Exp Neurol. 1966;16.
193. Kirino T, Sano K. Fine structural nature of delayed neuronal death following ischemia in the gerbil hippocampus. Acta Neuropathol. 1984a;62:209-18.
194. Kirino T, Sano K. Selective vulnerability in the gerbil hippocampus following transient ischemia. Acta Neuropathol. 1984b;62:201-8.
195. Kirino T, Tamura A, Sano K. Delayed neuronal death in the rat hippocampus following transient forebrain ischemia. Acta Neuropathol. 1984;64:139-47.
196. Corbett D, Crooks P. Ischemic preconditioning: a long term survival study using behavioural and histological endpoints. Brain Res. 1997;760:129-36.
197. Corbett D, Nurse S. The problem of assessing effective neuroprotection in experimental cerebral ischemia. Prog Neurobiol. 1998;54:531-48.
198. Corbett D, Nurse S, Colbourne F. Hypothermic neuroprotection. A global ischemia study using 18- to 20-month-old gerbils. Stroke. 1997;28:2238-42.
199. Nurse S, Corbett D. Direct measurement of brain temperature during and after intraischemic hypothermia: correlation with behavioral, physiological, and histological endpoints. J Neurosci. 1994;14:7726-34.
200. Block F. Global ischemia and behavioural deficits. Prog Neurobiol. 1999;58:279-95.
201. Colbourne F, Corbett D. Delayed and prolonged post-ischemic hypothermia is neuroprotective in the gerbil. Brain Res. 1994;654:265-72.
202. Mitani A, Andou Y, Masuda S, Kataoka K. Transient forebrain ischemia of three-minute duration consistently induces severe neuronal damage in field CA1 of the hippocampus in the normothermic gerbil. Neurosci Lett. 1991;131:171-4.

203. Colbourne F, Corbett D. Delayed postischemic hypothermia: a six month survival study using behavioral and histological assessments of neuroprotection. J Neurosci. 1995;15:7250-60.
204. Laidley DT, Colbourne F, Corbett D. Increased behavioral and histological variability arising from changes in cerebrovascular anatomy of the Mongolian gerbil. Curr Neurovasc Res. 2005;2:401-7.
205. Seal JB, Buchh BN, Marks JD. New variability in cerebrovascular anatomy determines severity of hippocampal injury following forebrain ischemia in the Mongolian gerbil. Brain Res. 2006;1073-4:451-9.
206. Reeves PG, Nielsen FH, Fahey GC. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr. 1993;123:1923-31.
207. Armitage P. Statistical methods in Medical Research. New York: Blackwell Scientific Publications; 1971.
208. Xu L, Sun J, Lu R, Ji Q, Xu JG. Effect of glutamate on inflammatory responses of intestine and brain after focal cerebral ischemia. World J Gastroenterol. 2005;11:733-6.
209. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. Anal Biochem. 1985;150:76-85.
210. Harmon M, Noyan-Ashraf M, Paterson PG. The effect of protein-energy malnutrition on inflammation and reactive gliosis following global ischemia in the gerbil. CSNS. Edmonton, AB, Canada; 2005.
211. Zhang Z, Juurlink BHJ, Lyon AW, Paterson PG. Effects of dietary protein and L-2-oxothiazolidine-4-carboxylate on rat brain glutathione concentration. Nutr Res. 2002;22:1475-86.
212. Bauman PF, Smith TK, Bray TM. The effect of dietary protein and sulfur amino acids on hepatic glutathione concentration and glutathionedependent enzyme activities in the rat. Can J Physiol Pharmacol. 1988;66:1048-52.
213. Leme-Brasil MR, Collares EF, B. VDMS, Garcia-Leme J. Acute inflammatory responses in rats with protein-calorie malnutrition. Agents Actions. 1980;10:445-50.
214. Truswell AS, Hansen JD, Watson CE, Wannenburg P. Relation of serum lipids and lipoproteins to fatty liver in kwashiorkor. Am J Clin Nutr. 1969;22:568-76.
215. Benjamin DR. Laboratory tests and nutritional assessment: Protein-energy status. Pediatr Cliln North Am. 1989;36:139-61.
216. Streit WJ, Hurley SD, McGraw TS, Semple-Rowland SL. Comparative evaluation of cytokine profiles and reactive gliosis supports a critical role for Interleukin-6 in neuron-glia signaling during regeneration. J Neurosci Res. 2000;61:10-20.
217. John GR, Lee SC, Brosnan CF. Cytokines: Powerful regulators of glial cell activation. Neuroscientist. 2003;9:10-22.
218. Cechetto D. Role of nuclear factor kappa B in neuropathological mechanisms. Prog in Brain Res. 2001;132:391-404.
219. Crack PJ, Taylor JM, Ali U, Mansell A, Hertzog PJ. Potential Contribution of NF-B in Neuronal Cell Death in the Glutathione Peroxidase-1 Knockout Mouse in Response to Ischemia-Reperfusion Injury. Stroke. 2006;37:1533-8.
220. Yu Z, Zhou D, Bruce-Keller AJ, Kindy MS, Mattson MP. Lack of the p50 subunit of nuclear factor-kappaB increases the vulnerability of hippocampal neurons to excitotoxic injury. J Neurosci. 1999;19:8856-65.

221. de Onis M, Frongillo EA, Blössner M. Is malnutrition declining? An analysis of changes in levels of child malnutrition since 1980. Bulletin of the WHO. 2000;78:1222-33.
222. Woodward B, Filteau SM. Immunoenhancement in wasting protein-energy malnutrition: assessment of present information and proposal of a new concept. Adv Nutr Res. 1990;8:11-34.
223. Bonatto F, Polydoro M, Andrades ME, da Frota Junior ML, Dal-Pizzol F, Rotta LN, Souza DO, Perry ML, Moreira JC. Effect of protein malnutrition on redox state of the hippocampus of rat. Brain Res. 2005;1042:17-22.
224. Hunter EA, Grimble RF. Dietary sulphur amino acid adequacy influences glutathione synthesis and glutathione-dependent enzymes during the inflammatory response to endotoxin and tumour necrosis factor-alpha in rats. Clin Sci. 1997;92:297-305.
225. Stipanuk MH, Londono M, Lee JI, Hu M, Yu AF. Enzymes and metabolites of cysteine metabolism in nonhepatic tissues of rats show little response to changes in dietary protein or sulfur amino acid levels. J Nutr. 2002;132:3369-78.
226. Adachi T, Yasutake A, Hirayama K. Influence of dietary protein levels on the fate of methylmercury and glutathione metabolism in mice. Toxicology. 1992;72:17-26.
227. Shivakumar BR, Kolluri SV, Ravindranath V. Glutathione and protein thiol homeostasis in brain during reperfusion after cerebral ischemia. Pharmacol Exp Ther. 1995;274:1167-73.
228. Shivakumar BR, Kolluri SV, Ravindranath V. Glutathione homeostasis in brain during reperfusion following bilateral carotid artery occlusion in the rat. Mol Cell Biochem. 1992;111:125-9.
229. Fechner A, Bohme C, Gromer S, Funk M, Schirmer R, Becker K. Antioxidant Status and Nitric Oxide in the Malnutrition Syndrome Kwashiorkor. Pediatr Res. 2001;49:237-43.
230. Shipp K, Woodward BD. A Simple Exsanguination Method That Minimizes Acute Pre-anesthesia Stress in the Mouse: Evidence Based on Serum Corticosterone Concentrations. Contemp Top Lab Anim Sci. 1998;37:73-7.