The Effect of Protein-Energy

Malnutrition on

Reactive Gliosis

Following Global Ischemia

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ABSTRACT

Protein-energy malnutrition (PEM) has been found in up to 16.3% of acute stroke

patients upon admission to hospital. Our laboratory has previously shown that PEM

impairs functional outcome in a gerbil model of global ischemia, but the mechanism

has not been established. The purpose of the current study was to characterize the

marked reactive gliosis apparent in a subset of these animals that could represent an

increased inflammatory response. A second objective was to validate a screening

protocol for assessing completeness of ischemia in this model.

Male Mongolian gerbils, aged 11-12 weeks, were randomized to PEM (2% protein)

or control diet (12.5% protein) for 28d. PEM animals lost 12.2% of their initial

body weight, and feed intake and serum albumin concentration were 12.3% and

17.8% lower than controls, respectively. At day 28, animals underwent 5 min

bilateral common carotid artery occlusion (ischemia) or sham surgery. Activity was

monitored using infrared beam interruptions for 20h post-surgery to screen for

complete ischemia on the basis of persistent hyperactivity. Brain sections were

stained with hematoxylin & eosin, and viable hippocampal CA1 neurons were

counted at 10d post-ischemia. Immunohistochemistry for glial-fibrillary acidic

protein (GFAP) and ricinus communis agglutinin -120 (RCA-120), markers for

astrocytes and microglia, respectively, and the inflammatory cytokine TNF-alpha

was performed on brain sections at 6h, 24h, 3d and 10d post-surgery (Ischemic, n=8;

Sham, n=3). The activity monitoring procedure for detecting complete ischemia

validated against hippocampal CA1 neuronal loss at 10d demonstrated an accuracy

of 84.6%. Temporal changes in GFAP and RCA-120 immunoreactivity

characteristic of reactive gliosis were demonstrated following ischemia, but this was not exacerbated by PEM. TNF-alpha immunoreactivity following ischemia was also unaltered by PEM. Ischemia significantly reduced surviving CA1 neurons at 10 days post-ischemia (two-way ANOVA; $p < 0.05$), but this was not influenced by PEM. Impaired functional outcome in PEM animals following global ischemia can not be accounted for by increased hippocampal CA1 neuron death or by altered glial response.

ACKNOWLEDGEMENTS

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AIN	American Institute of Nutrition
ANOVA	Analysis of variance
BCCAO	Bilateral common carotid artery occlusion
BMI	Body mass index
CON	Control
COX	Cyclooxygenase
DAB	3,3'-diaminobenzidine
DHT	Delayed hypersensitivity skin test
GFAP	Glial Fibrillary Acidic Protein
GSH	Glutathione
H&E	Hematoxylin and eosin
I	Ischemic
ICAM	Intracellular adhesion molecule
iNOS	Inducible-nitric oxide synthase
IL	Interleukin
LPS	Lipopolysaccharide
MCAO	Middle cerebral artery occlusion
MNA	Mini Nutritional Assessment
NAC	N-acetylcysteine
NF-κB	Nuclear factor-kappa B
PEM	Protein-energy malnutrition
PBS	Phosphate-buffered saline

RCA	Ricinus Communis Agglutinin
ROS	Reactive oxygen species
S	Sham
SGA	Subjective global assessment
TNF-α	Tumour necrosis factor-alpha

CHAPTER 1. INTRODUCTION

1.1 Rationale

The Canadian population is aging (1). It has been estimated that the proportion

of Canadians over the age of 65 will increase from 13% in 2001 to 21% by the year

2026 (1). After the age of 55, stroke risk doubles every 10 years (2). Stroke patients

may require long term care, and the quality of life of stroke survivors is often

reduced (2). One of the priority topic areas for the Institute of Aging of the

Canadian Institutes of Health Research is quality of life (1). To date there is no

effective treatment to prevent brain damage arising from stroke (3). In order to find

an effective treatment, it is crucial that factors that could worsen stroke outcome are

identified and their mechanisms elucidated. Recent studies suggest that stroke

patients that were malnourished before having a stroke had worse clinical outcomes

than patients that were well nourished (4-8). This suggests that undernutrition may

play a role in exacerbating brain injury following stroke.

A recent study conducted in our laboratory (9,10) has demonstrated a causal

relationship between protein-energy malnutrition (PEM) and poor stroke outcome in

an animal model of stroke. Gerbils with PEM that were subjected to global ischemia

had significantly worse behavioural outcomes, as assessed by open field, when

compared to ischemic animals fed control diet. Although PEM did not worsen the

loss of hippocampal CA1 neurons at day 10 post-ischemia, one third of the PEM

animals showed marked gliosis that completely disrupted the CA1 pyramidal neuron

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region. Sections immunostained with anti-glial fibrillary acidic protein (GFAP) antibody suggested that this subset of PEM ischemic animals had a large population of reactive astrocytes (unpublished observations). A limitation of this study is that the sections had first been stained with hematoxylin and eosin (H & E). This reduced sensitivity such that it was not possible to immunohistochemically detect whether there were also increased numbers of activated microglia present. Increased reactive gliosis post-ischemia, if confirmed, may be indicative of an increased inflammatory response in PEM (10).   In order to ascertain the effect of PEM on astrocytic and microglial activation following global ischemia, this study needs to be repeated with appropriate tissue sections.

At the time the study was conducted, evidence was also accumulating that cerebral vasculature was changing in gerbils from commercial suppliers, resulting in a marked increase in variability of ischemic brain damage in the model of global ischemia used in our laboratory (11). Thus, a method for selecting animals that had been exposed to complete (severe) ischemia also needed to be developed. Previous research suggested that the persistent hyperactivity that follows severe global ischemia in the gerbil (12) could provide a noninvasive way to identify these animals.

1.2 Hypothesis

PEM exacerbates reactive gliosis and inflammation occurring in response to global ischemia.

1.3 Objectives

The objectives of this research are as follows:

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1. To investigate the temporal response of markers of reactive gliosis and inflammation to 5 minute common carotid artery occlusion (global ischemia) in the gerbil as compared to sham controls.

2. To determine if PEM increases reactive gliosis in global ischemia.

3. To determine if PEM exacerbates inflammation in global ischemia.

4. To confirm the effect of PEM on hippocampal CA1 neuronal counts in global ischemia.

5. To validate the measurement of persistent hyperactivity against hippocampal CA1 neuronal counts as a method of screening for complete global ischemia in the gerbil.

CHAPTER 2. REVIEW OF LITERATURE

2.1 Introduction

Stroke, which is defined as a disruption in cerebral blood flow (13), afflicts between 40,000 and 50,000 Canadians every year (2). Cerebrovascular diseases rank as the third leading cause of death in Canada, following cancer and heart disease (14). Each year the Canadian Health Care System spends $2.7 billion on costs associated with stroke patients (2). To date there is no effective treatment to prevent brain damage arising from stroke (3). The only accepted therapy for acute stroke is intravenous recombinant tissue plasminogen activator administered within three hours of stroke, but this therapy is not neuroprotective and in order to be effective needs to be initiated within 3 hours of the start of the stroke (3). The limitations of this treatment mean that few patients qualify to be treated. In order to develop effective treatments, it is also crucial to identify factors that could exacerbate brain injury associated with stroke. Undernutrition, which is present in a large proportion of acute stroke patients, may play a role in stroke development and outcome (4,15).

A recent study conducted in our laboratory (9,10) has demonstrated a causal relationship between protein-energy malnutrition (PEM) and poor functional (behavioural) outcome in an animal model of stroke. Although the mechanism(s) for this are unknown, an increased inflammatory response may be a contributor (10).

2.2 Protein-Energy Malnutrition and Stroke

2.2.1 Prevalence of PEM in Stroke Patients

Most stroke patients are elderly (16). After the age of 55, stroke risk doubles every 10 years (2). This population is more at risk for undernutrition, including protein-energy malnutrition (PEM) (7). PEM is a comprehensive term used to refer to the situation when the requirement for protein and energy is not met (17).The tools used to measure PEM in the many different stroke studies are inconsistent, making it difficult to make comparisons between studies. Tests of PEM include dietary assessment, anthropometry, and static biochemical and functional indices (18). In hospitalized patients, the identification of PEM is difficult because many of the tests used to detect PEM can be affected by pre-existing conditions, such as inflammation (18). Tests of nutritional status using a single indicator have limited use for identifying PEM in patients; multi-parameter screening tests, or scoring systems, may be more effective for its identification (18). Serum albumin is the most common serum protein used to detect PEM (18). Although serum albumin is nonspecific, it has been found to be a strong independent predictor of mortality in hospitalized stroke patients (6,7). Other biochemical tests that can be used to detect PEM are serum transferrin, white blood cell count, total lymphocyte count, 24-hr urinary urea nitrogen, 24-hr urinary creatinine, creatinine height index, and more recently, serum transthyretin (18). Anthropometric indicators of PEM commonly used are weight loss, body mass index (BMI), triceps skin-fold thickness, and upper arm muscle circumference (18). Functional tests that can be used to detect PEM are tests of muscle weakness, such as hand grip strength, respiratory muscle weakness,

poor wound healing, depression, irritability and fatigue, and delayed hypersensitivity testing (18). Examples of multi-parameter screening tools used to detect PEM are the prognostic nutritional index, the nutrition risk index, the mini nutritional assessment (MNA), and subjective global assessment (18).

Despite these limitations in assessment tools used, several estimates of the prevalence of PEM in acute stroke patients have been made. In a study by Davalos *et al*. (4), 104 acute stroke patients were immediately assessed for nutritional status on admission and then weekly during hospitalization. Nutritional status was assessed by measuring triceps skin-fold thickness (TSF), mid-arm muscle circumference (MAMC), and serum albumin concentration. They found that 16.3% of patients at admission and 26.4% after the first week had protein-energy malnutrition. Malnutrition was associated with an increased stress reaction during the first week, increased incidence of respiratory and urinary tract infections and bedsores, increased mortality, worse outcomes and longer duration of hospital stay. Axelsson and colleagues (19) looked at the nutritional status of 100 stroke patients within the first 4 days of admission to hospital. Nutritional status was assessed by measuring body weight, triceps skin-fold thickness and upper arm muscle circumference (AMC) using the right arm, plasma albumin and prealbumin, as well as serum transferrin. Of the 100 patients, 16% were malnourished upon admission, and of the 78 patients remaining at discharge, 23% were malnourished. Unosson and colleagues (20) found that 8% of stroke patients had PEM and 62% had low serum albumin values within 48 hours of admission. The patients were classified as having PEM if they met certain criteria for the following variables: weight index,

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triceps skin-fold thickness and upper arm muscle circumfrence using the left arm except when the left arm was paralyzed, serum transthyretin, albumin and $\alpha 1$-antitrypsin, and delayed hypersensitivity skin testing (DHT). Choi-Kwon et al. (15) studied the nutritional status of 88 Korean female stroke patients (67 with cerebral infarction and 21 with intra-cerebral hemorrhage) admitted to hospital with their first stroke. Nutritional status was assessed by measuring BMI upon admission, TSF, sub-scapular skin-folds thickness (SSF), abdominal skin-folds thickness (ASF), and lean body mass (LBM) within one week of admission, and blood lymphocyte count, hemoglobin and serum albumin within 24 hours of admission. 62% of patients with intra-cerebral hemorrhage, 25% of patients with cerebral infarction and 13% of healthy age-matched controls were malnourished. This study found a very high incidence of malnutrition in female stroke patients compared to other studies, but this may be because the nutritional assessment was not conducted upon admission in all subjects; anthropometric measurements were taken up to one week following admission. Davis and colleagues (5) used subjective global assessment (SGA) within 24 hours of admission on 185 acute stroke patients and found that 16% of these patients were malnourished. Crude analysis found that malnourished patients were significantly more likely to die or have poor outcomes than the well-nourished patients; this was not significant after adjustment for other prognostic factors such as age, pre-morbid dependence, and stroke severity. Low serum albumin concentrations upon admission were significantly associated with pre-morbid nutrition and mortality at one month post-stroke, but not with poor outcome at the same time point.

Elderly patients are already at increased risk for co-existing PEM at the time of admission for stroke due to factors in their previous medical history. These can include reduced nutritional reserves, prolonged hospital stays, increased demands of repeated ill health, dysphagia, poor nutritional support, prolonged inactivity, and functional disability (4,6,7,15). The prevalence of PEM becomes even higher in the elderly post-stroke (4,6,7,19,21). One reason for this is decreased ability to eat following stroke (4,21).

Low serum albumin concentrations have been implicated with a negative prognosis in stroke patients (18). Serum albumin is used as a nonspecific marker for protein status because the latter is only one of several regulators of albumin synthesis (6,7). In two observational prospective studies (6,7) that measured serum albumin concentrations in patients admitted to hospital with stroke, low serum albumin concentration, after adjustment was made for other prognostic factors, was a strong predictor of death following hospitalization. These studies also looked at anthropometric measures such as triceps and biceps skin fold and mid arm circumference to assess nutritional status, but these variables were not correlated with stroke outcome. Preliminary analysis of the first 3012 patients admitted to hospital with recent stroke from the FOOD (Feed or Ordinary Food) trial (8), a series of international multi-centre randomized trials, suggested that undernourished patients had decreased chance of survival, functional ability and living circumstances at six months follow-up. Unfortunately, this series of studies had one major limitation, which was a lack of standardization for the nutritional assessment. Clinicians categorized the patients into underweight, overweight and normal based

on a bedside assessment and only in some instances did this include weight, height, dietary history or blood tests. Only 9% of the study population was judged to be malnourished upon admission to hospital. The low prevalence of PEM found in this study compared to other studies may be due to their poor nutritional assessment strategy. These studies also did not assess nutritional status after nutritional interventions, nor was actual nutrient intake recorded (22-24). Upon completion, the FOOD trials did not adequately test the hypothesis of a causal relationship between compromised nutritional status and stroke outcome (22,23). Thus, the full extent to which undernutrition and PEM contribute to stroke outcome is not yet known (7). However, a study from our laboratory in an animal model of stroke suggests that there is a direct link (10).

Although the tools used to assess PEM in elderly stroke patients have limitations, most of the studies estimating the prevalence of PEM arrive at a similar number. Of the tools that could be used to assess PEM, only the mini nutritional assessment was designed specifically for use in the elderly (18), but it has not been used to date in stroke studies. The original mini nutritional assessment uses 18 indices grouped into anthropometric assessment, general assessment, dietary assessment, and subjective assessment (18). The final score from these four groups is totaled and used to categorize patients as normal, at risk for malnutrition, or undernourished (18). A new version of the mini nutritional assessment uses a two step approach; the first step is used to screen for potential malnutrition and if patients fail this step, then the second step is used to assess malnutrition, and groups patients into either malnourished or at risk of malnutrition (18). The mini nutritional

assessment would be the best way to assess PEM in future studies with elderly stroke patients because it is the only one designed and validated for use in the elderly and it is a multi-parameter tool. If time is an issue, the new version of this tool may be more beneficial.

2.2.2 Modeling of PEM in Elderly Stroke Patients

PEM in industrialized countries occurs in the elderly, as well as in institutionalized and hospitalized adults (25-28). Decreased nutrient intake, physical activity levels, lean body mass and basal metabolic energy requirements occur naturally with healthy aging (29-34). As a result, elderly individuals can become protein-energy malnourished from decreased consumption of energy and protein; conversely, they may be subject to obesity if they continue to consume the same amount of energy as they did when they were younger (30). Physiological and pathophysiological changes that occur with aging that contribute to decreased energy intake are decreased olfactory and taste thresholds, gastro-intestinal changes, prevalence of chronic illness, physical impairments that hinder the ability to shop for and prepare food, increased medication use, poor dentition, depression, isolation and dementia (30-34). Chronic illness and medications can increase nutrient requirements as well (32,33).

In a study by De Castro (29), a convenience sample, which is a sample of volunteers, of 167 healthy adult males and 140 healthy adult females was used to study age-related changes in food intake. All subjects were instructed to keep a 7 day food diary detailing what they ate or drank, the time it was eaten, the amount eaten, how the item was prepared, the number of people that ate with them, their

subjective state of pre- and post-meal hunger, thirst, mental state and pre-meal activity levels. Subjects' intakes were verified by contacting individuals that had eaten with them during the study period. Subjects that were aged 50-80 ate 12% fewer Calories than subjects aged 20-49. This decrease in caloric intake was due to a decrease in both fat and protein intake and smaller meals, in general. The older subjects also reported feeling more elated at meal times, less anxious, and being less active than the younger groups. This may suggest that when the healthy elderly individual becomes unhealthy that they will be more likely to become malnourished. Overall, the reductions in energy intake were more marked for males than females. This study also found that external stimuli are more important for intake in the elderly than the subjective state of hunger. The results of this study do, however, have some limitations due to selection bias and self-reporting of results.

Endocrine changes may explain some of the decrease in food intake in the elderly population (31-34). Increased circulating concentrations of cholecystokinin, which is a satiating hormone, are found in the elderly (31-34). Dynorphin mediates the opioid feeding drive and participates in regulating fat intake (31,33,34). Aging is associated with decreased opioid receptors, which would lead to a decrease in the opioid feeding drive (31,33,34). Neuropeptide Y may be less effective in stimulating food intake in the elderly (31,34). Insulin increases with age, as a result of insulin resistance, may be satiating (31,34), and the co-secretion of amylin with age may act as an anorectic agent (31). In general, testosterone increases food intake and estrogen decreases it (31). Testosterone levels decrease with aging and may

play a role in the reduction in energy intake that occurs with aging, especially in men (31,34).

Common characteristics of PEM in the elderly are impaired muscle function, reduced heart muscle mass, decreased bone mass, immune dysfunction, poor wound healing, delayed surgical recovery, skin thinning, fatigue, apathy, hypothermia, anemia, reduced cognitive function, and increased mortality (34-36). Weight loss is also characteristic of PEM in the elderly; a 10% weight reduction from usual body weight may indicate PEM (33).

Current terminology utilizes the comprehensive term, protein-energy malnutrition, to refer to the situation when the requirement for protein and energy is not met (17). Upon diagnosis of PEM, it can be classified as mild, moderate or severe, and as acute or chronic (17). The diagnosis of severity and course or duration of PEM is usually done using anthropometry (17). Unfortunately, the available terminology and grading scheme for assessing the type and degree of PEM has been largely derived from studies of more severe malnutrition in children in which growth can be assessed, and thus these standards are not relevant for assessing PEM in elderly stroke patients. Weight for height is seen as the best measure to determine current nutritional status and height for age to determine past nutritional status (17). Wasting is the term used for a weight for height deficit and stunting is used for a deficit in height for age (17). Using these indexes, patients can be classified as i) normal, ii) wasted but not stunted (acute PEM), iii) wasted and stunted (acute and chronic PEM), and iv) stunted but not wasted (had PEM in the past, and are at present adequately nourished) (17). In adults, the BMI is the

anthropometric measure best used to assess severity of PEM (17). Weight loss is the

most predominant clinical manifestation of mild to moderate PEM, and adults show

a decrease in subcutaneous fat and lean body composition (17). Mild to moderate

PEM does not give consistent biochemical changes (17). Low protein intakes could

lead to low urinary excretion of creatinine, urea nitrogen and hydroxyproline, altered

plasma free amino acids, with a decrease of branched-chain essential amino acids

and slight decreases in serum transferrin and albumin, as well as a reduced number

of circulating lymphocytes (17). Studies focusing on PEM in stroke patients at

admission have not classified severity of PEM, although the data suggest that it is

mild to moderate.

In contrast to the milder forms of PEM described in elderly individuals in

North America, the terms, kwashiorkor and marasmus, are used to refer to the

extreme forms of PEM found most often in children of developing countries (26-

28,37). The diagnosis of severe PEM, including marasmus and kwashiorkor, is

based on dietary history and clinical features (17). Marasmus is the most common

form of malnutrition in times of famine and leads slowly to death (26).

Characteristics of marasmus are thinness, a weight loss of more than 20% of initial

body weight, fatigue, irritability, a triangular face, amenorrhea, extended abdomen,

anal or rectal prolapse, severe loss of muscle and adipose tissue, low body

temperature, decreased pulse and metabolic rate, constipation and occasionally

starvation diarrhea (26-28,37). Marasmus represents a form of adaptation to

starvation, it is normally well tolerated by adults (27,28). Kwashiorkor occurs in the

presence of severe protein deprivation and is not as well understood as marasmus

(26-28). Characteristics of kwashiorkor include edema, hair and skin discolouration, changes in mucous membranes, tongue papillae atrophy, loss of muscle tone, hepatomegaly, lethargy, apathy, irritability, severe immune deficiency, stunted growth, fatty liver, decreased serum transferrin and glutathione, and in its worst case coma and death (26-28,37). In contrast to marasmus, kwashiorkor can be seen as a failure to adapt to malnutrition (28).

When insufficient energy and protein are supplied (25,28), the initial source of amino acids for increased gluconeogenesis is smooth muscle from the gut; however, skeletal muscle quickly becomes the main source of amino acids (26,28). Decreased food intake leads to reduced plasma amino acid and glucose concentrations, which cause a reduction in insulin secretion and an increase in glucagon and epinephrine (17). The increase in glucagon and epinephrine also causes a reduction in insulin secretion (17). The declining insulin concentrations allow for increased lipolysis and ketogenesis (25,26,28,38), allowing adaptation to occur to maintain lean body mass (25,26,28). If starvation continues to the point where 50% of body protein stores have been used, the oxidation of lipids and the levels of ketones in the body are decreased (25). The resting metabolic rate also decreases, after an initial increase, when triiodothyronine and catecholamine levels decrease (28). If this process is not stopped, the eventual outcome will be death (25).

Although the need was pointed out in 1998 (39), there are no standards of nutritional status that allow investigators to choose experimental animal models to mimic specific categories of human PEM. In the absence of such information, the

parameters used to assess protein-energy status in animal models are typically decreased body weight, feed intake(40), serum albumin (6) and liver glutathione (40-42), and increased liver lipid (42). The two common experimental models of PEM in rodents are restricted intake of a nutritionally complete diet and ad libitum consumption of a low-protein diet (39). Previously, a gerbil model of PEM has been characterized for use in global ischemia studies in our laboratory (9,10). In this model of PEM, gerbils fed a low protein diet (2% protein) voluntarily reduce their feed intake, making the model a combination of both protein and energy malnutrition (9,10). Feeding the 2% protein diet for 4 weeks in the gerbil previously induced a 17%, 15%, and 49% reduction in body weight, feed intake, and liver glutathione, respectively, as well as a 66% increase in liver lipids compared to controls (9,10).

2.3 Mechanisms of Ischemic Brain Damage

The mechanisms that cause damage following ischemic stroke are excitotoxicity, peri-infarct depolarizations, oxidative stress, inflammation and programmed cell death or apoptosis (13,43). Disruption of cerebral blood flow interrupts the delivery of oxygen and glucose, which are substrates the brain needs for energy production (13,44). When cellular energy decreases, the cells in the brain, neurons and glia, depolarize (13,44). This causes the activation of voltage-gated Ca^{2+} channels and the release of excitatory neurotransmitters, such as glutamate (13,44). Glutamate accumulates in the extracellular space which causes the activation of NMDA receptors and metabotropic glutamate receptors which also leads to Ca^{2+} efflux (13,44). Na^+ and Cl^- enter neurons due to extracelluar glutamate

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accumulation (13,44). This is followed by water rushing into cells which causes edema (13,44). Activated leukocytes cause the activation of phospholipase and cyclooxygenase (COX) which causes the generation of free radicals (13,44). Activated phospholipase also causes the upregulation of leukotrienes, eicosanoids, prostaglandins and platelet-activating factor which leads to vasoconstriction and a subsequent increase in platelet aggregation (44). Increased free radical generation under ischemic circumstances exhausts endogenous scavengers of free radicals (13). This leads to lipid peroxidation and cell membrane damage (13). Oxygen free radicals also trigger the initiation of the inflammatory response and apoptosis (13).

There are two categories of human ischemic stroke (43,45). Focal stroke is when there is a loss of blood flow, due to a clogged artery, in a focalized area of the brain (43,45). Global ischemia occurs when there is a complete loss of blood flow to a hemisphere of the brain, which is what happens during cardiac arrest (43,45). Although global ischemia models, such as the one used in this thesis, have been criticized for mimicking cardiac arrest rather than focal stroke, they share many of the same pathophysiological mechanisms (43,46) yet provide more consistent injury (47). Thus animal models of global ischemia are often used to investigate problems relevant to patients with focal ischemia. As described below, the pathophysiological mechanisms of interest in this thesis, oxidative stress, inflammation, and reactive gliosis are all present in models of global ischemia (48) although inflammation to a lesser degree than in focal ischemia (49).

Primary damage from brain ischemia begins with a disruption of blood flow to the brain, which results in the depletion of oxygen and glucose, necessary

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substrates for the production of ATP, thus depleting the brain's supply of ATP (50-52). The depletion of ATP leads to the reduced activity of ATP-dependent ion pumps, in particular, Na^+/K^+-ATPase, which leads to membrane depolarization (50-52). Reduction in Na^+/K^+-ATPase activity leads to an increase in extracellular K^+ and intracellular Na^+, Cl^- and Ca^{2+} (50-52). The influx of Ca^{2+} leads to an increased release of excitatory amino acids, such as glutamate (50,51). The excess glutamate over-stimulates NMDA receptors and leads to Na^+, Cl^-, Ca^{2+} and water accumulation which leads to cellular swelling and cytotoxic edema (50,51). The increase in intracellular Ca^{2+} leads to the activation of phospholipase A2, COX, neuronal nitric-oxide synthase (nNOS) and inducible nitric-oxide synthase (iNOS), which consequently produce free radicals (50). Excess intracellular Ca^{2+} also leads to a calcium overload in mitochondria, inhibited ATP production, breakdown of macromolecules such as phospholipids, proteins and nucleic acids (51).

Secondary brain damage occurs hours or days after the initial ischemic event, and is when symptoms worsen and brain damage progresses (52). Transient global ischemia can give rise to delayed neuronal cell damage or death, which is the case in the CA1 region of the hippocampus following global ischemia (52).

2.3.1 Role of Cytokines and Glial Cells in Global Ischemia

An inflammatory response has been found in approximately 75% of acute stroke patients (53). Prolonged inflammation in acute stroke has been associated with a poor prognosis (53). Following an ischemic episode, leukocyte adhesion molecules such as intercellular adhesion molecule (ICAM)-1, P-selectin, and E-selectin are rapidly induced on microvascular endothelium (44). Peripheral

inflammatory cells can migrate into the CNS after the leukocyte adhesion molecules adhere to the microvascular endothelium (54). Proinflammatory cytokines are produced by activated leukocytes (44). Cytokines are also produced secondary to ischemia and reperfusion (55). Endothelial cell exposure to cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-1β upregulate ICAM-1, E-selectin and leukocyte adhesion molecule generation (54-56).

Inflammation is a major component of the brain damage caused in global ischemia. In a study by Saito *et al.* (57), female Mongolian gerbils underwent bilateral common carotid artery occlusion, and regional levels of TNF-α, IL-1β and interleukin (IL)-6 were measured by enzyme-linked immunoassays. After 5 minutes of occlusion, the greatest increase in cytokine concentration compared with shams was found in the hippocampus at 6 hours post-reperfusion. Peripheral immune cells usually do not migrate into the damaged CNS before 24 hours post-ischemia in global ischemia models as they do in focal ischemia (45); therefore these early increases in cytokine concentrations are most likely due to activated microglia and astrocytes. In another study, male and female Mongolian gerbils were subjected to 10 minutes of bilateral common carotid artery occlusion (58). Localization of IL-6, which has been shown to have neuroprotective effects, was examined using immunohistochemistry and Western blot analysis. IL-6 was localized in the CA1 region of the hippocampus, peaked at 3-6 hours after reperfusion, and then steadily declined until 3 days, by the last measurement at 7 days, only a few IL-6 immunoreactive cells remained. Activated astrocytes were shown to increase from 24 hours to 3 days after reperfusion in the CA1 cell layer. These activated

astrocytes, detected by increased numbers of GFAP positive astrocytes, did not

demonstrate IL-6 immunoreactivity. IL-6 immunoreactivity was also found to

significantly decrease at 12 hours after reperfusion. This decrease is most likely due

to CA1 vulnerability to injury. COX-2 is induced by inflammation,

proinflammatory cytokines and reactive oxygen species (ROS) (59). Maslinska *et*

al. (60) found COX-2 immunopositive hypertrophic astrocytes around the edges of

the necrotic tissue in human brains of patients that had died 2 or more weeks after

resuscitation from global ischemia.

Reactive gliosis, which is caused by brain injury, is an increase in the size of

the astrocyte cell body and its processes accompanied by an increase in GFAP (61).

Reactive gliosis also causes both astrocytes and microglia to proliferate around the

site of injury (62). There is considerable controversy as to whether astrocytes and

microglia are protective or detrimental in brain injury. The end product of reactive

gliosis is the glial scar, which is composed mainly of astrocytes, and may possibly

function to isolate the region of injury from healthy neural tissue (62). Microglia are

often activated before the activation of astrocytes which suggests that microglia may

be a trigger for reactive gliosis (61). In the rat brain, microglia respond within the

first 20 minutes after transient global ischemia (63). After 2-4 days, neural damage

is obvious in the CA1 region of the hippocampus and the microglia are activated

further which worsens the inflammatory response (63). Proinflammatory cytokines

induce the activation of glial cells, which then produce cytokines (64). Despite some

studies showing the temporal response of microglia and astrocytes to ischemic injury

up to 7 days following global ischemia (65-68), the temporal response from the time

following ischemia to a later time point, such as 10 days post-ischemia has not yet

been shown, and the effect of PEM on microglial and astrocytic activation following

global ischemia is not yet known.

Whether astrocytes provide a protective role for neurons and/or an injurious

role is controversial (69). Astrocytes release compounds such as neurotrophins

which are protective (69). On the other hand, astrocytic swelling, which occurs early

after ischemia in humans, is a cause of delayed neuronal death (62). Activated

microglia stimulate inflammation and secrete toxic compounds such as TNF-α, IL-

1β and NO which promote neuronal injury (69,70). Chronic neurological stress

causes microglia to become activated by both the signal produced by the injury and

inflammatory cells (70). The activated microglia secrete proinflammatory

compounds and chemokines which cause other microglia to migrate to the area (70).

This process perpetuates the damage (70). Microglia can also transform into

macrophages and present antigens to T cells (49).

Reactive gliosis is a general feature of rodent models of global ischemia

(67,68,71). This reactive gliosis has also been described in a number of studies

using the gerbil model of bilateral common carotid artery occlusion (48,66,69,72).

Kato *et al*. (69) looked at astrocyte and microglial activation in gerbils that

underwent 2 minutes of ischemic preconditioning, a brief ischemic insult that does

not produce significant brain damage and protects the brain from later ischemic

insults, before a 3 minute ischemic episode, compared with animals that had a sham

operation followed by the 3 minutes of ischemia. In animals with ischemic

tolerance, the animals that have had the ischemic preconditioning, there were

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moderate increases in astrocyte and microglial staining. After the 3 minute ischemic episode in the preconditioned animals, GFAP staining increased in the hippocampus. The animals without ischemic tolerance had microglial activation with pronounced staining using isolectin-B4 from *Griffonia simplicifolia*, followed by neuronal destruction by day 7 post reperfusion, whereas preconditioned animals had no CA1 neuronal injury. This suggests that astrocyte activation may be protective in the CA1 hippocampal region. Another study (66) looked at whether doxycycline and monocycline, which are antibiotics with anti-inflammatory properties, had neuroprotective effects. Male Mongolian gerbils were subjected to 6 minutes of bilateral common carotid artery occlusion. They found that minocycline blocked the activation of microglia and reduced the induction of iNOS. They found no difference in astrogliosis between treated and non-treated animals. This demonstrates that microglia have more injurious effects in global ischemia than astrocytes. Liu *et al*. (72) found pronounced microglial/macrophage proliferation after 5 minutes of bilateral common carotid occlusion in male Mongolian gerbils. The length of ischemia in these animals caused the death of most of the CA1 pyramidal neurons. They also found microglia throughout the CA3 region and dentate granule cell neuron layer, which is normally resistant to global ischemia (12).

Domanska-Janik *et al*. (48) looked at the role of nuclear factor (NF)-κB, a protein transcription factor which promotes the transcription of a number of pro-inflammatory molecules (48), in activation of glial cells. Mongolian gerbils underwent bilateral common carotid artery occlusion followed by 3-4 days of

reperfusion. They found that GFAP levels were near control levels for the first 24 hours, and the strongest reaction was seen at day 7 post reperfusion in the CA1 hippocampal region. Ricinus communis agglutinin (RCA)-120 lectin staining for microglia was apparent in the first 24 hours in the CA1 region of the hippocampus and had increased by day 3. The apoptotic neurons had disappeared by day 7, but GFAP and RCA-120 lectin were still expressed by astrocytes and microglia, respectively. They also found that NF-κB levels were increased early in the CA1 region. This demonstrates a relationship between NF-κB and the early activation of microglia in the CA1 region of the hippocampus.

2.3.2 Proposed Mechanism of Influence of PEM on Brain Damage in Stroke

Our laboratory has previously shown that protein-energy malnourished gerbils that were subjected to global ischemia had significantly worse behavioural outcomes, as assessed by open field, when compared to ischemic animals fed control diet (10). An open field test is conducted by placing animals in a novel environment 3-5 days post-ischemia, animals that fail to habituate to this environment by decreasing their activity levels, are assessed as having functional deficits in the CA1 region of the hippocampus (12). Although PEM did not worsen the loss of hippocampal CA1 neurons at day 10 post-ischemia, one third of the PEM animals showed marked gliosis that completely disrupted the CA1 pyramidal neuron region. Sections immunostained with anti-glial fibrillary acidic protein (GFAP) antibody suggested that this subset of PEM ischemic animals had a large population of reactive astrocytes (unpublished observations). A limitation of this study is that the hippocampal sections had first been stained with hematoxylin and eosin (H & E).

This reduced sensitivity such that it was not possible to immunohistochemically detect whether there were also increased numbers of activated microglia present. Reactive astrocytes and microglia can cause an increased inflammatory response post-ischemia (49,67,69,73). Therefore, increased reactive gliosis post-ischemia, if confirmed, may be indicative of an increased inflammatory response in PEM (10). In order to ascertain the effect of PEM on astrocyte and microglial activation following global ischemia, this study needs to be repeated with appropriate tissue sections.

One potential mechanism by which PEM may increase the inflammatory response of the brain to stroke is by increasing activation of the protein transcription factor, NF-κB. NF-κB, which promotes transcription of proinflammatory molecules, is found in all cell types (74,75). Proinflammatory molecules transcribed by NF-κB include ICAM-1, iNOS, COX-2, IL-1β, IL-6 and TNF-α (74). Activated NF-κB has been found in high concentrations in brain tissue of rodent stroke models (75). Recently, a colleague in our laboratory, using the bilateral common carotid artery occlusion model of global ischemia in the gerbil, has shown that PEM, independent of ischemia, causes increased NFκB, as assessed by electrophoretic mobility shift assay, activation at 6hr post-surgery, and a trend at 24hr towards an increase (76). Li *et al*. (77) examined the effect of lipopolysaccharide (LPS), a bacterial endotoxin, on inducing NF-κB activation in the liver of protein-malnourished mice. They found that mice with protein malnutrition had significantly increased activation of NF-κB and also increased levels of its transcription products IL-1β and TNF-α. This study also found that early supplementation with N-acetylcysteine (NAC), a cysteine

pro-drug that has been found to increase reduced-glutathione (GSH) levels, to

protein malnourished mice normalized the GSH levels and the activation of NF-κB.

GSH is an endogenous antioxidant that has been found to decrease in tissues, such as

liver, lung, colon, heart and spleen, under conditions of protein malnutrition (77,78).

Brain concentrations of glutathione in protein-malnourished gerbils are not reduced,

which may indicate that the brain has tighter control over glutathione concentrations

than other body tissues (10). Although brain glutathione concentration remains

unchanged in PEM, there may be increased oxidative stress driving NF-κB

activation, independent of glutathione (10). In support of this idea, increased

inflammatory mediators, such as IL-6, C-reactive protein, the soluble receptors for

TNF-α, and leukotrienes, have been reported in children with PEM (79,80). PEM

has also been associated with an increased inflammatory response in patients with

chronic obstructive pulmonary disease, chronic heart failure, chronic renal failure

and rheumatoid arthritis (81).

2.4 Animal Models of Stroke

Animal models of brain ischemia are crucial to the study of stroke because

they can be controlled, produce reproducible results and are important for studying

the mechanisms involved in ischemia (82,83). Treatments for cerebral ischemia also

need to be tested and proven safe and effective in animal models prior to being

tested in clinical trials (84).

2.4.1 Animal Models of Focal and Global Ischemia

Animal models of ischemia can be classified as either focal or global (82-84).

Rodents are used to study brain ischemia because of low cost, homogeneity between

strains, similarity of cerebrovascular anatomy to that of higher species, small brain size, and their use is more ethically acceptable than the use of higher species (82). Physiological monitoring is important in all models of brain ischemia to ensure accurate and reproducible results (82-84).

Focal ischemia more closely mimics what is seen in human stroke (82-84). The most widely used model of focal ischemia is the occlusion of the middle cerebral artery (MCAO) in the rat, which can be either transient or permanent (82-84). MCAO produces a targeted infarct in the area where the artery would normally deliver blood flow as well as a penumbra surrounding the necrotic tissue that is potentially salvageable (82-84). The middle cerebral artery can be either clipped or ligated transcranially, postorbitally or transorbitally, which are all invasive and require surgery (82-84). Recently, intraluminal methods of MCAO have replaced the more invasive methods (82-84). This method involves the placement of fine-nylon threads with a silicon-thickened tip into the external carotid artery to obstruct the flow of blood to the middle cerebral artery (82-84). This model can become transient by the removal of the threads (82-84).

Global ischemia is usually transient and affects tissue more globally than the focal models (82-84). Global ischemia mimics human cardiac arrest more than human stroke (82-84). As noted above, focal stroke and global ischemia share many of the same pathophysiological mechanisms, with the benefit of global ischemia models providing more consistent injury (82-84). A widely used model of global ischemia has been the bilateral common carotid artery occlusion (BCCAO) in the gerbil. This involves clamping the bilateral common carotid arteries for a

predetermined period of time followed by the removal of the clamps to allow reperfusion (82-84). This model allows for delayed neuronal death (82-84). In rats, the two-vessel occlusion of the bilateral common carotid arteries produces global ischemia when combined with systemic hypotension (82-84). This model also produces delayed neuronal death (82-84). The major disadvantage of this model is that because of the hypotension, physiological variables are more difficult to control (82-84). The four-vessel occlusion model of forebrain ischemia is another model of global ischemia used in the rat (82-84). This method is technically more difficult, but is applied to freely moving, awake animals (82-84). This procedure requires 2 stages (82-84). The first stage has a high fatality rate and involves the permanent occlusion of the anterior vertebral arteries by electrocauterization under anaesthesia as well as the placement of a clasp around the bilateral common carotid arteries that is placed through a ventral midline neck incision (82-84). One day later, forebrain ischemia is produced by the tightening of the clasps around the bilateral common carotid arteries (82-84). These clasps are removable if reperfusion is desired (82-84). Due to the high degree of technical difficulty of this procedure, the failure rate can be as high as 25% (82).

2.4.1.1 Gerbil Model of Global Ischemia

Historically, the bilateral common carotid artery occlusion (BCCAO) in the gerbil was an excellent model of global ischemia due to the gerbil's incomplete Circle of Willis. The gerbil lacked posterior communicating arteries such that BCCAO led to delayed neuronal cell death and severe forebrain ischemia that was reproducible with low variability (11,82-84). The gerbil model had also been

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characterized for functional outcome (behavioural) tests, including the open field and the t-maze, and has the advantage of being less invasive (12,83). The latter translates into less of a stress response in the animal which is important for use in nutritional studies (12,83).

Recently, variability in neuronal cell death in the gerbil BCCAO model has been increasing and has led to the discovery that a high percentage of these animals are developing posterior communicating arteries that are large enough to allow for significant blood flow, thereby completing the Circle of Willis (11,85). A recent study conducted using gerbils from two different suppliers, Charles River and High Oaks, showed that gerbils from these suppliers had bilateral or unilateral posterior communicating arteries in 61% and 16%, respectively (11). Ischemic gerbils from High Oaks showed 95% loss of CA1 hippocampal neurons, while gerbils from Charles River showed only a 71% loss of CA1 hippocampal neurons (11). Also, in open field testing, High Oaks gerbils exposed to BCCAO showed impaired habituation on days 3, 7, and 10 post-ischemia, while gerbils from Charles River did not, and performance in the open field predicted the CA1 hippocampal neuronal loss (11). A study using gerbils from the two suppliers in the United States, Charles River and Harlan Sprague-Dawley, found that 90% of their gerbils had posterior communicating arteries (85). These studies demonstrated a definite need to screen gerbils for severity of ischemia following BCCAO in order for the model to continue to be used.

2.4.1.1.1 Post-Ischemic Hyperactivity as a Screening Tool for Severity of Ischemia

Directly following the ischemic episode, there is a period of inactivity that lasts from when the animal regains consciousness and their righting reflex for about 30 minutes to a few hours (11). Immediately following this phase, there is a period of persistent hyperactivity that can last for several days post-ischemia depending on the severity of the ischemic episode (11). Gerbils with a severe ischemic insult display consistent hyperactivity during this period (11,86,87). Therefore, an effective, non-invasive method of screening for complete ischemia would be to monitor post-ischemic activity. Another objective of this thesis describes validation of a method of screening for complete ischemia based on hyperactivity.

CHAPTER 3. MATERIALS AND METHODOLOGY

3.1 Animals and Diet

Male Mongolian gerbils (*Meriones unguiculatus*, Charles River Canada, Saint-Constant, QC, Canada), aged 11-12 weeks, were acclimated for 3-7 days, and then randomized to control (CON, n=44) or protein deficient (n=45) pelleted diets (Dyets, Inc., Bethlehem, PA, USA), which contained 12.5% and 2% protein as casein (10), respectively (Table 3.1). When gerbils are fed this low protein diet, they voluntarily reduce their feed intake, making them both protein and energy malnourished (PEM) (10). During the acclimatization period (4-7d), the animals were all kept on the basal (CON) diet. The basal diet was modified from the American Institute of Nutrition (AIN)-93M rodent diet (88) to contain no tertiary- butylhydroquinone, an antioxidant. Animals were housed in climate controlled facilities on a 12 hour light/dark cycle in shoebox cages with CareFRESH® (Absorption Corp, Bellingham,WA, USA) bedding in groups of three or four with free access to feed and water. During the 28 day feeding period, weekly body weights (OHAUS, Triple Beam Balance 700 Series 2610 g, Florham Park, NJ) and daily feed intakes (Sartorius 1401 MP 1500,0 g, Germany) were recorded. The white pelleted diet was easily seen and removed from the dark bedding in order to measure feed spillage. Body weights were also recorded on the final day of the study. All animal care and procedures were in accordance with the Canadian Council on Animal Care

guidelines (89) and were approved by the University of Saskatchewan Committee on Animal Care and Supply.

Table 3.1. Modified AIN-93M Rodent Diet[¥]

Component	Adequate Protein[§] (Control, CON) g/kg	Protein Deficient[§§] (PEM) g/kg
Vitamin free casein	140	22.4
L-cysteine	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

[¥]Diet formulation provided by Dyets, Inc.
[§]Control diet was formulated to contain 12.5% protein, 10% fat, 78% carbohydrate.
[§§]PEM diet was formulated to contain 2% protein, 10% fat, 88% carbohydrate.
[*]AIN-93M mineral mix (88).
[**]AIN-93M modified mineral mix: calcium and phosphate deleted, potassium citrate increased from 28 to 226.55g/kg, sucrose increased from 209.806 to 618.256g/kg mineral mix.
[¶]AIN-93M vitamin mix (88).

3.1 Surgical Procedures

At day 28, animals underwent 5 minutes of bilateral common carotid artery occlusion or a sham surgery as described by Dowden and Corbett (90), generating four experimental groups (Figure 3.1): 1) Control-Sham (CON-S) (n=12); 2) Control-Ischemic (CON-I) (n=32); 3) PEM-Sham (PEM-S) (n=12); 4) PEM-

Ischemic (PEM-I) (n=32). Each group had outcome measures after ischemia (n = 8) or sham (n=3) surgery at 4 timepoints: 6hr, 24hr, 3d and 10d. Animals were anaesthetized with 1.5–2.0% isoflurane with 1L/min oxygen, at which time rectal and tympanic membrane temperature probes were inserted. The rectal probe with a homeothermic blanket (Harvard Apparatus Canaday, Saint-Laurent, QC, Canada) was used to monitor core body temperature and keep the animal warm during anesthesia. The tympanic probe (Barnant Type T Digi-Sense Thermometer) with a Mul-T-Pad® water-heated blanket wrapped around the head was used to approximate and maintain the brain temperature at $36.5 \pm 1.4°C$, with a 6 point average between $36.3°C$ and $36.7°C$.   During the surgical period, under anaesthesia, a ventral midline incision was made and the common carotid arteries were isolated by passing silk surgical thread under both arteries; this allowed for gentle lifting of the arteries. Upon stabilization of tympanic temperature, the arteries were occluded with micro-aneurysm clips that provided 60g of pressure (World Precision Instruments Inc., Sarasota, FL, USA) for five minutes. Occlusion of blood flow was visually assessed. During the five minute occlusion, core and tympanic temperatures were monitored continuously and recorded once a minute. After 5 minutes the clips were removed and blood reflow through the carotid arteries was visually assessed. The incision was then closed using Novafil™ sutures (4-0) and Xylocaine® 2% was used as a topical analgesic. Sham surgeries were identical with the exception of artery occlusion. Immediately following removal from the anesthetic machine, animals were individually placed in a recovery cage under a red

40 watt bulb for two hours; once mobile, animals had the choice to access the lamp or not.
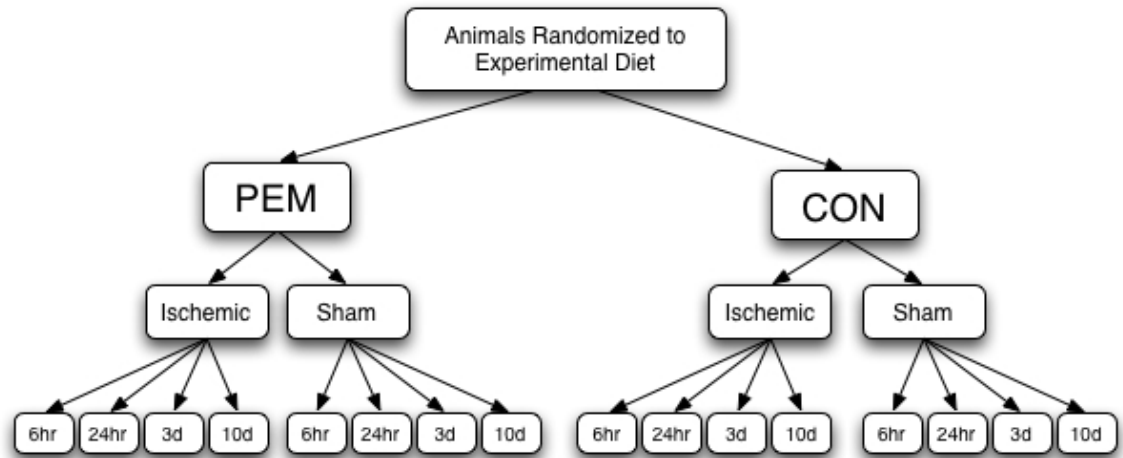
Figure 3.1. Overview of experimental factorial design. The time-points refer to times at which brains were collected for immunohistochemistry.

3.2 Animal Activity Monitoring

Since gerbils with complete global ischemia show persistent hyperactivity (86), this characteristic was used to screen out animals with incomplete ischemia. Immediately following the two hour recovery period, all animals having outcome measurements at 24hr, 3d, and 10d post-surgery had their activity monitored and recorded for 20 hours. The animals with outcome measurements at 6hr were monitored for 4 hours only. Activity was monitored using Opto-M3 Activity Meter (Columbus Instruments, Columbus, OH, USA). This equipment uses infrared sensing on a 16"x16" grid to detect the movement of the animals. Activity data were collected at 15 minute intervals for the duration of the monitoring period. Animals had access to both feed and water during this period. After the allotted monitoring period, animals were returned to their home cage, or in the case of the six hour time point, immediately perfused.

To screen out animals with incomplete ischemia, the following two criteria were proposed and applied to gerbils subjected to bilateral common carotid artery occlusion:

1) Individual mean activity/20hr of these gerbils had to be greater than three standard deviations above the mean activity of a sham group. The rationale for this criterion is that, assuming a normal distribution, the mean + 3 standard deviations will encompass greater than 99% of the sham population (91). Data from a study conducted by Colbourne et al. (87) also suggests that ischemic gerbils would be correctly classified on the basis of this criterion. The area under the curve for the activity pattern over 20 h was also tested in place of mean activity/20h, but identified the same animals for exclusion.

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 only ischemia affects activity values (Data from the 2 combined experiments: p<0.001; PEM-I, n = 35; CON-I, n = 32; PEM-S, n = 15; CON-S, n = 15;), while there is no effect of PEM (p= 0.875) nor is there an interaction between ischemia and PEM (p= 0.686).

2) Activity had to be persistently elevated over the course of the monitoring period (86). The rationale for this criterion is that gerbils with incomplete ischemia can have high initial activity that subsequently decreases (11).

Animals that did not meet these criteria were screened out of the experimental group for immunohistochemical and histological assessment.

The 6h animals could have activity monitored for only 4 hours, which was deemed an insufficient amount of time to assess ischemic severity since the persistence of the hyperactivity could not be assessed (86). Consequently, all 6h animals were included in the results. In interpreting the results, it was taken into account that these animals would likely have a variable ischemic insult.

3.3 Assessment of Protein-Energy Malnutrition

Assessment of PEM was previously defined in the group fed the protein-deficient diet in our laboratory on the basis of reduced feed intake, body weight, and liver glutathione, and elevated liver lipid (10). In the current study, PEM was further characterized by measuring serum albumin concentration. Blood was collected by cardiac puncture at 3 days post-ischemia before tissue perfusion and allowed to sit on the bench top at room temperature for 30 minutes to coagulate. Blood was then spun for 10-15 min at 1500 x g before harvesting serum to be frozen at -80°C until all samples were collected and sent to Prairie Diagnostic Services Veterinary Clinical Pathology Laboratory for analysis. Serum albumin was analyzed using the bromcresol green methodology (92) of detection on a Hitachi 912 colorimeter (Hitachi, Ltd., Japan).

3.4 Immunohistochemistry

At 6hr, 24hr, 3d and 10d post-surgery, animals were anaesthetized with 4.5-5.0% isoflurane with 1L/min oxygen and perfused trans-cardially at 10 mL/min with heparinized saline for 4 minutes followed by 4% paraformaldehyde for 8 minutes. Heads were then removed and placed in jars with 4% paraformaldehyde for 24 hours. Brains were then removed and placed in jars of 4% paraformaldehyde until

they were sent for paraffin embedding, at which point they were placed in phosphate buffered saline (PBS) (pH 7.4) until processing. Brain processing was conducted by the Histology Laboratory in the Department of Veterinary Biomedical Sciences. After paraffin embedding, brains were sectioned using a microtome to 10μm and placed on Precleaned Superfrost® Plus slides (VWR International). Consecutive sections of the entire hippocampus were taken for each immunostain. For each time point, sections were stained with antibodies to glial fibrillary acidic protein (GFAP) (DAKO, Denmark), a marker of astrocytes (48,61), and biotinylated ricinus communis agglutinin I (RCA-120) (Vector Laboratories, Burlingame, CA, USA), a marker of activated microglia (93,94). Sections were also stained with antibodies against the cytokine tumour necrosis factor-alpha (TNF-α) (Biosource International, Camarillo, CA, USA). Approximately 18 sections per antibody were examined to draw conclusions. As a result of moving the tissue sections from the water bath to the slide, at times the sections show tissue folds.

Sections were first incubated overnight at 4°C in 10% horse serum (Vector Laboratories, Burlingame, CA, USA) to block non-specific reactions, followed by overnight incubation at 4°C in the respective primary antibody at dilutions of 1/500 for rabbit polyclonal anti-bovine GFAP, 1/200 for RCA-120 and 1/500 for rabbit polyclonal anti-mouse TNF-α. With the exception of sections being stained for RCA-120, all sections were incubated in biotinylated goat anti-rabbit secondary antibody diluted 1/1000 (Vector), followed by incubation in H_2O_2 to quench endogenous peroxidase activity and washing in PBS (pH 7.4). Sections were then incubated in avidin-biotin peroxidase (ABC, Vector Laboratories, Burlingame, CA,

USA) and the peroxidase reaction was visualized using 3,3'-diaminobenzidine

tetrachloride and hydrogen peroxide from the DAB Substrate Kit for Peroxidase

(Vector Laboratories, Burlingame, CA, USA). $NiCl_2$ from the DAB Substrate Kit

for Peroxidase was used to intensify staining only for RCA-120. Both RCA-120 and

TNF-α protocols required antigen retrieval, techniques used to unmask antigens after

fixation (95), using citrate buffer (96) and pepsin (97), respectively. For citrate

buffer antigen retrieval, citrate buffer was heated in an oven to 50°C, at which point

the rehydrated sections were added and the solution was then heated to 90°C and left

for 25 minutes. Sections were allowed to cool down before continuing with

immunohistochemistry. For the pepsin antigen retrieval, rehydrated sections were

incubated for 15 minutes at room temperature with 2mg/ml pepsin in 0.1N HCl.

After each method of antigen retrieval, sections were washed in PBS prior to

continuing with immunohistochemistry. The specificity of the immunoreactions was

verified by incubating sections without the primary antibody.

Other antibodies to pro-inflammatory cytokines were also tested on brain

sections from the gerbil, but the results were not of high enough quality to proceed.

One potential reason is paraffin embedding of tissue sections. Markers of

proinflammatory cytokines that were tried without success were rabbit anti-mouse

polyclonal COX-2 (Cayman Chemical, Ann Arbor, MI, USA), rabbit monoclonal

COX-2 (Clone SP21) (Lab Vision Corporation, Fremont, CA, USA), rabbit

polyclonal anti-mouse IL-1β (Serotec, Oxford, UK), mouse monoclonal iNOS

(Clone NOS-IN) (Sigma, St. Louis, MO, USA), as well as another iNOS antibody

(Santa Cruz, Santa Cruz, CA, USA). All antibodies were used as described in

methods for immunohistochemistry using antigen retrieval with alterations in protocol being made as follows: 1) primary antibody concentration; 2) length of blocking time; 3) length of time for incubation in ABC; 4) with and without $NiCl_2$; 5) length of incubation time for secondary antibody; and 6) citrate buffer, trypsin, and pepsin antigen retrieval methods.

3.5 Histology

Every tenth section of the brains collected at 10d post-surgery for immunohistochemistry was dedicated for histological analysis. Sections were placed on gelatin subbed slides and stained with hematoxylin and eosin (H&E). The investigator was blinded to slide identity to avoid bias. Viable neurons, non-eosinophilic cells with a defined cell membrane and nucleus, were counted bilaterally at 400x magnification using a 200μm square grid (10 x 10) at the median, middle and lateral sectors of the CA1 region of the hippocampus at levels 'A' (anterior, ~-1.7mm from bregma), and 'B' (middle, ~-2.2mm from bregma), and in the middle sector at level 'C' (posterior, ~-2.7mm from bregma) at 400x magnification, in order to cover the anterior-posterior axis of the hippocampus (Figure 3.2), as previously described (10).
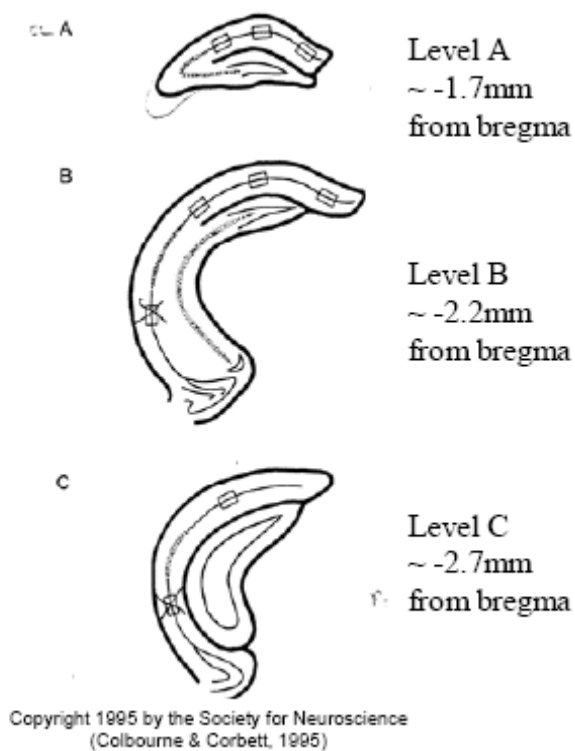
Figure 3.2. Hippocampal CA1 neuron regions for histological assessment (98).

3.6 Validation of Activity Screening for Complete Ischemia

To validate the method of activity screening proposed to identify complete ischemia, the activity data from the 10d animals were checked against the hippocampal CA1 neuron counts from the same animals.

3.7 Statistics

When quantitative data were collected post-surgery, statistics were performed using a 2-way analysis of variance (ANOVA), which tested for independent effects from ischemia and PEM as well as the interaction between ischemia and PEM. Pre-surgery feed and weight data were analyzed using unpaired T-test. Statistical significance was set at the level of $p<0.05$. Post-surgery feed data are shown

descriptively because sample size was insufficient for statistical analysis. Statistics

were analyzed using SPSS for Windows version 13.0.

CHAPTER 4. RESULTS

4.1 Feed Intake and Weight Gain

Initial body weight did not differ between control and PEM groups (p= 0.183) (Table 4.1). At day 28, PEM animals had significantly lower body weight when compared to the control group (p< 0.001), and had lost 12.2% of their initial body weight. Average feed intake in the PEM group per animal per cage (n=2-4 animals/cage) was 10.7% lower than that of the control group for the 28 day feeding period; this difference was significant (p< 0.001).

Table 4.1. Effect of diet on pre-ischemic body weight and feed intake

	Control	PEM
Initial Body Weight (g)	62.3 ± 0.5 (44)	63.2 ± 0.4 (45)
Body Weight on day 28 (g)	65.7 ± 0.5 (44)	55.4 ± 0.5 (45)*
Feed Intake/28d (g)[¶]	108.5 ± 1.8 (11)	96.9 ±1.6 (12)*

Mean ± SEM(n).
* p<0.05, significance determined by unpaired student's t-test.
[¶] Average feed intake per animal per cage (2-4 animals/cage).

Table 4.2 shows body weight data by cage for the post-ischemic period. At 6h and 10d post-ischemia only PEM significantly decreased body weight. At 3d post-ischemia, both PEM and ischemia independently decreased body weight. There was no interaction between diet and ischemia. The data from the 24h PEM-S animals was not collected; therefore statistics could not be performed on the 24h post-surgery group. Figure 4.1 shows feed intake post-ischemia for animals from

the 10d time-point. Feed intakes of ischemic and sham animals fed control diet had

returned to normal by 3-4 days post-surgery. Feed intake of PEM ischemic and

sham animals remained depressed post-surgery. The pattern of feed intake in

animals from the 1d and 3d time-points was similar (data not shown). Animals from

the 6hr time-point ate on average 0-0.1g during the 6 hours.

Table 4.2. Pattern of body weight (g) post-ischemia*

Time-point	PEM-I	PEM-S	CON-I	CON-S
6hr[¶]	54.9 ± 0.9 (8)	57.2 ± 0.7 (3)	63.9 ± 1.2 (8)	63.7 ± 2.6 (3)
24hr	53.5 ± 1.4 (8)	___ [†]	62.5 ± 1.4 (8)	65.9 ± 0.8 (3)
3d[¶§]	51.9 ± 1.2 (8)	53.7 ± 2.0 (3)	59.2 ± 3 (4)	67.6 ± 1.8 (3)
10d[¶]	49.4 ± 0.8 (9)	52.3 ± 0.7 (3)	68.1 ± 1.6 (8)	66.3 ± 1.2 (3)

*Mean ± SEM(n)
[¶] Significant effect of PEM determined by 2-way ANOVA ($p < 0.05$).
[§] Significant effect of ischemia determined by 2-way ANOVA ($p < 0.05$).
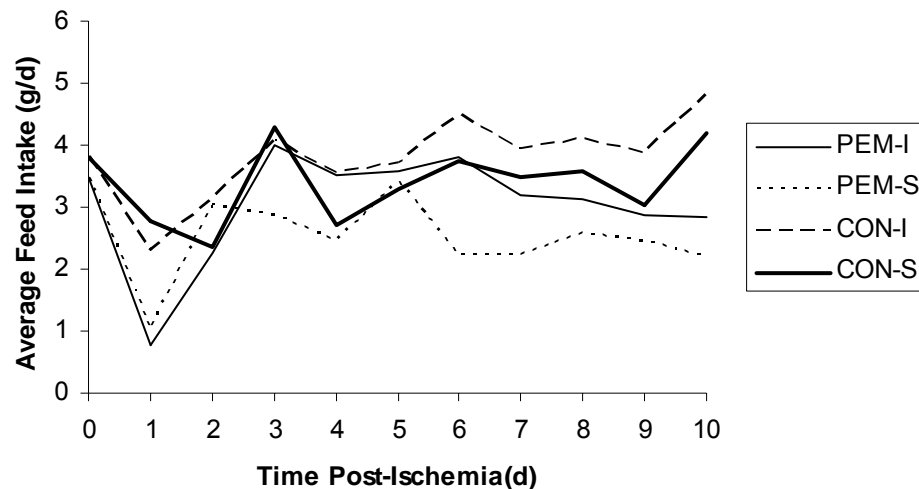[†] Data were not collected for this group.

Figure 4.1. Pattern of 10d post-ischemic feed intake. Average feed intake/day was
calculated as total feed intake/day/number of animals per cage (I, n=4; S, n=3). For comparison at
time 0, average intake/animal/day during the 28d pre-ischemia feeding period was 3.5g/d for PEM
and 3.8g/d for CON groups.

4.2 Serum Albumin

Mean (±SEM) serum albumin concentration (g/L) at 3d post-surgery were as follows: PEM-I, 28.5 ± 0.5; PEM-S, 28.1 ± 0.5; CON-I, 34.8 ± 0.5; CON-S, 33.8 ± 0.5. Protein-energy malnutrition decreased serum albumin by 17.8% (p<0.001), while ischemia had no effect. There was no interaction between the two independent variables. Table 4.3 shows serum albumin concentrations for individual animals for all experimental groups. The variability within groups was small.

Table 4.3. Serum albumin concentration (g/L) for individual animals

PEM-I	29	29	29	28	29	27	
PEM-S	29	29	27	29	29	28	26
CON-I	34	33	36	35	35	36	
CON-S	36	33	32	33	35		

4.3 Activity Monitoring

4.3.1 Screening for Complete Ischemia

Ischemic animals that remained in the study after screening were : 10d PEM-I, n = 7; 10d CON-I, n = 5; 3d PEM-I, n = 8; 3d CON-I, n = 5; 24h PEM-I, n = 5; 24h CON-I, n = 3. However, unfortunately, the activity data from 7 of these animals were lost (3 animals from the 10d PEM-I group and 4 animals from the 3d CON-I group). Immunohistochemical data from these 7 animals were still examined. Of these 7 animals, 4 had unilateral damage as determined by inspection of the H&E stained CA1 region of the hippocampus, one from the 10d PEM-I group and 3 from the 3d CON-I group. Using the exclusion criteria, 70% of the animals screened were labeled as truly ischemic. Table 4.4 shows individual animal inclusions or

exclusions on the basis of mean activity/20hr. Figure 4.2 shows the hippocampus of a 10d animal with incomplete ischemia, in which one side of the hippocampus has an intact CA1 region and the other side of the hippocampus has almost complete damage to the CA1 region.

4.3.2 Validation of Exclusion Criteria

From the 16 ischemic animals at 10d, activity monitoring was collected for 13; the data from 3 animals were lost. After examining the CA1 neuronal cell counts, 11/13 animals were correctly classified by our screening protocol. For the two errors, one was a false positive and one was a false negative result. That is, one animal was excluded on the basis of activity but had complete bilateral and extensive damage on histological assessment. One animal was included but had only unilateral damage detected by histology. Our method of screening for complete ischemia thus had an 84.6% success rate.

4.4 Hippocampal CA1 Neuron Cell Counts

Ischemia significantly reduced the total number of surviving CA1 neurons ($p<0.001$), whereas PEM had no effect ($p = 0.091$) (Figure 4.3 and 4.4). There was no interaction between diet and surgery ($p=0.972$).   The increased number of H&E stained glia apparent in the PEM sections is due to inter-ischemic variation and is not representative of the effect of PEM. The statistical results for the individual hippocampal levels were identical to those for the total cell counts (Table 4.5). The temporal loss of CA1 neurons following ischemia is demonstrated in Figure 4.5. There were no apparent differences between PEM animals and those fed the CON diet.

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Table 4.4. Individual study inclusions and exclusions on the basis of activity

Animal#	Activity[a]	Pass/Fail	Animal#	Activity[a]	Pass/Fail
	10d PEM-I			3d CON-I	
C1L-7	1657	Pass	C2L-1	570	Fail
C1N-7	423	Fail	C2N-1	508	Fail
C1R-7	924	Pass	C2R-1	2397	Pass
C2N-7	1681	Pass	C2B-1	507	Fail
C2R-7	2310	Pass	C2N-2	N/A	Pass
C1N-2	N/A[b]	Pass	C2B-2	N/A	Pass
C1B-2	N/A	Pass	C2R-2	N/A	Pass
C1R-2	N/A	Pass	C2L-2	N/A	Pass
	10d CON-I			1d PEM-I	
C1R-3	328	Fail	C3R-3	1524	Pass
C1L-3	3292	Pass	C3B-3	543	Fail
C1B-3	3433	Pass	C3L-3	1013	Pass
C1N-3	438	Fail	C3N-3	95	Fail
C1N-4	973	Pass	C3L-4	1086	Pass
C1L-4	4284	Pass	C3R-4	3200	Pass
C1R-4	2004	Pass	C3N-4	1297	Pass
C1B-4	237	Fail			
	3d PEM-I			1d CON-I	
C2R-5	2150	Pass	C4L-1	506	Fail
C2B-5	1340	Pass	C4B-1	72	Fail
C2L-5	1773	Pass	C4R-1	2300	Pass
C2N-5	1338	Pass	C4N-1	112	Fail
C2L-6	2584	Pass	C3N-2	544	Fail
C2R-6	2136	Pass	C3L-2	3500	Pass
C2N-6	2457	Pass	C3B-2	2259	Pass
C2B-6	1630	Pass	C3R-2	450	Fail

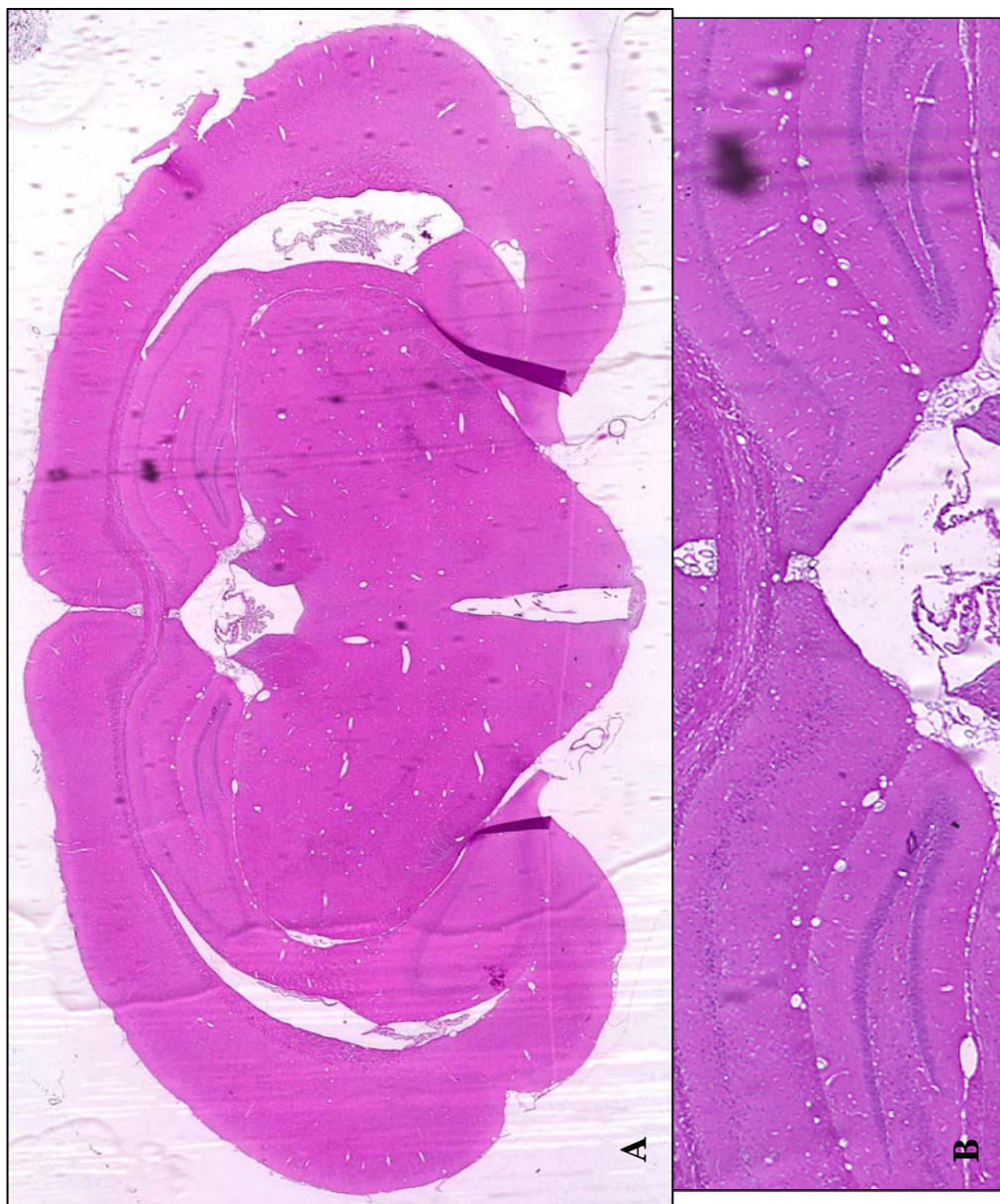[a] Mean counts/20h.
[b] Data not available.

Figure 4.2. Hippocampus displaying unilateral CA1 neuronal damage. Right side of brain is intact resembling that of a sham animal, and the left side is damaged, resembling that of an ischemic animal. A, Scan of brain (resolution 4000 pixels/inch). B, Magnification of A.
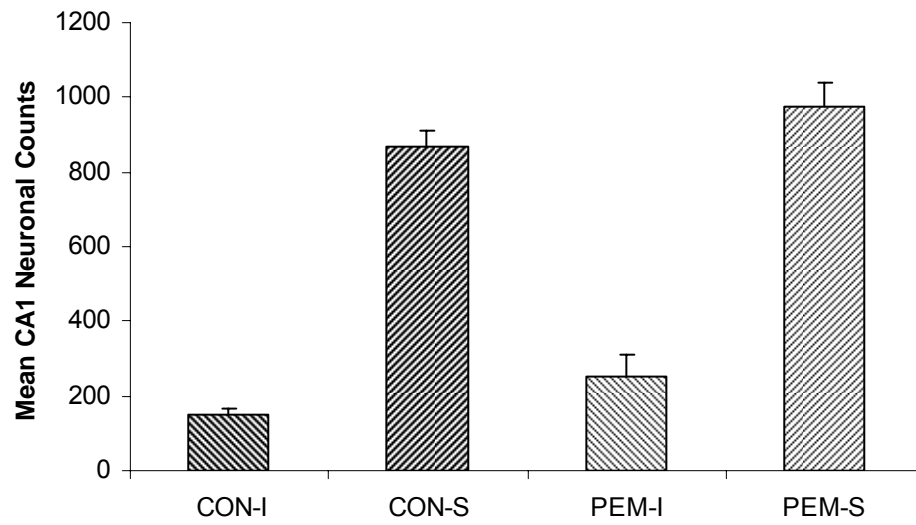
Figure 4.3. Total hippocampal CA1 neurons at 10d post-ischemia. Data are expressed as mean ± SEM. There was a significant effect of ischemia detected by 2-way ANOVA ($p<0.05$).
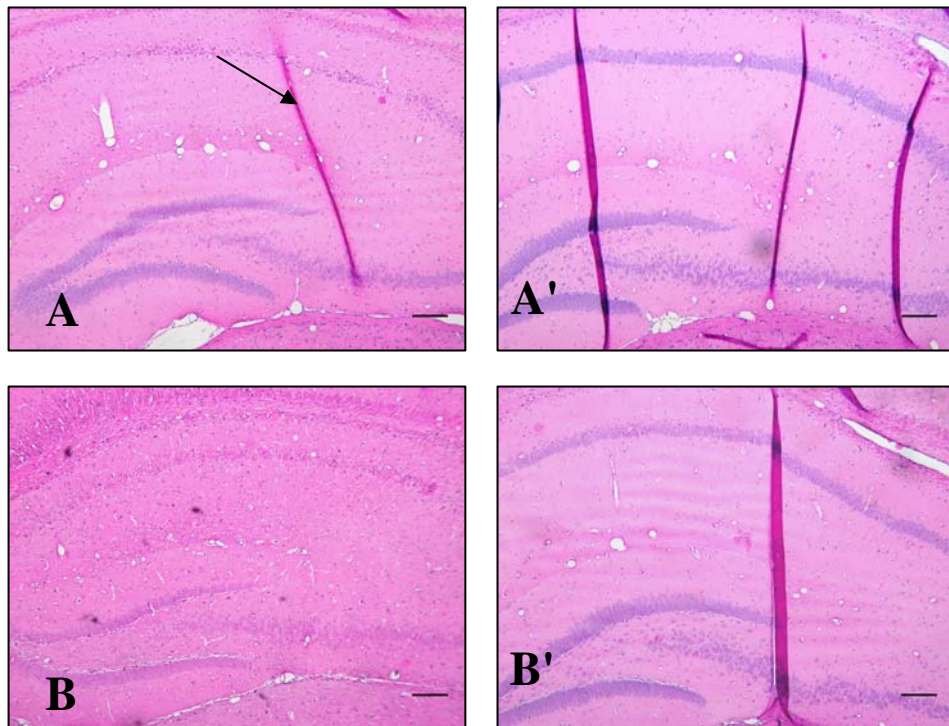

Figure 4.4. Comparison of PEM and CON hippocampus at 10d post-ischemia. A = PEM-I, A' = PEM-S, B = CON-I, B' = CON-S. Arrow indicates tissue fold. Scale bar = 150μm.

Table 4.5. Neuronal cell counts at individual sectors of the hippocampal CA1 region

	PEM-S[a]	PEM-I[b]	CON-S[a]	CON-I[c]
Level A*	389 ± 8	95 ± 24	348 ± 26	60 ± 8
Level B*	440 ± 40	111 ± 27	389 ± 14	61 ± 9
Level C*	145 ± 18	46 ± 10	130 ± 5	28 ± 5
Total*	974 ± 66	252 ± 59	867 ± 42	149 ± 19

Mean ± SEM; [a] n= 3, [b] n= 7, [c] n= 5.
*Significant effect of ischemia determined by 2-way ANOVA ($p<0.05$).

4.5 Immunohistochemistry

Semi-quantification of the immunohistochemical results was not undertaken due to variability in staining among slides. Temporal expression of GFAP and RCA-120 following global ischemia was demonstrated (Figures 4.6 and 4.7 and Figures 4.8 and 4.9, respectively). Figures 4.6 and 4.8 show higher magnification photographs of the hippocampus and Figures 4.7 and 4.9 show lower magnification photographs of the CA1 region of the hippocampus. PEM did not cause any apparent increase in expression of GFAP or RCA-120 compared to controls (Figures 4.6-4.9). Although reactive gliosis was demonstrated, the pronounced reactive gliosis previously seen in a subset of ischemic protein-energy malnourished gerbils in our laboratory was not demonstrated. PEM also did not appear to cause obvious earlier activation of glial cells, or prolong their activation.
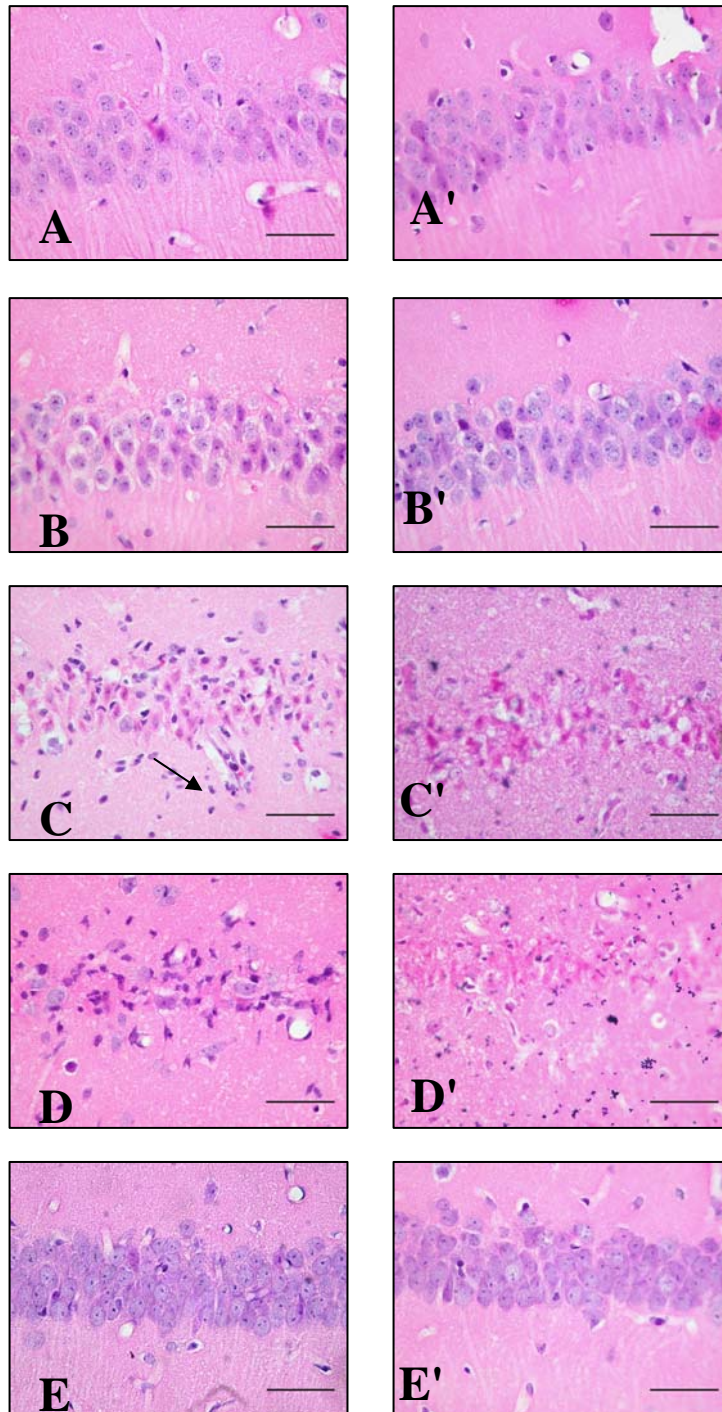
Figure 4.5. Temporal loss of hippocampal CA1 neurons following global ischemia. A-E, PEM; A'-E', CON. A-D and A'-D', ISCH; E and E', Sham. A, A' = 6hr, B, B' = 24hr, C, C' = 3d, D, D' = 10d, and E, E' = 10d. Arrow indicates H&E stained glial cell. Differences in H&E stained glia between PEM and CON are due to picture selection. Scale bar = 100µm.

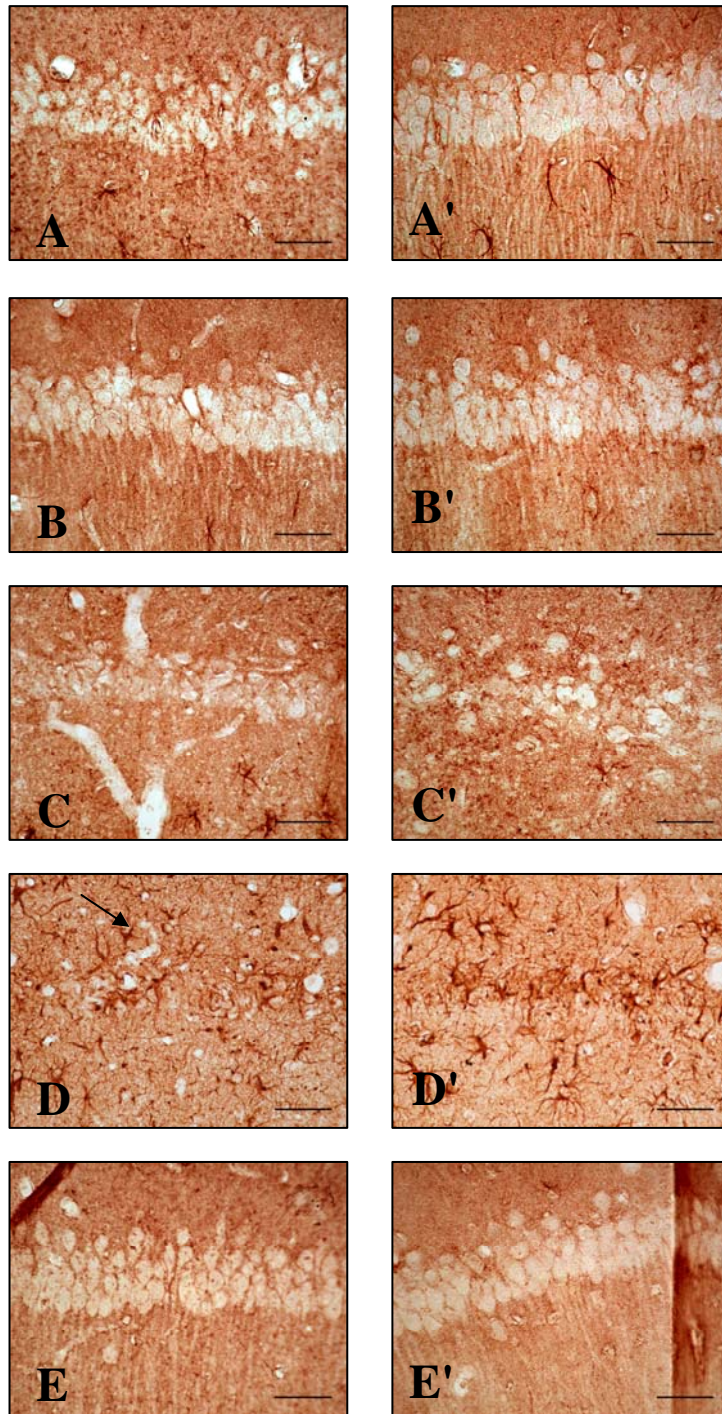**Figure 4.6.** Temporal expression of GFAP in the CA1 hippocampal region following global ischemia. A-E, PEM; A'-E', CON. A-D and A'-D', ISCH; E and E', Sham. A=6hr, B=24hr, C=3d, D=10d, and E=10d. Arrow indicates GFAP stained astrocyte. Scale bar = 100μm.

Figure 4.7. Expression of GFAP in the hippocampus at 10d post-ischemia. A and B, ISCH; A' and B', Sham. A, PEM; B, CON. Scale bar = 150µm.

Figure 4.8. Temporal expression of RCA-120 in the CA1 hippocampal region following global ischemia. A-E, PEM; A'-E', CON. A-D and A'-D', ISCH; E and E', Sham. A=6hr, B=24hr, C=3d, D=10d, and E=3d. Arrow indicates RCA-120 stained microglia. Scale bar = 100μm.
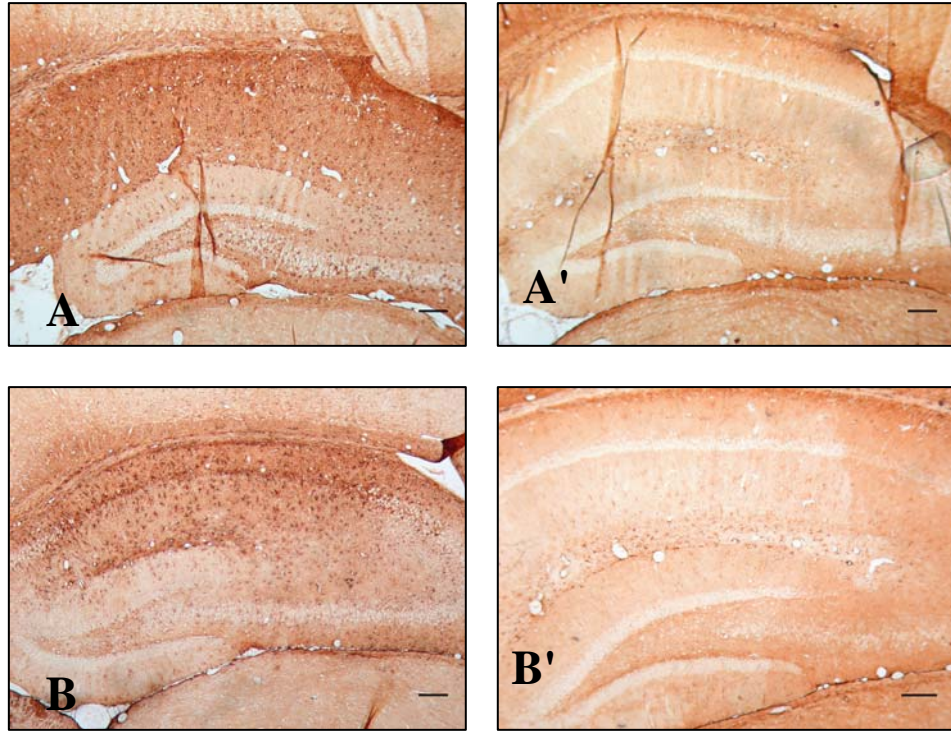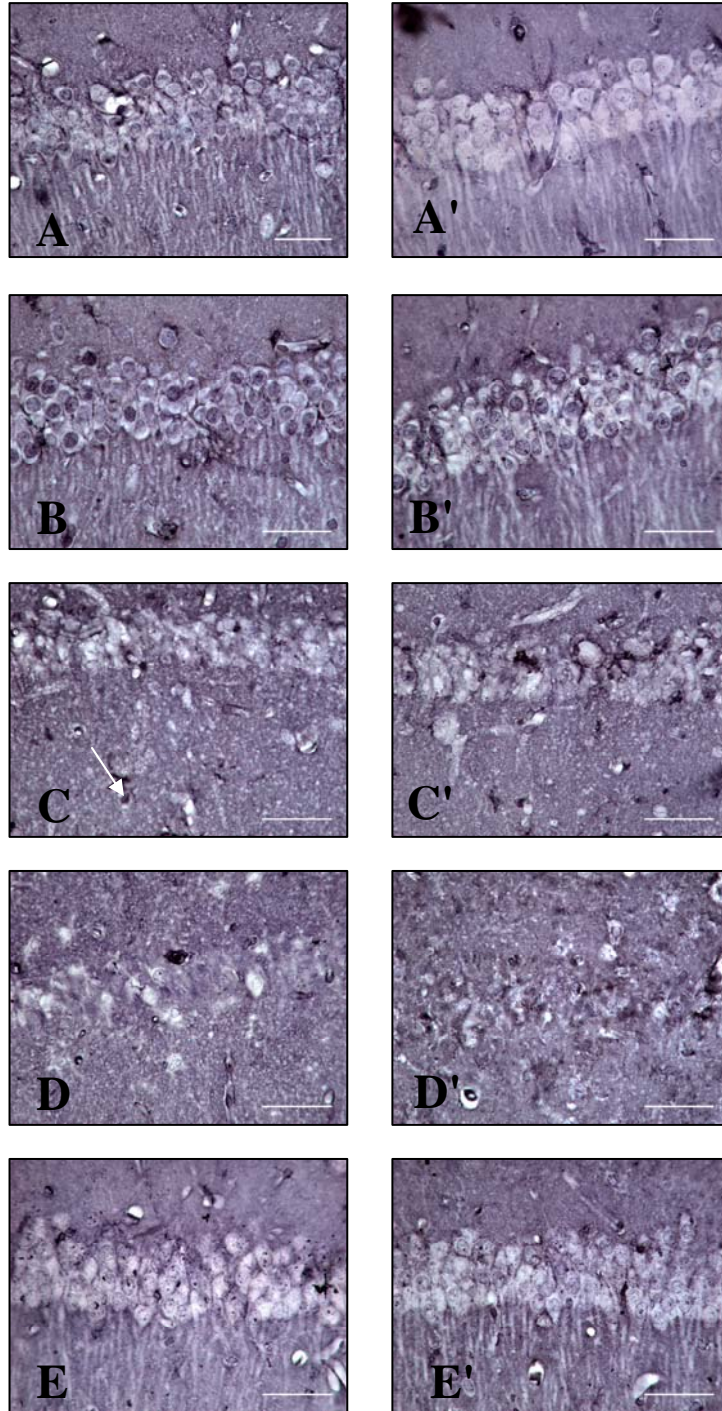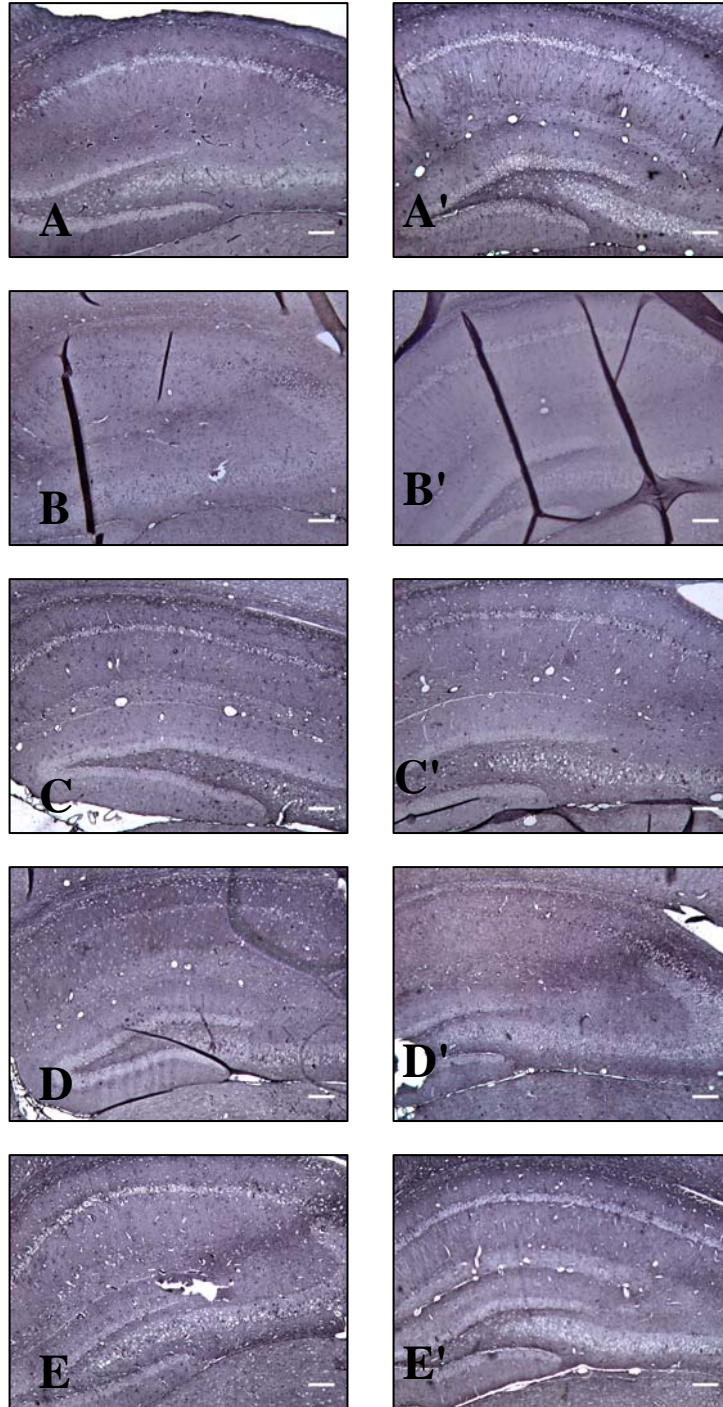
Figure 4.9. Temporal expression of RCA-120 in the hippocampus following global ischemia. A-E, PEM; A'-E', CON. A-D and A'-D', ISCH; E and E', Sham. A=6hr, B=24hr, C=3d, D=10d, and E=3d. Scale bar = 150μm.

Astrocytes and microglia are seen throughout the hippocampus. For GFAP, expression was at its peak at 10d post-ischemia (Figure 4.7). Expression of GFAP at 6h and 24h was slightly more than that seen in shams, and noticeably different than staining in shams by 3d post-ischemia (Figure 4.6). The morphology of astrocytes had obviously changed by 10d post-ischemia; the astrocytes at this time-point were hypertrophic, which is typical of reactive astrocytes (69,99). Expression of RCA-120 was at its peak at 3d post-ischemia (Figures 4.8 and 4.9). RCA-120 expression was slightly more than seen in sham animals at 6h post-ischemia, and had slightly increased by 24h. By 10d post-ischemia, expression of RCA-120 had almost completely returned to sham levels. The morphology of microglia at the 6hr and 24hr time-points was that of early activated microglia, and by 3d had changed to bushy activated microglia (100).

Temporal expression of TNF-α following global ischemia was also demonstrated in the hippocampus (Figure 4.10) and hippocampal CA1 region (Figure 4.11). TNF-α immunoreactivity was seen in the neurons of the CA1, CA2 and CA3 regions of the hippocampus and the dentate gyrus in all sham animals (Figure 4.11). The staining was similar at all time-points in sham animals. At 6h post-ischemia, TNF-α was no longer expressed in the CA1 neurons of the hippocampus, but was still present in the CA2 and CA3 neurons and dentate gyrus. Expression of TNF-α slowly returned to the CA1 neurons of the hippocampus at 24h and 3d post-ischemia. By 10d post-ischemia the death of neurons in the CA1 region of the hippocampus is nearly complete (Figure 4.5), and accordingly the expression

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of TNF-α was no longer seen in the CA1 neurons at this time-point. There was no

apparent effect of PEM on expression of TNF-α in either sham or ischemic groups.

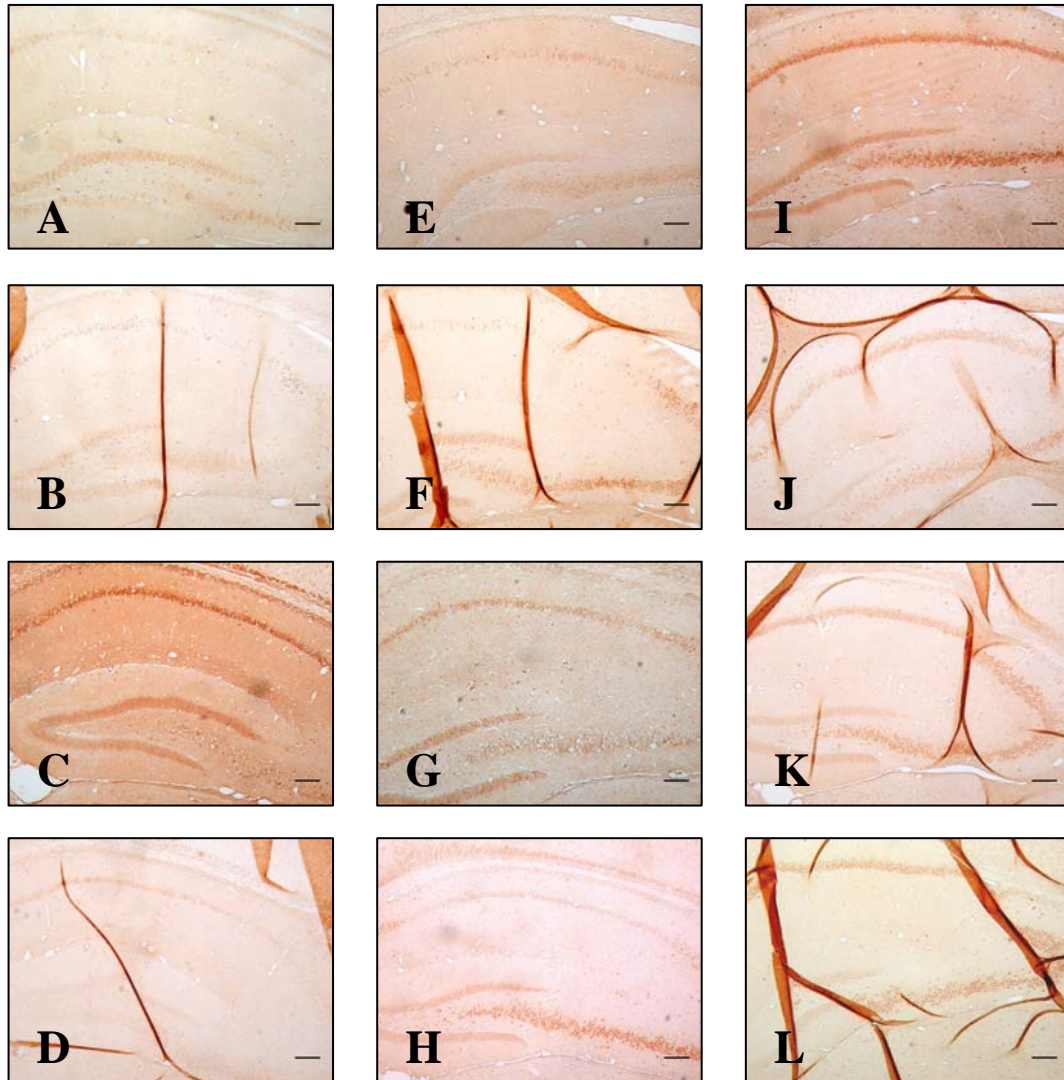**Figure 4.10.** Temporal expression of TNF-α in the hippocampus following global ischemia. A-D, PEM-I; E-H, CON-I; I-L, CON-S. A, E, I=6hr, B, F, J=24hr, C, G, K=3d, and D, H, L=10d. Scale bar = 150µm.
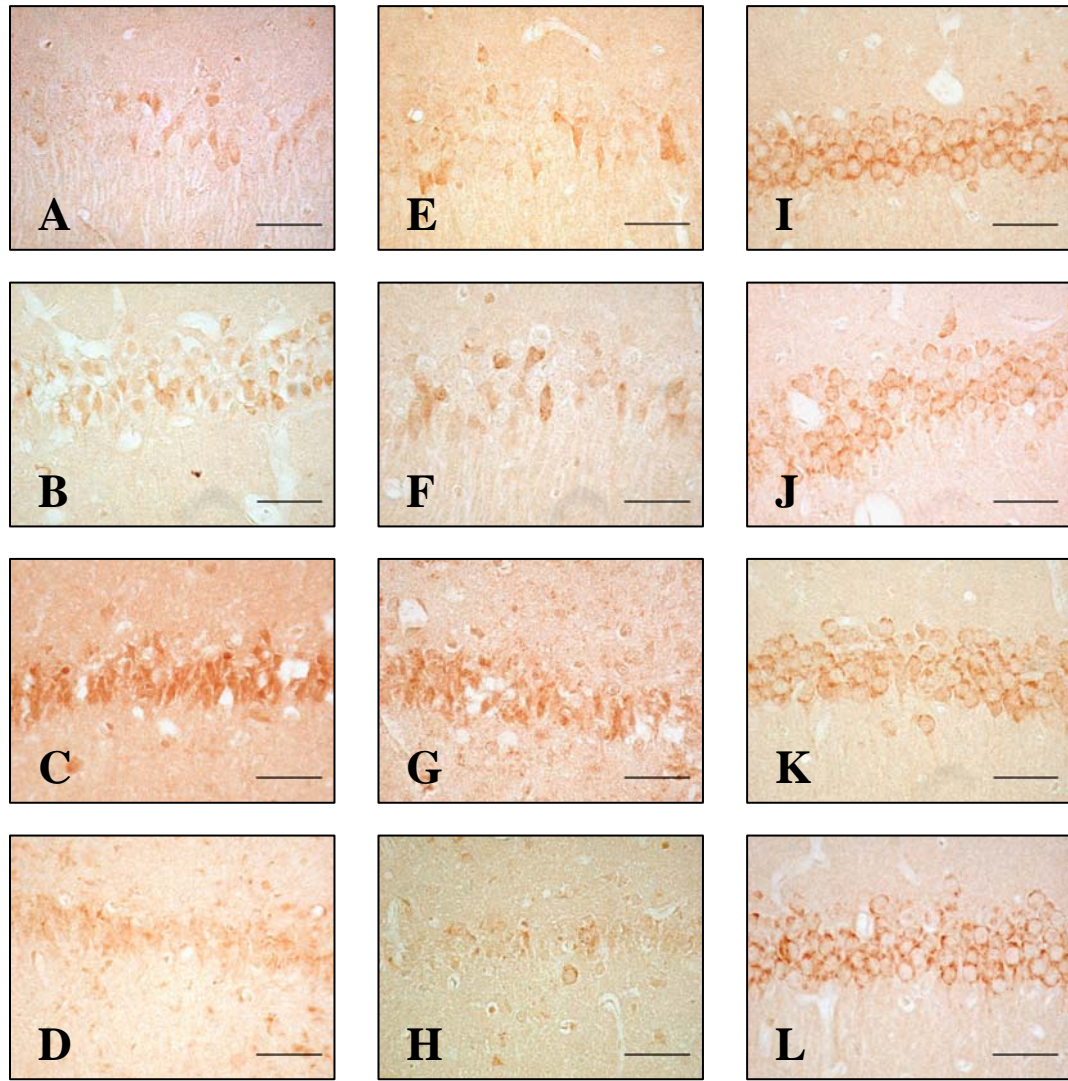
Figure 4.11. Temporal expression of TNF-α in the CA1 hippocampal region following global ischemia. A-D, PEM-I; E-H, CON-I; I-L, CON-S. A, E, I=6hr, B, F, J=24hr, C, G, K=3d, and D, H, L=10d. Scale bar = 100μm.

CHAPTER 5. DISCUSSION

Previously, our laboratory had shown that a subset of gerbils with PEM that had undergone 5 minutes of BCCAO had what appeared to be a dramatic exacerbation of reactive gliosis in the hippocampal region of the brain (9). The major goal of the current study was to confirm these results and investigate the extent of astrocytic versus microglial activation. A second goal was to determine whether PEM also exacerbated inflammation, as measured by TNF-α, in response to global ischemia.

In order to replicate and expand these findings, an identical feeding protocol and experimental design were utilized. Previously our laboratory had shown that a 2% protein diet fed for 28d caused mild to moderate PEM assessed by reduced feed intake, body weight and liver glutathione, and elevated liver lipid (9). To verify the mild to moderate PEM that was previously seen in this model, both feed intake and body weight were measured in this study. Both were depressed in the pre-surgical period as well as following surgery in both sham and ischemic groups fed the 2% protein diet. To further characterize this model of PEM, serum albumin was measured in the current study and found to be reduced by 17.8%. Serum albumin is generally not decreased in early PEM because of its long half-life of 14-20 days (18). As PEM progresses, and protein depletion worsens, serum albumin concentration, along with that of other serum proteins, decreases because of decreased protein synthesis (17).

Exposure to ischemia decreased feed intake in both PEM and CON groups up to 3 days post-surgery. Accordingly, ischemia independently decreased body weight by the 3d time-point. Sham surgery caused a smaller depression in feed intake in the CON group with little weight loss. After 3 days, the feed intake of all CON animals, both sham and ischemic, returned to normal, and their body weights started to increase. Post-ischemic body weight and feed intake are important variables to measure in rodent models of stroke, as they may influence the extent of brain damage, but they are not often reported.

In order to undertake the major research objectives, it was necessary to develop methodology for identifying those animals that had a severe ischemic insult so that others could be screened out of the study. This became necessary because of recent published evidence for a high incidence of a full or partial Circle of Willis that makes the variability in ischemic insult unacceptably high in gerbils commercially available in North America (11,85). Thus, a third goal of this study was to validate criteria for identifying persistent hyperactivity against hippocampal CA1 neuronal cell counts that could serve as a noninvasive method for screening for complete global ischemia in the gerbil. Using the criteria that hyperactivity would be defined as activity greater than 3 standard deviations above the mean activity of a sham group resulted in correct identification of complete ischemia in 84.6% of cases. Of the 2 of 13 animals that were incorrectly classified, one was a false positive and one a false negative. Although it does not frequently occur, gerbils with severe global ischemia can sometimes have damage in the striatal area of the brain, and these animals can show decreased activity despite extensive damage to the

hippocampal CA1 region (101); although striatal damage was not evaluated, this may explain the false negative result. Since the sample size used for validation was relatively small, it is possible that the accuracy of the criteria for identifying complete ischemia has been either over- or underestimated.

For those laboratories continuing to use the gerbil BCCAO model of global ischemia, the method of screening for complete global ischemia developed here would be a very good option for decreasing the variability in CA1 hippocampal neuron death occurring when animals have either a full or partial Circle of Willis. One limitation of this method is that it does not work for assessing outcome in the first few hours after global ischemia since evidence must be obtained that activity is persistently elevated. This is because gerbils with incomplete ischemia can have high initial activity that subsequently decreases (86). In this experiment, animals at the 6hr time-point were monitored for 4hr following ischemia so that they would be treated in the same manner as the rest of the experimental groups. However, these data were not used to assess ischemic insult in these gerbils, and thus the immunohistochemical data collected at this early time-point suffers from this limitation. A more precise method, that would also allow for animals to be assessed at any post-ischemic time-point is Doppler flowmetry as suggested by Laidley and colleagues (11). This technique ensures blood flow is sufficiently reduced in the brain region of interest. Even if one of these methods is used to reduce variability, a high number of animals will be unusable and this will lead to unnecessary costs and use of animals. For short-term studies, for example, when doing follow-up work to a previous study done in the gerbil, these methods would be practical. To continue

using the gerbil model of global ischemia in the long term, however, a colony of gerbils would need to be bred which consistently have an incomplete Circle of Willis. The other, and more practical, option is to switch to rat global ischemia models of 2- or 4-vessel occlusion (82,84).

The influence of PEM on the temporal response of astrocytes and microglia to global ischemia was demonstrated for the first time in this study at 6hr, 24hr, 3d and 10 d post-ischemia. GFAP staining was at its peak at 10d following ischemia. Expression was slightly increased over what was seen in shams at 6 and 24hr post-ischemia, with a larger increase by 3d post-ischemia. This pattern has been previously demonstrated in the BCCAO model of global ischemia in the gerbil (48). By 10d post-ischemia, the morphology of the astrocytes was hypertrophic, as is typical of reactive astrocytes (69,99). Expression of RCA-120 peaked at 3d post-ischemia, as has been previously documented following global ischemia in the gerbil (48). At 6hr post-ischemia, RCA-120 staining was slightly higher in ischemic animals than in shams, and at 24hr following ischemia, this difference was more noticeable. By 10d, RCA-120 staining in ischemic animals had returned to sham levels. The morphology of the microglia at 6hr and 24hr post-ischemia was that of early activated microglia, and by 3d, this had changed to bushy activated microglia, which is what is described in the literature (100).

Under the conditions of this study, PEM did not exacerbate reactive gliosis compared to CON animals. There was no obvious increase in staining of astrocytes or microglia nor did PEM hasten or prolong the rate of activation of glial cells compared to CON animals. These findings are contrary to those previously

described in our laboratory by Bobyn *et al.* (9). There are a number of possible explanations for the disagreement between the two studies, as both have limitations. After excluding animals with incomplete ischemia in the current study, the sample size may have been too small to see an effect of PEM on reactive gliosis, particularly since Bobyn *et al.* (9) reported the observation in only one third of the 12 ischemic animals with PEM. The quality of the immunohistochemistry in the current study was not high enough to use quantitative methods to assess the degree of staining or to count astrocytes and microglia. This may have detected a small effect of PEM that could be missed visually. Although brain temperature is monitored and controlled during the ischemic period in our laboratory, a limitation of both studies was the inability to monitor brain or core temperature during the post-ischemic period. Proper control of brain temperature both during and after brain ischemia is crucial because hypothermia (32-34°C) is neuroprotective (82,83,86,87,98,102) and hyperthermia aggravates the neuronal damage (102). It is possible that the subset of PEM animals with marked reactive gliosis from the previous study had post-ischemic hyperthermia, causing a more marked glial response. Another difference between the two studies is that regulation of brain temperature during the ischemic period in the current study was not kept in as narrow a range as in the study by Bobyn *et al.* (9). Although it is possible that this masked a marked glial response, there was no bias in temperature regulation between the PEM or CON groups, and the brain temperatures recorded in the current study were well above what is considered mild hypothermia and below the hyperthermic range. Finally, the results from the current study, with the exception of data collected at 6hr following

ischemia, may be seen as more reliable since animals were screened for complete ischemia and removed from the study if they did not meet criteria. Although the previous study conducted in our laboratory was conducted before the prevalence of a complete Circle of Willis was as high in the gerbil, Bobyn *et al.* (9) used all animals regardless of completeness of ischemia, although variability in the model was clearly increasing. Another limitation of both studies is the use of young gerbils. The majority of the population that suffers from stroke are elderly, and there are differences in outcome between young and old animals (103). Therefore, a future direction should be to use aged animals.

Given the limitations of the gerbil model of global ischemia, a rat model of global ischemia, either the 2- or 4-vessel occlusion model (82,84), should be used to confirm whether or not reactive gliosis is exacerbated by PEM post-ischemia. There are more markers for glial cells validated for use in the rat. Staining of microglia with RCA-120 in the gerbil gives less than optimal immunohistochemistry. Other markers of microglia that could be used in the rat are OX42 and ED1, both of which have been tested in the rat and give clear results (63,104). Another possibility to improve the quality of immunohistochemistry would be to use frozen sections instead of paraffin embedded sections.

In agreement with the previous study published from our laboratory (9), PEM did not exacerbate hippocampal CA1 neuronal loss at 10d following global ischemia in the gerbil. However, Bobyn *et al.* (9,10) reported that animals with PEM had significantly worse behavioural outcomes, as assessed by open field testing, compared to the CON animals. It is known that neurons can remain viable

after a brain insult, but not be able to function properly (12). In this model of global ischemia, CA1 neurons have delayed cell death, and these viable neurons could be functionally abnormal prior to death or remain functionally abnormal but alive (12). Therefore, the functional results reported are more meaningful than the neuronal cell counts in relation to neuronal damage (12). More functional testing is indicated in future studies to fully understand the extent of the abnormalities in PEM following global ischemia. Whether functional impairments persist beyond the recovery period is also of interest.

TNF-α immunoreactivity was employed as a single marker of inflammation in this study and was seen in all sham animals in the neurons of all hippocampal regions. There was no detectable difference between the CA1 hippocampal neurons and the other neurons of the hippocampus, in all sham animals. The pattern of staining is similar to what has previously been reported in the rat (105,106). One study found that sham animals showed TNF-α immunoreactivity in the CA1 through CA3/hilar regions of the hippocampus, dentate gyrus, cortex and corpus callosum, as well as a weak TNF-α mRNA signal in the same brain regions, as well as the caudate putamen. There was an even stronger signal for the mRNA of the TNF-α receptor, TNFR1, in the entire hippocampus of sham animals (106). A second study in rats showed that sham animals had a few scattered TNF-α immunoreactive cells in the hippocampus (105). Some possible explanations for the differences of TNF-α immunoreactivity seen in sham animals between studies are differences in animals used, either species or strain, differences in tissue processing and the antibody used (106).

At 6hr post-ischemia, the TNF-α immunoreactivity had disappeared from the CA1 neurons of the hippocampus, but was still present in the CA2 and CA3 neurons and dentate gyrus. Staining was again seen in the CA1 neurons at 24hr and was still present at 3d post-ischemia. By 10d post-ischemia, the majority of neurons in the CA1 region of the hippocampus have died, and accordingly, the TNF-α staining was also gone, but remained in the CA2 and CA3 neurons and the dentate gyrus. In a rat 2-vessel occlusion model of global ischemia in which animals are exposed to 20 minutes of bilateral common carotid artery occlusion combined with hypotension, Sairanen *et al.* (106) reported peak TNF-α mRNA expression at 8hr post-reperfusion, with a decrease to that of shams by 24hr after reperfusion. However, neuronal TNF-α immunoreactivity following global ischemia was similar to that of sham animals (106). One explanation given for the mismatch between protein and mRNA levels is that the mRNA following ischemia may not be as stable for translation into protein (106). Also, it was suggested, that the TNF-α, if attached to its receptors, might not be as available to the antibody (106). These findings are in contrast to those obtained using a cruder model of transient global ischemia in which the intensity of the TNF-α immunoreactivity increased and reached its peak at 3d post-ischemia (107). A third study using a mouse model of 20 minutes of bilateral common carotid artery occlusion reported that hippocampal TNF-α mRNA expression was increased at 3hr post-ischemia, returned to control levels and then was increased again by 36hr post-ischemia (107). Contrary to our findings, they found that TNF-α protein levels were only significantly elevated compared to shams at 6 hours post- ischemia, as was TNFR1 protein (57). In the one study investigating

TNF-α immunoreactivity in a 5-minute global ischemia gerbil model, it was reported, using enzyme-linked immunoassay, that TNF-α immunoreactivity was only elevated in the hippocampus only at 6hr post-ischemia (57). This study also examined 10 minutes of global ischemia, which caused TNF-α immunoreactivity to increase in the hippocampus by 1hr post-ischemia, with a return to sham levels by 12hr, and an increase again at 24hr post-ischemia (106). However, a 10-minute occlusion period generally results in post-ischemic seizures (12), which confounds the results. As this was the only other study looking at TNF-α expression after 5 minutes of BCCAO in the gerbil, we expected to see similar results. Although this was not the case, there were limitations in the data collected in the current study. This included inconsistencies related to technical problems in sectioning and staining, which also made it unclear as to when expression was the highest following global ischemia.

Contrary to our hypothesis, PEM did not appear to alter the pattern of TNF-α immunoreactivity in sham animals at any time-point following global ischemia. This suggests that the inflammatory response to global ischemia is not increased as a result of PEM. Recently, a colleague in our laboratory, using the same model described here, has shown that PEM, independent of ischemia, causes increased NFκB activation at 6hr post-surgery, and a trend at 24hr towards an increase (76). TNF-α is one pro-inflammatory molecule whose transcription is increased by NFκB (74). Therefore, the finding here, that PEM has no effect on TNF-α immunoreactivity was surprising. As an increase in TNFR1 has been reported following global ischemia in the rat, it is possible that increased TNF-α

immunoreactivity was not detected because it was already bound to its receptor (106). Conclusions are also limited since only one marker of inflammation was employed, and future studies should examine additional inflammatory markers such as IL-1β and COX-2. The finding that PEM did not alter the pattern of TNF-α immunoreactivity, however, is consistent with the finding that PEM did not increase reactive gliosis following global ischemia. As previously discussed, reactive astrocytes and microglia can cause an increased inflammatory response post-ischemia (49,67,69,73)

In conclusion, the influence of PEM on the temporal development of microglia and astrocytic activation was investigated along with TNF-α, as a marker of inflammation, in the hippocampus following global ischemia in the gerbil. The data suggest that PEM neither alters glial activation nor increases the inflammatory response after global ischemia. However, the results are not definitive due to limitations of the study, including small sample size after excluding animals with incomplete forebrain ischemia and inability to quantify immunohistochemical results. A model of screening animals for complete forebrain ischemia on the basis of persistent hyperactivity was successfully validated against 10d hippocampal CA1 neuronal cell counts. The finding of Bobyn *et al.* (9,10) that PEM does not exacerbate hippocampal CA1 neuronal cell death at 10d following global ischemia was confirmed. Due to the current high incidence of a Circle of Willis in the gerbil (11,85), an alternate animal model of ischemia will need to be used in future studies investigating the mechanisms by which PEM worsens functional outcome, as has been previously reported in both gerbils (4,5,8) and humans (11,85).

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