Cerebral ischemia/reperfusion injury in rats: Oxidative stress and role of antioxidants

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by

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Abbreviations

ALP	Alkaline phosphatase
AMPA	2-Amino-3-(3-hydroxy-5-methylisoxsazol-4-yl) propionate
ATP	Adenosine 5'-tri phosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
Ca^{2+}	Calcium ions
CAMP	Cyclic adenosine mono phosphate
Cat	Catalase
CDNB	1-Chloro 2,4-dinitro benzene
Cl^-	Chloride ion
CPCSEA	Committee for the Purpose of Control and Supervision On Experiments on Animal
CT	Computer aided Tomography
Cyt-C	Cytochrome-C
Da	Dalton
DNA	Deoxy ribose nucleic acid
DTT	1,4-Dithio-DL-threitol
dUTP	deoxy uridynyl tri phosphate
ε	Extinction coefficient
EDTA	Ethylenediaminetetraaceticacid
Fe^{3+}	Ferric ion

Fe^{2+}	Ferrous Ion
FITC	Fluorescein isothiocyanate
GABA	γ- aminobutyric Acid
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidized)
GST	Glutathione S-transferase
GTP	Guanosine 5'-tri phosphate
H+	Hydrogen ion
H_2O	Water
H_2O_2	Hydrogen peroxide
HCl	Hydrogen Chloride
iNOS	inducible Nitric Oxide Synthase
KCl	Potassium Chloride
kDa	Kilo Dalton
LDL	Low density lipoproteins
LPO	lipid peroxidation
MCA	Middle Cerebral Artery
MDA	Malondialdehyde
mg	Milligrams
$MgCl_2$	Magnesium Chloride
mGluR	Metabotropic Glutamate Receptor

mM	Millimolar
Mn	Manganese
MRI	Magnetic Resonance Imaging
Na^+	Sodium ion
Na_2EDTA	Ethylenediaminetetraacetic acid disodium salt dihydrate
NaCl	Sodium Chloride
NAD+	Nicotinamide adenine dinucleotide
NADP+	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NaEGTA	Ethyleneglycol-bis(β-aminoethylether)-N,N,N',N',-tetraacetic acid tetrasodium salt
NaOH	Sodium hydroxide
NBT	Nitro blue tetrazolium
NEM	N-Ethylmaleimide
NIH	National Institute of Health
NINDS	National Institute of Neurological Diseases and Stroke
nM	Nanomolar
NMDA	N-methyl D-aspartate
nNOS	Neuronal Nitric Oxide Synthase

NO	Nitric oxide
NOS	Nitric Oxide Synthase
O2-	Superoxide ion
OH^-	Hydroxyl ion
CI_2	Chlorine
$ONOO^-$	Peroxy nitrite
OPT	O-pthaldehyde
PADRT	Poly (ADP- ribosyl) transferase.
PANT	Positive DNA polymerase I-mediated biotin-dATP nick-translation
PARP	Poly (ADP-ribose) Polymerase
PBN	N-tert-butyl-alpha-phenylnitrone
PBS	Phosphate Buffered Saline
PGG	Prostaglandin G
pH	Negative logarithm of hydrogen ion concentration
PI	Propidium Iodide
PLA_2	Phospholipase A2
PMSF	Phenylmethylsufonyl fluoride
PUFA	Poly Unsaturated Fatty Acids
ROS	Reactive Oxygen Species
rtPA	Recombinant tissue Plasminogen Activator

SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulphate
Se	Selenium
SOD	Superoxide Dismutase
SVNs	Selectively Vulnerable Neurons
TBA	Thiobarbituric acid
Tdt	Terminal deoxy nucleotidyl transferase
TE	Tris EDTA
TMP	1,1,3,3,-tetramethoxypropane
TNFα	Tumor necrosis factor α
tPA	Tissue Plasminogen Activator
Tris	Tris (hydroxy methyl) amino methane
TUNEL	Terminal deoxy nick end labeling
Vit C	Vitamin C
Vit E	Vitamin E
w/v	Weight /Volume
2VO	Two-vessel occlusion
4VO	Four-vessel occlusion

More than 2,400 years ago the father of medicine, Hippocrates, recognized and described stroke as the sudden onset of paralysis. In ancient times stroke was called *apoplexy,* a general term that physicians applied to anyone suddenly struck down with paralysis. Until recently, modern medicine has had very little power over this disease, but the world of stroke medicine is changing and new and better therapies are being developed every day. Today, some people who have a stroke can walk away from the attack with no or few disabilities if they are treated on time. Studies using animals have shown that brain injury occurs within minutes of a stroke and can become irreversible within as little as an hour. In humans, brain damage begins from the moment the stroke starts and often continues for days afterward.

Incidence of Stroke

Brain stroke has emerged as the third-leading cause of death all over the world and the incidence in India is put at about 800 per 100,000 individuals. Worldwide stroke mortality is 1 million per year and stroke incidence is around 25 millions. More than half a million Americans die of stroke every year.

Stroke

Stroke occurs when the blood supply to part of the brain is suddenly interrupted or when a blood vessel in the brain bursts, spilling blood into the

space surrounding brain cells. Brain cells die when they no longer receive oxygen and nutrients from the blood. *Ischemia* is the term used to describe the loss of oxygen and nutrients for brain cells when there is inadequate blood flow. Ischemia ultimately leads to *infarction*, the death of brain cells, which are eventually replaced by a fluid-filled cavity (or *infarct*) in the injured brain (Fig In 1). When blood flow to the brain is interrupted, some brain cells die immediately, while others die over a period of time. These cells make up the *ischemic penumbra* and can linger in a compromised state for several hours; with timely treatment these cells can be saved.

Types of stroke

There are two forms of stroke: *ischemic* - blockage of a blood vessel supplying the brain, and *hemorrhagic* - bleeding into or around the brain.

Ischemic Stroke

An ischemic stroke occurs when an artery supplying blood to the brain is blocked causing a brain infarction. This type of stroke accounts for approximately 80 percent of all strokes. Blood clots are the most common cause of artery blockage and brain infarction. Normally clotting is necessary and beneficial throughout the body because it stops bleeding and allows repair of damaged areas of arteries or veins. However, when blood clots develop in the wrong place within an artery they can cause devastating injury by

interfering with the normal blood flow. Problems with clotting become more frequent as people age. Blood clots can cause ischemia and infarction in two ways. A clot that forms in a part of the body other than the brain can travel through blood vessels and become lodged in a brain artery. This free-roaming clot is called an *embolus* and often forms in the heart. A stroke caused by an embolus is called an *embolic stroke*. The second kind of ischemic stroke, called a *thrombotic stroke*, is caused by *thrombosis*, the formation of a blood clot in one of the cerebral arteries that stays attached to the artery wall until it grows large enough to block blood flow. Ischemic strokes can also be caused by *stenosis*, or a narrowing of the artery due to the buildup of *plaque* (a mixture of fatty substances, including *cholesterol* and other lipids) and blood clots along the artery wall. Stenosis can occur in large arteries and small arteries and is therefore called *large vessel disease* or *small vessel disease*, respectively. The most common blood vessel disease that causes stenosis is atherosclerosis. In atherosclerosis, deposits of plaque build up along the inner walls of large and medium-sized arteries, causing thickening, hardening, and loss of elasticity of artery walls and decreased blood flow.

Hemorrhagic Stroke

In a healthy, functioning brain, neurons do not come into direct contact with blood. The vital oxygen and nutrients the neurons need from the blood reaches the neurons across the thin walls of the cerebral capillaries. The glia

(nervous system cells that support and protect neurons) form a blood-brain barrier, an elaborate meshwork that surrounds blood vessels and capillaries and regulates which elements of the blood can pass through the neurons. When an artery in the brain bursts, blood spews out into the surrounding tissue and upsets not only the blood supply but the delicate chemical balance neurons require to function. This is called a hemorrhagic stroke. Such strokes account for approximately 20 percent of all strokes.

Hemorrhage can occur in several ways. One common cause is a bleeding *aneurysm,* a weak or thin spot on an artery wall. Over time, these weak spots stretch or balloon out under high arterial pressure. The thin walls of these ballooning aneurysms can rupture and spill blood into the space surrounding brain cells. Hemorrhage also occurs when arterial walls break open, plaque-encrusted artery walls eventually lose their elasticity and become brittle and thin, prone to cracking. *Hypertension*, or *high blood pressure*, increases the risk that a brittle artery wall will give way and release blood into the surrounding brain tissue.

Symptoms of stroke

Symptoms of stroke appear suddenly.

- Sudden numbness or weakness of the face, arm, or leg, especially on one side of the body.
- Sudden confusion, trouble talking, or understanding speech.

- Sudden trouble seeing in one or both eyes.
- Sudden trouble walking, dizziness, or loss of balance or coordination.
- Sudden severe headache with no known cause.

Diagnosis of Stroke

Physicians have several diagnostic techniques and imaging tools to help diagnose the cause of stroke quickly and accurately. Blood tests, an electrocardiogram, and CT scans will often be done. One test that helps doctors judge the severity of a stroke is the standardized NIH Stroke Scale, developed by the NINDS. Health care professionals use the NIH Stroke Scale to measure a patient's neurological deficits by asking the patient to answer questions and to perform several physical and mental tests. Other scales include the Glasgow Coma Scale, the Hunt and Hess Scale, the Modified Rankin Scale, and the Barthel Index.

Imaging for the Diagnosis of Acute Stroke

Health care professionals also use a variety of imaging devices to evaluate stroke patients. The most widely used imaging procedure is the computed tomography (CT) scan. Another imaging device used for stroke patients is the magnetic resonance imaging (MRI) scan.

Risk factors of stroke

Some people are at a higher risk for stroke than others. Unmodifiable risk factors include age, gender, race/ethnicity, and stroke family history. In contrast, other risk factors for stroke, like high blood pressure or cigarette smoking, can be changed or controlled by the person at risk.

Unmodifiable Risk Factors

Age: The *incidence* of stroke is increasing proportionately with the increase in the elderly population. When the baby boomers move into the over-65 age group, stroke and other diseases will take on even greater significance in the health care field.

Gender: Gender also plays a role in risk for stroke. Men have a higher risk for stroke, but more women die from stroke. The stroke risk for men is 1.25 times that for women. But men do not live as long as women, so men are usually younger when they have their strokes and therefore have a higher rate of survival than women. In other words, even though women have fewer strokes than men, women are generally older when they have their strokes and are more likely to die from them.

Stroke seems to run in some families. Several factors might contribute to familial stroke risk. Members of a family might have a genetic tendency for stroke risk factors, such as an inherited predisposition for hypertension or

diabetes. The influence of a common lifestyle among family members could also contribute to familial stroke.

Other Risk Factors

The multiple risk factors compound their destructive effects and create an overall risk greater than the simple cumulative effect of the individual risk factors.

Hypertension

Of all the risk factors that contribute to stroke, the most powerful is hypertension, or high blood pressure. People with hypertension have a risk for stroke that is four to six times higher than the risk for those without hypertension.

Heart Disease

After hypertension, the second most powerful risk factor for stroke is heart disease, especially a condition known as *atrial fibrillation*. Atrial fibrillation is irregular beating of the left atrium, or left upper chamber, of the heart. This leads to an irregular flow of blood and the occasional formation of blood clots that can leave the heart and travel to the brain, causing a stroke.

Diabetes

Diabetes is another disease that increases a person's risk for stroke. People with diabetes have three times the risk of stroke compared to people without diabetes. The relative risk of stroke from diabetes is highest in the fifth and sixth decades of life and decreases after that.

Blood Cholesterol Levels

High levels of cholesterol contribute to the risk of atherosclerosis and thickening of the arteries and there by stroke.

Modifiable Lifestyle Risk Factors

Cigarette smoking is the most powerful modifiable stroke risk factor. *High alcohol consumption* is another modifiable risk factor for stroke. *The use of illicit drugs*, such as cocaine and crack cocaine, can cause stroke. Cocaine may act on other risk factors, such as hypertension, heart disease, and vascular disease, to trigger a stroke.

Injuries to the head or neck may damage the cerebrovascular system and cause a small number of strokes. Recent viral and bacterial infections may act with other risk factors to add a small risk for stroke.

Stroke Therapies

Generally there are three treatment stages for stroke: prevention, therapy immediately after stroke, and post-stroke rehabilitation. Therapies for stroke include medications, surgery, or rehabilitation.

Medications

Medication or drug therapy is the most common treatment for stroke. The most popular classes of drugs used to prevent or treat stroke are *antithrombotics* (*antiplatelet agents* and *anticoagulants*), *thrombolytics*, and *neuroprotective agents*.

Antithrombotics prevent the formation of blood clots that can become lodged in a cerebral artery and cause strokes. Antiplatelet drugs prevent clotting by decreasing the activity of platelets, blood cells that contribute to the clotting property of blood. These drugs reduce the risk of blood-clot formation, thus reducing the risk of ischemic stroke. In the context of stroke, physicians prescribe antiplatelet drugs mainly for prevention. The most widely known and used antiplatelet drug is aspirin. Other antiplatelet drugs include clopidogrel and ticlopidine. The NINDS sponsors a wide range of clinical trials to determine the effectiveness of antiplatelet drugs for stroke prevention.

Anticoagulants reduce stroke risk by reducing the clotting property of the blood. The most commonly used anticoagulants include *warfarin* (also known as *Coumadin)* and *heparin*. Thrombolytic agents are used to treat an ongoing, acute ischemic stroke caused by an artery blockage. These drugs halt the stroke by dissolving the blood clot that is blocking blood flow to the brain. Recombinant tissue plasminogen activator (rt-PA) is a genetically engineered form of t-PA, a thombolytic substance made naturally by the body. It can be effective if given intravenously within 3 hours of stroke symptom onset, but it should be used only after a physician has confirmed that the patient has suffered an ischemic stroke. Neuroprotectants are medications that protect the brain from secondary injury caused by stroke. There are several different classes of neuroprotectants that show promise for future therapy, including calcium antagonists, glutamate antagonists, opiate antagonists, antioxidants, apoptosis inhibitors, and many others.

Surgery

Surgery can be used to prevent stroke, to treat acute stroke, or to repair vascular damage or malformations in and around the brain. There are two prominent types of surgery for stroke prevention and treatment: carotid endarterectomy and *extra cranial/intracranial (EC/IC) bypass*.

Post-Stroke Rehabilitation

Type	**Goal**
Physical Therapy (PT)	Relearn walking, sitting, lying down, switching from one type of movement to another
Occupational Therapy (OT)	Relearn eating, drinking, swallowing, dressing, bathing, cooking, reading, writing, toileting
Speech Therapy	Relearn language and communications skills
Psychological/Psychiatric Therapy	Alleviate some mental and emotional problems

Disabilities From a Stroke

Although stroke is a disease of the brain, it can affect the entire body. Some of the disabilities that can result from a stroke include paralysis, cognitive deficits, speech problems, emotional difficulties, daily living problems, and pain.

Molecular mechanisms in cerebral ischemia

The principle pathophysiological processes leading to cell death after cerebral ischemia/reperfusion are energy failure, loss of cell ion homeostasis, acidosis, exitotoxicity, generation of reactive oxygen species (ROS) and Ca^{2+} influx in to cells. All these processes are interrelated. The main route of Ca^{2+} entry is through channels gated by glutamate receptors. The reuptake of extracellular glutamate and the Ca^{2+} pump function are decreased during energy failure. Intracellular Ca^{2+} overload results in activation of lipases, proteases, kinases, phosphatases and endonucleases in potentially harmful metabolic cascades. Loss of ion homeostasis is accompanied by osmotical H_2O- influx leading to cytotoxic oedema that aggravates ischemia. Under acidotic conditions Ca^{2+} ions are released from their intracellular storage leading to a rise in free cytosolic Ca^{2+} concentration with subsequent Ca^{2+} overload of mitochondria. This interferes with ATP production and enhances anaerobic glycolysis. Both intracellular calcium overload and acidosis enhance the production of ROS, thus predisposing to the free radical damage (Fig In 2).

Mechanisms contributing to the formation of free radicals *(Fig In 3)*

Increased decomposition of ATP via AMP leads to an accumulation of hypoxanthine and xanthine. Further, metabolism of hypoxanthine and xanthine by xanthine oxidase via uric acid leads to the generation of $O_2^{-\cdot}$ (Kinuta *et al* 1989; Atlante *et al* 1997). Another superoxide generating step is initiated by

phospholipases. Cellular phospholipase A2 (PLA2) is activated by increase in $Ca_2^{+,}$ leading to the increased release of free fatty acids, especially arachidonic acid. The accumulation and decomposition of free fatty acids leads to a further release of superoxide anions (Kuehl Faj and Egan 1980). On the other hand, the conversion of arachidonic acid to prostaglandins, leukotreines and thromboxanes by cyclooxygenase and lipoxygenase causes vasoconstriction and aggravates ischemia. Activation of NOS results in increased NO production. NO can react with superoxide anions to form the highly reactive peroxy nitrite ($ONOO^-$) (Bolanos *et al* 1997). Free iron or iron chelates participate in free radical reactions at several levels. The autooxidation of Fe^{2+} results in the formation of $O_2^{-\cdot}$. Enzymatic removal of $O_2^{-\cdot}$ by SOD leads to the formation of hydrogen peroxide (H_2O_2) and highly reactive hydroxyl radicals ($^{\cdot}OH$) via the well-known iron dependent Fenton or Haber weiss reaction (Halliwell 1992). Enhanced acidosis is detrimental because it accelerates delocalization of protein bound iron, with ensuing free radical damage to membrane lipids and proteins. Lipid peroxidation occurs when ROS attack unsaturated fatty acids of membrane lipids, resulting in the formation of lipid radicals. Once lipid peroxidation begins, iron may participate in driving the process, lipid hydroperoxides formed are decomposed by reactions with Fe^{2+}, $Fe^{3+,}$ or their chelates (Florian ringel and Robert Schmid - Elsaesser 2001).

Historical observations of major phenomena in brain ischemia and reperfusion

Four major observations have provided the foundation for investigation of brain injury by ischemia and reperfusion (Krause *et al* 1988): (1) rapid loss of high-energy phosphate compounds during ischemia followed by their recovery within the first 15 min of reperfusion (2) morphological evidence that most structural damage occurs during reperfusion, especially in selectively vulnerable zones (3) progressive brain hypoperfusion during post-ischemic reperfusion and (4) prolonged suppression of protein synthesis in selectively vulnerable neurons (SVNs).

High-energy phosphate compounds

The brain's ATP supply is dependent on continuous perfusion and approaches zero within about 4 min of complete ischemia (Krause *et al* 1988). This energy depletion is associated with depolarization and loss of the normal 10,000/1 reperfusion partition of Ca^{2+} between the extracellular fluid and the cytosol. Depolarization and the increase in intracellular Ca^{2+} sets off a chain of events including lipolysis during ischemia followed by free fatty acid metabolism and generation of superoxide radical ($O_2^{-\cdot}$) during reperfusion (Krause *et al* 1988). During post-ischemic reperfusion, even after ischemic periods up to 1hr, the high-energy phosphate 'charge' recovers rapidly and approaches normal within 15min of reperfusion (Hossman *et al* 1976; Siesjo *et al* 1973).

Reperfusion injury and selective vulnerability

Negovskii (Negovskii 1962) proposed that the extent of tissue injury observed following ischemia might largely reflect damage incurred during reperfusion. Morphologic studies provided direct support for this hypothesis (Jenkins *et al* 1981; Kumar *et al* 1987) and also identified SVNs (hippocampal hilar and CA1 pyramidal neurons and cortical pyramidal neurons in layers 3 and 5). Based on conventional staining, reperfusion damage in the SVNs is observed in two phases (Sato *et al* 1990). Cytosolic microvacuolation is noted within the first 15 min of reperfusion, although a substantial degree of normalization is observed by 1hr of reperfusion. Morphologic evidence of progressive damage to these neurons is then seen the following 6hr, and by 48 to 72hr the neurons are disintegrated.

Post-ischemic brain hypoperfusion

Capillary hypoperfusion was first shown qualitatively (Ames *et al* 1968), and subsequent quantitative studies demonstrated that cortex capillary perfusion was only 30% normal by 90 min of reperfusion following 10 to 20 min of global ischemia (Krause *et al* 1988). This phenomenon occurred without a change in intracranial pressure and was originally taken to represent 'secondary ischemia,' thought to lead to failure of high-energy metabolism and neuronal death during reperfusion. However, by the mid-1980s it was clear that calcium antagonists (Krause *et al* 1988), superoxide dismutase and deferoxamine (Cerchiari *et al* 1987), or U74006F (Hall *et al* 1988) (a lipid

peroxidation chain terminator (Braughter *et al* 1987) inhibited post-ischemic brain hypoperfusion, but had little effect on neurologic outcome. Moreover, tissue high-energy charge, which approaches zero within 4 min of complete ischemia (Krause *et al* 1988), recovers rapidly during reperfusion following ischemia, and this recovery is apparently not adversely affected by the post-ischemic hypoperfusion (Hossman and Kleihues 1973; Meis *et al* 1990; White *et al* 1985; White *et al* 2000). This evidence argues that post-ischemic hypoperfusion is an epiphenomenon of more fundamental biochemical events and is not likely to be itself involved in neuronal death that occurs during reperfusion.

Inhibition of brain protein synthesis

Protein synthesis in vulnerable neurons is substantially suppressed during post-ischemic reperfusion (Hossman and Kleihues 1973; Cooper *et al* 1977; Dienel *et al* 1980). Protein synthesis is a fundamental cellular activity, and various mechanisms are involved in inhibition of protein synthesis

Classical mechanisms implicated in selective vulnerability

Two major hypotheses emerged during the 1980s from efforts to explain the phenomenon of selective vulnerability. One is the exitotoxic neurotransmitter hypothesis directed largely at events during ischemia, and the other is free radical hypothesis directed largely at events during reperfusion.

Excitotoxic amino acid neurotransmitters

The first of these suggests that SVN cell bodies and dendrites receive projections that release large amounts of important amino acid neurotransmitter (i.e. glutamate) during ischemia induced depolarization (Siesjo 1992). There are two classes of glutamate receptors, the ionotropic receptors, which are the ligand-gated ion channels, and the metabotropic receptors (mGluR), which are coupled to cellular effectors via GTP-binding proteins. On SVNs there are two subtypes of glutamate ionotropic receptors that are distinctively activated by either *N*-methyl-D-aspartate (NMDA) or α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA). NMDA receptor activation opens a Ca^{2+} channel, allowing Ca^{2+} influx, and AMPA receptor activation opens a Na^{+} channel, allowing Na influx. The increased intracellular Na concentration (Na^{+}) is to induce reversal of normal Ca^{2+} extrusion through a 3 Na^{+} /Ca^{2+} antiporter, thereby again resulting in increased Ca^{2+} , which is thought to exert cytotoxic effects. The mGluRs are coupled second messenger systems, including those involved in the activation of phosphoinositide hydrolysis, phospholipase D activation, regulation of cAMP formation, and ion (calcium and potassium) channel modulation. The mGluR agonists have two unusual effects, (1) reduction of transmission at excitatory amino acid (glutamate) and inhibitory synapses and of inhibitory post-synaptic potentials (probably via decreased GABA release) in the striatum and hippocampus, and (2) in the hippocampal CA1 region induction of long-term synaptic potentiation,

thought to be mediated by protein kinase C-induced increases in NMDA receptor currents (Schoepp and conn 1993). Intraocular injection of metabotropic receptor agonists protected against retinal damage induced by NMDA, but intra-hippocampal injections of mGluR agonists caused massive hippocampal damage (Siliprandi *et al* 1992; Lipartiti *et al* 1993).

Both glutamate release (Siesjo 1992) and massive increases in $Ca2^{+}$ (Erecinska and Silver 1992) (from ~70 nM to ~30 mM) occur during complete global ischemia, and the Ca^{2+} influx itself stimulates glutamate release from presynaptic vesicles (Krause *et al* 1988). Re-uptake of glutamate is inhibited by arachidonic acid, lipid peroxidation (Chan *et al* 1983) or products of lipid peroxidation (Braughter 1985). Magnesium inhibits synaptic transmitter release, it is a natural calcium antagonist, it inhibits cell death *in vitro* (Ames 1983), NMDA receptor antagonists have generally failed to salvage neurons following complete global ischemia. Furthermore, H^{+} (accumulated during ischemia) inhibits the NMDA receptor (Traynelis and Cull – Candy 1990), and there is little evidence for persistently increased extracellular glutamate during reperfusion. These observations raise the question of whether 'exitotoxicity' at NMDA receptors is causally related to morphologic evidence of damage progression during reperfusion; there is better evidence that AMPA receptor blockade is neuroprotective following complete global ischemia (Nellgard and Wieloch 1992). In any case, glutamate exitotoxicity seems only to provide special mechanisms in SVNs for ischemia-induced Ca over loading, which

occurs anyway due to the large transmembrane ionic gradient and ATP depletion induced failure of the calcium pumps. Perhaps the most important thing about this hypothesis is that it represents an early insight that cell-signaling mechanisms are involved in neuronal death.

Lipolysis, free radicals, and lipid peroxidation

Free fatty acids (FFAs), and in particular free arachidonic acid, are released during brain ischemia as a consequence of the activity of both phospholipase C (activated by depolarization) and phospholipase A (activated by increased Ca^{2+}) (Drenth *et al* 1976; Moskowitz *et al* 1984), and elevated concentrations of these FFAs persist into the early recirculation phase (Abe *et al* 1987; Rehncrona *et al* 1982; Bazan 1970; Yasuda *et al* 1985; Yoshida *et al* 1980; Katsuki and Okuda 1995; Umemura 1990). The rate of lipolysis during ischemia in the selectively vulnerable zones is significantly greater than in other areas of the brain (Umemura 1990). Reperfusion-initiated metabolism of free arachidonic acid may be the major source of $O_2^{-\cdot}$. Cyclooxygenase catalyzes the addition of two molecules of $O_2^{-\cdot}$ to an unsaturated fatty acid, like arachidonic acid, and produces prostaglandin PGG, which is rapidly peroxidized to PGH with concomitant release of $O_2^{-\cdot}$ (Krause *et al* 1988).

The morphological progression of injury during reperfusion (Negovskii 1962; Jenkins *et al* 1981; Kumar *et al* 1987) led to the hypothesis (Demopoulos *et al* 1980) that accelerated structural damage during reperfusion is a consequence of excessive generation of oxygen radicals

followed by lipid peroxidation. The free radical hypothesis suggests that the SVNs are especially prone to radical-induced damage (specifically lipid peroxidation) during reperfusion because (1) they are deficient in glutathione peroxidase (Ushijima *et al* 1986) and (2) they are surrounded by iron-laden supporting cells that release this iron during ischemia and reperfusion (Krause *et al* 1987; Zaleska and Floyd 1985). The changes in the conformation of the fatty acids resulting from lipid peroxidation would then alter the permeability and fluidity of the membrane. In addition, and probably more importantly, membranes contain receptors, ion channels, and other proteins whose functions are likely to be compromised by alterations in the lipid membrane.

Lipid peroxidation (Fig In 4) is a set of radical-mediated chemical chain reactions whereby the double bonds in the polyunsaturated fatty acid (PUFA) side chains are rearranged (Halliwell and Gutteridge 1984; Freeman and crapo 1982). In the presence of a transition metal (such as ferrous iron), lipid peroxidation chain reactions can expand geometrically (Aust and Morehouse 1985). The brain glia has abundant stores of oxidized (ferric) iron (Harrison *et al* 1968), mostly in ferritin and transferrin (Crichton 1978). Although $O_2^{-\cdot}$ is not itself a potent oxidizer, it does promote reduction of ferric and release of ferrous iron from ferritin (Thomas *et al* 1985), and iron delocalization into low-molecular-weight species is seen in the brain during post-ischemic reperfusion (Krause *et al* 1987). Peroxynitrite, formed by the reaction of $O_2^{-\cdot}$ with NO [produced by neuronal constitutive (nNOS) or inducible (iNOS) nitric oxide

synthetase], is implicated radical as the lipid peroxidation-initiating radical species during reperfusion (Beckman 1991; Samdani 1997). Peroxynitrite-mediated nitrosylation of tyrosine residues is also seen in reperfused SVNs (Tanaka *et al* 1997).

Membrane lipids are extensively peroxidized by iron dependent radical reactions during reperfusion (Zaleska 1985; Kogure *et al* 1982; Watson *et al* 1984; Bromont *et al* 1989; Sakamoto *et al* 1991;Rosenthal *et al* 1992). Formation of PBN (a radical spin trap) adducts peaks at 5 min reperfusion following 10 or 20 min of carotid occlusion in rats, and total PBN adduct increases with increasing ischemic time (Sakamoto *et al* 1991). Lipid peroxidation generates PUFA hydroperoxides and alcohols that are subsequently degraded into several aldehydic products, including malondialdehyde (Janero 1990) and 4-hydroxynonenal (Lukacova *et al* 1998). Such lipid peroxidation products are seen by 15–30 min reperfusion (Sakamoto *et al* 1991; Lukacova *et al* 1998), persist for up to 72 h (Bromont *et al* 1989), and are associated with substantial loss of PUFAs, development of gaps in the plasmalemma, and failure of ion partitioning (Kumar *et al* 1987). These markers of progressive membrane damage are substantially inhibited by pharmacologic iron chelators (White *et al* 1993). The lipid peroxidation products appear to accumulate in the Golgi apparatus, an important site of membrane recycling (Farquhar 1978; Voelker 1990).

Scope of the present study

In view of the key role played by ROS upon ischemia and reperfusion injury the present study is undertaken to study the amount of free radical damage and status of antioxidant defense mechanisms during varying periods of reperfusion in bilateral carotid artery occlusion in male rats. Due to the selective vulnerability of brain regions the study was done simultaneously in three different regions i.e. cerebral cortex, cerebellum and hippocampus. Several pharmacological agents (including antioxidants) have been tested in animal models for stroke prevention and therapy. Finally multifactorial strategies are been employed for stroke treatment. We have carried out the present study to check the potent role of a plant extract, known to have several medicinal properties for its efficacy in minimizing the ischemia reperfusion injury.

Animal Models of Cerebral Ischemia (Fig In 4)

Although stroke has been studied in many species (for example rabbits, dogs, cats, and baboons), rats and mice are the most widely investigated (Ginsberg1997). Several well-established models are available to study *global ischemia*. In a "four-vessel occlusion model" (4VO), flow in both carotid arteries and vertebral arteries are blocked for a specified time period ("Pulsinelli-Brierley"-model) (Pulsinelli *et al* 1982). In the two-vessel occlusion model (2VO), which is also referred to as "severe forebrain ischemia", only the carotid arteries are temporarily occluded, sometimes along with mild

hypotension (Fujii *et al* 1997). In these models, injury develops selectively in cells most vulnerable to ischemic damage such as in the CA1 sector in hippocampus, medium-sized neurons in the striatum, and Purkinje cells in cerebellum. Neurons are more susceptible than glial cells, and die over hours to days after the insult; hence the term "delayed neuronal death". Experimental *focal ischemia* is most commonly studied during permanent or transient occlusion of a middle cerebral artery (MCA) (Belayev *et al* 1995). Proximal MCA occlusion can be induced by an intraluminal suture (so-called filament model) or with a vascular clip and causes injury to cortex and deep structures (striatum).

Animal model for the present study

For the present study the 2 vessel occlusion model in rats is chosen, rats are easy to handle, maintenance is cost effective, extremely resistance to infections and the value of the model lies in the selective cell death produced in the CA1 neurons of hippocampus and cortex (Smith *et al* 1984). A transient reduction in cerebral blood flow induced by bilateral clamping of rat carotid arteries causes functional impairment (Jaspers *et al* 1990). In this model of 2-vessel occlusion (2VO), cerebral blood flow in the rat is reduced by about 50% (Eklof and Siesjo 1972). After 20 minutes of bilateral carotid occlusion, striatal cerebral blood flow decreased to 9.1 +/- 1.5 and 3.9 +/- 2.0 ml/100g/min in aged and adult rats, respectively, and hippocampal cerebral blood flow

decreased to 8.6 +/- 2.4 and 5.7 +/- 2.4 in aged and adult rats, respectively (Yao *et al* 1991). Three regions of the brain were chosen for the present study, cerebral cortex, cerebellum and hippocampus.

Cerebral cortex: The cerebrum or cortex is the largest part of the human brain, associated with higher brain function such as thought and action. The cerebral cortex is divided into four sections, called "lobes": the frontal lobe, parietal lobe, occipital lobe, and temporal lobe. Frontal Lobe- associated with reasoning, planning, parts of speech, movement, emotions, and problem solving. Parietal Lobe- associated with movement, orientation, recognition, perception of stimuli. Occipital Lobe- associated with visual processing . Temporal Lobe- associated with perception and recognition of auditory stimuli, memory, and speech

Cerebellum: The cerebellum, or "little brain", is similar to the cerebrum in that it has two hemispheres and has a highly folded surface or cortex. This structure is associated with regulation and coordination of movement, posture, and balance

Hippocampus: The portion of the cerebral hemispheres in basal medial part of the temporal lobe. This part of the brain is important for learning and memory, for converting short-term memory to more permanent memory, and for recalling spatial relationships in the world about us.

Animals

The experiments were performed on male *Wistar* rats (100 ± 20 grams) aged 1 month, obtained from National Institute of Nutrition, Hyderabad, India. Experimental protocols were performed according to Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA 2003) and were approved by institutional animal ethics committee. Prior to the experiment animals were housed individually in the animal house maintaining 12 hr day and night rhythm. Further, these animals had free access to pelleted diet (NIN, INDIA) and water, *ad libitum*.

Animal model

In the present study a transient incomplete global ischemia model in male *Wistar* strain rats has been employed. This model bilateral carotid-artery occlusion was done to significantly reduce blood flow.

Briefly, rats were anesthetized with ether. A small midline neck incision was made on the ventral side of the animal, the carotid artery which lies medial to and below the jugular vein was exposed by blunt dissection between the sterno hyoid, sterno mastoid and omo hyoid muscles (Fig M1). Care was taken that the adjacent vagus nerve was dissected away from the artery. The carotid arteries were then occluded for 20 minutes using temporary aneurysm clips to create ischemia and the clips were slowly removed to allow reperfusion.

All the surgeries were performed in four groups of animals.

Sham operated: Animals were anesthetized; carotid arteries were traced out and sutured back without occlusion.

0hr reperfusion: Animals were anesthetized; carotid arteries were traced out and occluded for 20 minutes. Animals were sacrificed immediately without allowing any reperfusion.

1hr reperfusion: Animals were anesthetized; carotid arteries were traced out and occluded for 20 minutes. Animals were sacrificed after allowing reperfusion for 1 hour.

24hr reperfusion: Animals were anesthetized; carotid arteries were traced out and occluded for 20 minutes. Animals were sacrificed after 24 hours of reperfusion.

Animals were decapitated, brains were removed, and cerebral cortex, cerebellum and hippocampus regions were separated out, frozen in liquid nitrogen and stored at -80^0C until use.

Histopathology

Transcardial perfusion:

Rats were anesthetized with an overdose of pentobarbital and the ribcage was cut carefully. The diaphragm was removed to expose the heart a fine syringe needle was inserted in the left ventricle and perfused with saline +heparin an incision was made in the right atrium to flush the blood out of the whole body. Then, freshly prepared solution of 4% (w/v) paraformaldehyde in

PBS (phosphate Buffered saline) was infused to fix the organs *in vivo*. After perfusion brains were removed, and 3mm sections were cut using acryl brain matrix, which were post fixed overnight in 4%paraformaldehyde.

Preparation of brain sections:

The post-fixed regions of brain were dehydrated through increasing concentrations of isopropanol (80%, 90%, 95%, 95%, 100%, 100%, 100% for 1hour each), followed by clearing the tissue using three changes of chloroform 1hour each. The tissue blocks were infiltrated with 2 changes of molten paraffin wax at 55^0C – 60^0 C for 3hours each.

Thin sections of 3µ were made using Leica microtome (RM2145) on to microscopic slides precoated with gelatin (0.5 % gelatin, 0.05% chrome alum). The sections were fixed on to slides by keeping them at 40^0C for 1hour. After drying the sections can be processed for staining or can be stored at – 20^0C until use.

Haematoxylin and eosin staining:

The sections were dewaxed in 2 changes of xylene (7min each), cleared in acetone (2min), Rehydrated in 95% ethanol (2min) and stained with Haematoxylin and eosin as reported earlier by Geiger *et al* (1995) to observe the cell morphology under light microscope.

DNA Isolation:

200mg of tissue was homogenized in eppendorfs tubes with 200µl of buffer A (40mM Tris pH 8.0), 300µg of proteinase K and a final concentration

of 2%SDS were added and incubated at 37^{o}C for overnight. 200 µl of buffer B (4mM Tris pH 8.0, 1.5 mM NaCl and 1mM EDTA pH 8.0) was added and mixed. To this 100 µl of buffer C (5M sodium perchlorate) was added and mixed well. Equal volume of phenol chloroform : isoamylalcohol (24:1) was added and centrifuged at 6000rpm for 5 min. to the supernatant equal volume of chloroform: isoamylalcohol was added and centrifuged at 6000rpm. Double the volume of absolute alcohol was added to the supernatant to precipitate the DNA and centrifuged at 10,000 rpm for 15 min. the pellet was washed with 70% alcohol, dried and dissolved in 200µl of TE (Tris EDTA). The isolated DNA was electrophoresed in 1.2% Agarose gel to check for the DNA fragmentation.

TUNEL assay:

The assay is based on terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end-labeling (TUNEL). TdT catalysis incorporation of fluorescein-dUTP at the free 3' –hydroxyl ends of the fragmented DNA. Fluorescein-labeled DNA can be detected via fluorescence microscopy. Briefly the protocol is as follows.

The sections were dewaxed in 2 changes of xylene, followed by rehydration in a graded series of ethanol (100%, 95%, 85%, 70%, 50%). Proteinase k treatment was given followed by incubation in equilibration buffer containing nucleotide mix and TdT enzyme. The sections were double stained with propidium iodide and observed under fluorescence microscope using

FITC filters. Apoptotic cells exhibit strong green fluorescence (520±20nm) and cells stained with PI exhibit red fluorescence at >620nm.

Determination of Oxidative stress

Estimation of Glutathione levels: Glutathione levels were estimated according to the protocol of Hissin and Hilf (1976).

Briefly, tissue (250 mg) was homogenized in 3.75 ml of the phosphate-EDTA buffer and 1ml of 25% HPO_3 which was used as a protein precipitant. The total homogenate was centrifuged at 4^0 C at 100,000g for 30 min to obtain the supernatant for the assay of GSSG and GSH.

GSH assay : The assay mixture contained 100 ml of the diluted tissue supernatant, 1.8 ml of phosphate- EDTA buffer, and 100 ml of OPT solution, containing 100 mg of OPT. After thorough mixing and incubation at room temperature for 15 min, the solution was transferred to quartz cuvette, fluorescence at 420 nm was determined with the activation at 350 nm.

GSSG assay : A 0.5 portion of original 100,000g supernatant was incubated at room temperature with 200 ml of 0.04 M NEM for 30 min to interact with GSH present in the tissue. To this mixture, 4.3 ml of 0.1N NaOH was added. A 100 ml portion of this mixture was taken for measurement of GSSG, using the procedure outlined above for GSH assay, except that 0.1 N NaOH was employed as diluent rather then phosphate EDTA buffer. Protein Estimation was done by the method of Bradford (1976).

Different concentrations of GSH and GSSG were used as standards.

Estimation of Lipid peroxides:

Lipid peroxides were measured using the protocol of Ohkawa *et al* (1978). Briefly tissue homogenates were prepared in a ratio of 1gram of wet tissue to 9 ml of 1.15% KCl by using a glass potter elvehjem homogeniser. The homogenate was then centrifuged at 2000g for 10 min. The resultant supernatant was used for further assay.

To samples less than 0.2 ml of 10% (w/v) tissue homogenate were added 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 with NaOH, and 1.5 ml of aqueous solution of Thiobarbituric acid. The mixture was made up to 4.0 ml with distilled water, and then heated in an oil bath at 95^0 C for 60 minutes using a glass ball as a condenser. After cooling with tap water, 1.0 ml of distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15:1, v/v) were added and shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was taken and its absorbance at 532 nm was measured. TMP was used as an external standard, and level of lipid peroxides was expressed as nano moles of MDA (malondialdehyde) per gram wet tissue.

Antioxidant enzyme assays

Catalase (Cat) (E.C 1.11.1.6) activity assay :

Catalase activity was measured as described by Chance and Maechly (1955). TA 1ml of reaction mixture contained 50µl of enzyme in phosphate

buffer pH 7.0 and 10mM hydrogen peroxide. The absorbance was read at 240 nm against a blank. Extinction coefficient of H_2O_2 was taken as 43.6 $mM^{-1}cm^{-1}$.

Activity was calculated according to the following equation.

$$\frac{\text{Difference in absorbance for 1 min x Volume of the reaction mixture in ml}}{\varepsilon\ H_2O_2\ (43.6) \text{ x Volume of the enzyme in ml}}$$

The enzyme activity was expressed as µmoles of H_2O_2 metabolized/mg protein/min.

Glutathione S-Transferase (GST) (2.5.1.18) activity assay :

One unit of GST activity was defined as the amount of enzyme that catalysis the formation of 1 µM of S-2, 4-Dinitophenyl glutathione per minute at 30^0C in a reaction mixture containing 1mM GSH, 1mM CDNB in 100mM potassium phosphate buffer (pH6.5) (Habig *et al* 1981). The reaction was monitored spectrophotometrically at 340 nm. Extinction coefficient of CDNB was taken as 9.6 mM^{-1} cm^{-1}

CDNB + GSH $\longrightarrow$ S-2, 4 dinitrophenyl glutathione + H^+ + Cl^-

Activity was calculated according to the following equation.

$$\frac{\text{Difference in absorbance for 1 min x Volume of the reaction mixture in ml}}{\varepsilon\ \text{CDNB}(9.6) \text{ x Volume of the enzyme in ml}}$$

Specific activity is expressed as units per mg of protein.

Glutathione Reductase (GR) (E.C 1.6.4.2) activity assay:

GR activity was estimated according to the method described by Carlberg and Mannervik (1975). A unit of enzyme activity is defined as the amount of enzyme that catalysis the oxidation of 1nm of NADPH in a reaction mixture containing 200 mM phosphate buffer (pH 7.0) with 2mM EDTA, 2mM NADPH in 10 mM Tris buffer pH 7.0 and 20 mM GSSG. The reaction was initiated by the addition of 50 µl of enzyme sample and the oxidation of NADPH was recorded as decrease in absorbance at 340 nm for 60 sec. Extinction coefficient of NADPH was taken as 6.2 mM^{-1} $cm^{-1.}$

Specific activity is expressed as units/mg of protein, where 1 unit is defined as 1nmole of NADPH oxidized per minute.

$NADPH + H^+ + GSSG \longrightarrow NADP^+ + 2\ GSH.$

Activity was calculated according to the following equation.

$$\frac{\text{Difference in absorbance for 1 min x Volume of the reaction mixture in ml}}{\varepsilon\ \text{NADPH (2.6) x Volume of the enzyme in ml}}$$

Specific activity is expressed as units/mg of protein, where 1 unit is defined as 1nmole of NADPH oxidized per minute

Glutathione peroxidase (GPx) (E.C 1.11.1.9) activity:

GPx activity was estimated according to the method described by Reddy *et al* (1981). Tissue was homogenized (10% wt/vol) in 50 mM phosphate buffer (pH 7.0) containing 1mM EDTA, 1mM PMSF and 250 mM

sucrose. The cytosolic fraction was used as the enzyme source to estimate the peroxidase activity. The activity was measured by monitoring oxidation of NADPH at 340 nm in a reaction mixture containing 50 µl of enzyme, 250mM phosphate buffer (pH 7.0), 2.5 mM EDTA, 2.5 mM sodium azide, 1mM GSH, 2mM NADPH, 1unit of GR incubated for 5 min at room temperature. GSH Px activity was assayed by using 12mM hydrogen peroxide. Extinction coefficient of NADPH was taken as 6.2 mM^{-1} $cm^{-1.}$

$$ROOH + 2GSH \longrightarrow ROH + H_2O + GSSG$$

$$NADPH + H^+ + GSSG \xrightarrow{GR} NADP^+ + 2\ GSH.$$

Activity was calculated according to the following equation.

$$\frac{\text{Difference in absorbance for 1 min x Volume of the reaction mixture in ml}}{\varepsilon\ \text{NADPH (2.6) x Volume of the enzyme in ml}}$$

Specific activity was expressed as units/mg of protein, where 1 unit is defined as 1nm of NADPH oxidized per minute.

Subcellular Fractionation of Nuclei, Membrane and Cytosol:

The nuclei, membrane and cytosolic fractions were prepared essentially as described by Kim *et al* (1997). Briefly, cerebral cortex, cerebellum and hippocampus from all the four groups of rats were homogenized in an isolation buffer containing 0.32M sucrose, 10mM Tris Hcl buffer (pH 7.4), 1.5mM MgCl2, 1mM Na_2EDTA, 1mM Na_2EGTA, 1mM dithiotrietol (DTT), 1mM PMSF, Leupeptin (2µg/ml) and aprotinin (2µg/ml) by douncing 30 times in a glass homogenizer using pestle B (Wheaton scientific

USA). Homogenization with pestle B facilitates disruption of tissue without disrupting the nuclei. The homogenate was filtered through nylon mesh (pore diameter 10 µM), centrifuged 1000 X g for 7 min, the pellet thus obtained was crude nuclear pellet. The supernatant was further centrifuged at 12,300 X g for 15 min to separate out the membrane fraction as pellet. The resultant supernatant was considered as cytosol enriched fraction.

Protein estimation :

Protein estimation was done according to Lowry *et al* (1951) which depends on quantitating the colour obtained from the reaction of folin ciocalteau phenol reagent with the tyrosyl residues of an unknown protein using bovine serum albumin as external standard.

SDS – PAGE:

Vertical slab gel electrophoresis (SDS - PAGE) was performed according to the method of Laemmli *et al* (1970). The separated protein bands were stained with coomassie blue.

Western Blotting:

Proteins resolved on SDS – PAGE were electroblotted on to nitrocellulose membranes by wet transfer in 25mM Tris – HCl (pH 8.3) buffer containing 192mM glycine and 20% methanol (Towbin, 1979). The membrane was incubated in blocking solution (5% dried minimal fat milk and 0.25% Tween 20 in TBS) for 2 hour followed by primary antibody solution in 5% Fat free milk at 4^{o} C overnight. The blot was washed with TBS and probed with

secondary antibody (1:1000 goat anti-rabbit IgG linked to alkaline phosphatase [ALP] in 5% fat free milk. The blot was developed using specific substrates, 0.033% nitro blue tetrazolium chloride and 0.0165% 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in ALP buffer (100mM Tris and 5mM $MgCl_2$; pH 9.2).

Collection of plant material :

Whole plants were collected and identified by one of the authors in and around the campus of University of Hyderabad, Hyderabad, Andhra Pradesh, India. The plants were washed in tap water, and air-dried. The dried plant material is made in a fine powder in a mortar and pestle. The powder was stored at 4 ^{o} C until use.

Preparation of Plant Extracts:

Aqueous extracts were prepared by macerating 5 g of powered plant material for 30 min in boiling water. The mixture was filtered through cheesecloth. The supernatant was stored at 4 ^{o} C until use.

Animal treatment with antioxidants:

Animals were pretreated orally with Vitamin C (100 mg/ kg bodyweight), Vitamin E (100 mg/kg body weight) or aqueous extract of *Phyllanthus* (100mg/ kg body weight) for one week prior to induction of 20 minutes ischemia and 1hr reperfusion.

Title: Extent of oxidative stress in cerebral ischemia and varying periods of reperfusion

Introduction

Oxidative stress occurs as a consequence of an alteration in the equilibrium of the production of reactive oxygen species (ROS) and antioxidative processes in favour of the production of ROS (Dringen 2000). Reactive oxygen species include non-organic molecules such as the superoxide radical ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$), as well as organic molecules such as alkoxyl and peroxyl radicals. ROS are continuously generated during oxidative metabolism (Dringen 2000). Mechanisms exist within the cells to combat the ROS effect. Compared to other organs the brain has some disadvantages regarding the generation and the detoxification of ROS.

(1) The cells of the mammalian brain utilize 20% of the oxygen consumed by the body constitute only 2% of the body weight (Clark and Sokoloff, 1999) indicating the potential generation of a high quantity of ROS during oxidative phosphorylation in the brain.

(2) A high content of iron has been reported for some brain areas (Gerlach *et al* 1994), which can catalyze the generation of ROS.

(3) The brain is rich in lipids with unsaturated fatty acids, targets for lipid peroxidation (Porter *et al* 1984, Halliwell *et al* 1992)

(4) The brain contains only low to moderate activities of superoxide dismutase (SOD), Catalase and Glutathione peroxidase (GPx) compared to other organs (Cooper *et al* 1997, Ho *et al* 1997).

In addition, the loss of neurons in adult brain cannot be regenerated.

In recent years, the oxidative stress theory has gained more and more interest in the development of tissue damage after CNS ischemia or trauma (Siesjo *et al* 1989, Lewen *et al* 2000). Evidence is growing that glutathione plays an important role in the detoxification of ROS in brain (Dringen 2000). Therefore, in the present study extent of oxidative stress was investigated in transient bilateral carotid artery occlusion in rats.

Oxidative stress and Glutathione

The tripeptide Glutathione (GSH; γ-L-glutamyl-L-cysteinylglycine) is a cellular thiol present in concentrations up to 12 mM in mammalian cells (Cooper *et al* 1997). It has an important function as antioxidant, it is a reaction partner for the detoxification of xenobiotics, it is a cofactor in isomerisation reactions, and it is a storage and transport form of cysteine (Cooper *et al* 1997).

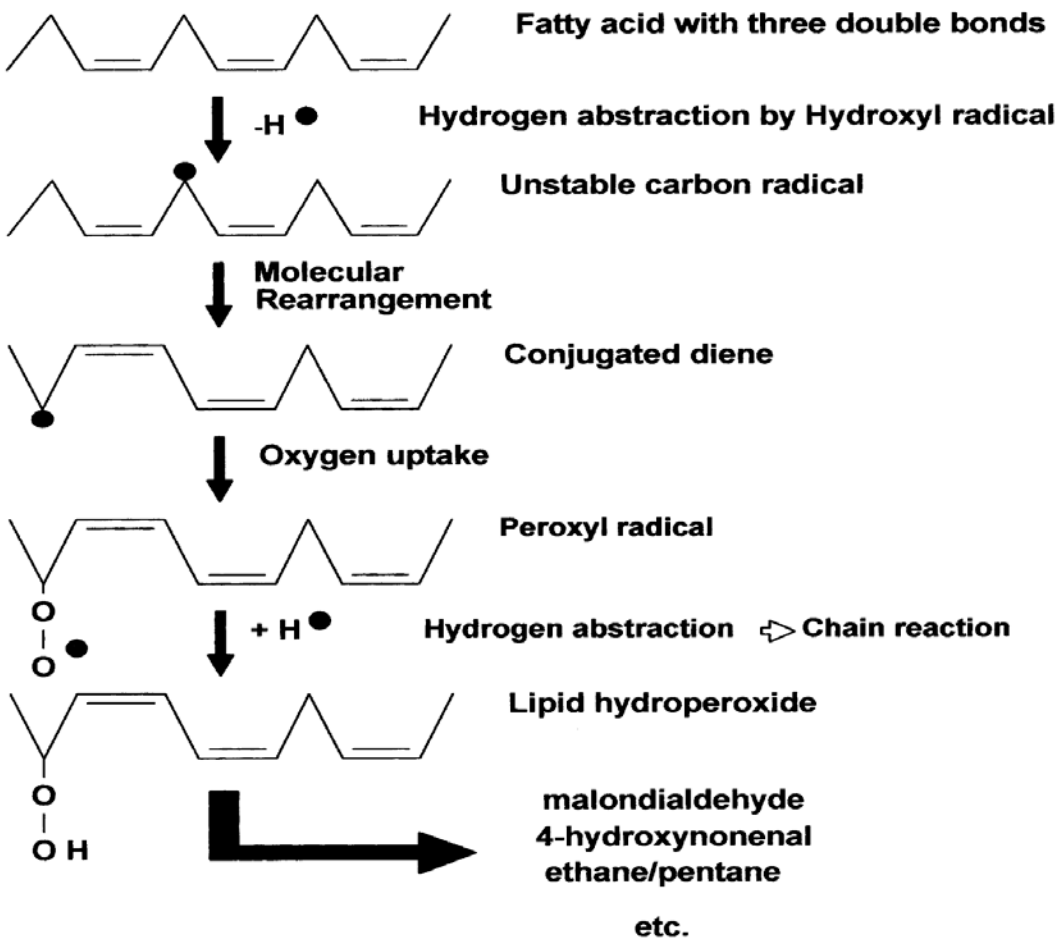
The glutathione system is very important for the cellular defense against ROS. A high intracellular concentration of Glutathione protects against a variety of different ROS. GSH reacts directly with radicals in non-enzymatic reactions (Saez *et al* 1990; Winterbourn and metodiewa 1994) and is also an electron donor in the reduction of peroxides catalyzed by GPx (Chance *et al* 1979). The final product of the

oxidation of GSH is Glutathione disulphide (GSSG). Determination of the ratio between reduced (GSH) and oxidized Glutathione is an important parameter to study the extent of oxidative stress, and this is chosen as a parameter for our present study.

Oxidative stress and Lipid peroxidation

The enhanced production of free radicals can overwhelm the endogenous antioxidative capacity of the brain and lead to cell damage by lipid peroxidation (LPO), protein oxidation and oxidation of nuclei acids. Lipid peroxidation is a self-propagating process that damages lipid membranes. Free radicals attack double bonds in unsaturated fatty acid side chains of membrane lipids, resulting in the formation of lipid radicals. These lipid- radicals mostly react with oxygen to form peroxyl radicals, which subsequently can attack membrane proteins and adjacent membrane lipids, thus resulting in a chain reaction of lipid and protein peroxidation. The ongoing destruction of membrane lipids and proteins leads to loss of membrane proteins, altered membrane fluidity, membrane disintegration and finally cell death (Farooqui *et al* 1998).

As depicted in the figure malondialdehyde is one of the intermediates of lipid peroxidation. Thus, quantitating malondialdehyde is a measure of lipid peroxidation and there by oxidative stress. The lipid



peroxidation may be the direct consequence of the action of free radicals formed during ischemia and reperfusion periods in bilateral carotid artery occlusion in rat (Atsuhiro Sakamoto *et al* 1991).

Objective The aim of this study was to induce incomplete global moderate ischemic and reperfusion in rat brain and to determine the Glutathione (GSH : GSSG) ratio and level of lipid peroxidation in brain tissues in order to investigate the extent of oxidative stress in brain ischemia with short and long- term reperfusions.

Results

Animal Model **:** Animal model for cerebral ischemia reperfusion was developed in male *Wistar* strain rats. Bilateral carotid arteries were occluded for 20 minutes and varying periods (0hr, 1hr and 24hr) reperfusion was allowed.

Cell Morphology

To examine whether apoptosis is involved in neuronal cell death induced by ischemia, the cellular morphology of brain cells on sections from cerebral cortex, cerebellum and hippocampus is monitored. The results of Haematoxylin – eosin staining indicated that a large number of degenerative cells (pyknosis of nuclei, disruption of eosinophilic cytoplasm) appeared in the cortex, cerebellum and hippocampus after ischemia and 24hr reperfusion (Fig 1.1b, 1.2b, 1.3 b). Little or no signs of degeneration were seen in the cells from the brains of control rats (Fig 1.1a, 1.2a, 1.3a). The primary difference between normal and apoptotic cells was observed in the nucleus. Healthy neurons exhibited a lightly stained nucleus and a darkly stained nucleolus. In contrast, the nuclei of apoptotic cells were darkly stained, particularly the chromatin at the periphery of nuclei. Staining of the cell body was very light, and in some cases absent. In more advanced stages of apoptosis, darkly stained chromatin was observed in the nucleus, and staining of the cell body was usually absent. Haematoxylin – eosin staining clearly showed the shrunken apoptotic neurons with eosinophilic cytoplasm and

a large, round, basophilic chromatin clumps in ischemia cortex, cerebellum and hippocampus. The present results indicate that bilateral occlusion of carotid arteries for 20min followed by reperfusion for 24hrs induces neurodegeneration in rat brain.

DNA Damage

Apoptosis is characterized by a number of intracellular phenomenon such as membrane blebbing, chromatin condensation and nuclear DNA fragmentation. Fragmentation can be visualized by Agarose gel electrophoresis following DNA extraction and also by *in situ* DNA nick end labeling. In DNA nick end labeling method, labeled nucleotides are incorporated in to the 3' hydroxyl ends of the fragmented DNA using terminal deoxy nucleotidyl transferase enzyme (TdT) followed by detection of the labeled molecules using fluorescence microscope. This type of assay, often referred to as the TUNEL assay, allows monitoring of apoptosis in cell samples or in the tissue sections, providing histological localization of apoptotic cells. The TUNEL assay done in cerebral cortex, cerebellum and hippocampus of control and ischemic rats showed a positive signal in ischemic treatment (Fig 1.4 a, b).

DNA ladder formation in cells is an index of apoptosis. To demonstrate the occurrence of apoptosis as characterized by internucleosomal DNA fragmentation, DNA isolated from cerebral cortex, cerebellum and hippocampus of control and ischemic rats was subjected to Agarose gel electrophoresis. DNA appeared as a single band in

control samples (Fig 1.5, lanes 1,5 and 9). DNA from ischemic samples appeared as cleaved and smear suggesting DNA fragmentation (Fig 1.5 lanes 2,3,4,6,7,8,10,11 and 12).

Oxidative Stress parameters

Glutathione plays an important role in protecting cells against oxidative stress, hence determining the levels of reduced and oxidized forms of glutathione were chosen as parameters to study the defensive status of cells against oxidative stress. Increased ROS induce lipid peroxidation, and so LPO levels was chosen as index for oxidative stress.

Glutathione content in cerebral cortex :

Reduced glutathione (GSH) content did not show any significant variation in cerebral cortex after ischemia /reperfusion when compared to that of sham operated samples (Fig 1.6a, Table 1.1). Oxidized glutathione (GSSG) content increased after 0hr, 1hr and 24hr reperfusion following 20min ischemia in cerebral cortex samples compared to that of sham operated samples (Fig 1.6b, Table 1.1). The increase was 23.87%, 26.02% and 34.04% respectively of that of sham - operated samples.

Lipid peroxidation in cerebral cortex :

Lipid peroxidation increased significantly after 1hr reperfusion (57.55% of control) following 20min ischemia. 0hr and 24hr samples did

not show any significant variation when compared to that of sham operated cerebral cortex samples (Fig 1.6c, Table 1.1).

A decrease in GSH, an increase in GSSG and lipid peroxidation indicates the induction of oxidative stress in cerebral cortex after ischemia and reperfusion.

Glutathione content in cerebellum :

GSH content increased by 10% after 20min ischemia without reperfusion, Whereas 20min ischemia followed by 1hr reperfusion showed a small but significant decrease (6.4% of control) in GSH content when compared to sham operated cerebellum (Fig 1.7a, Table 1.2). 20min ischemia followed by 24hr reperfusion increased GSH content by 37.4% of that of sham operated. GSSG content increased after 1hr and 24hr reperfusion following 20min ischemia. The increase was 12.4% and 15.1% respectively of that of sham operated cerebellum samples (Fig 1.7b, Table 1.2).

Lipid peroxidation in Cerebellum:

Lipid peroxidation increased significantly after 1hr and 24hr reperfusion (70% and 69.4% respectively) following 20min ischemia compared to sham operated cerebellum samples (Fig 1.7c, Table 1.2).

An increase in GSSG and lipid peroxidation indicates the induction of oxidative stress in cerebellum after ischemia and reperfusion

Glutathione content in hippocampus:

20min ischemia without any reperfusion decreased GSH level (31.1% of control) in hippocampus when compared to sham operated. This decrease continued for 1hr reperfusion samples whereas GSH level in 24hr reperfusion following 20min ischemia were same as compared to sham operated (Fig 1.8a, Table 1.3). GSSG levels did not show any significant variation in 0hr, 1hr and 24hr reperfusion following 20min ischemia when compared to sham operated hippocampus samples (Fig 1.8b, Table 1.3).

Lipid peroxidation in hippocampus:

A significant increase of lipid peroxides was observed in 20min ischemia without reperfusion hippocampus samples when compared to that of sham operated (Fig 1.8c, table 1.3). 1hr and 24hr reperfusion after 20min ischemia showed not much alteration in lipid peroxidation when compared to that of sham operated.

Discussion:

The neuropathological changes observed during brain injury, trauma, stroke, and epileptic associated brain damage have all been ascribed to enhanced oxidative stress and related alterations to lipid protein and DNA molecules (Srinivasan *et al* 2002). One of the mechanisms responsible for the delayed neuronal injury after global cerebral ischemia is the membrane alteration. The most important cause

for membrane alteration is the lipid peroxidation due to a relatively excess generation of free radicals.. Many mechanisms involving free radical production have been documented both during ischemia and reperfusion but their actual importance is still not clear (Nita 2001). When free radical production exceeds the buffering capabilities, LPO levels increase while non-enzymatic scavengers (GSH) and antioxidant enzymes decrease. A decrease in free radical production was followed by a gradual normalization of LPO and both GSH and antioxidant enzymes (Nita 2001). Experimental ischemia and reperfusion models, such as transient focal/global ischemia in rodents, have been thoroughly studied and cumulative evidence suggests involvement of oxygen radicals in the pathogenesis of ischemic lesions (Sugawara *et al* 2004). However, in transient bilateral carotid artery occlusion during various reperfusion time periods, oxidative stress and antioxidant status has not been well established. Hence the present study was taken up to address the above issues.

Reoxygenation during reperfusion provides a substrate for numerous enzymatic oxidation reactions. Lipid peroxidation is a major contributor to ischemic damage, but the underlying mechanisms are poorly understood (Eileen *et al* 2001). The rate of lipolysis during ischemia in selectively vulnerable zones is significantly greater than in other areas of the brain (White *et al* 2000). Studies involving animal models for ischemia reperfusion have been extensively done in focal

Ischemia and global Ischemia (Sugawara *et al* 2003) where global ischemic models involved the occlusion of carotid arteries and vertebral arteries (complete global ischemia) or occlusion of carotid arteries along with hypotension (incomplete global ischemia). To our knowledge this is for the first time the studies pertaining to oxidative stress in bicarotid artery occlusion for 20min followed by varying periods of reperfusion is being reported.

Many contradictory reports have emerged regarding the status of GSH during reperfusion injury in brain. Investigations by Folbergrova *et al* (1979) led to the observation that the GSH and GSSG levels were unaltered in brain during hypoxia. Further, it was observed that the brain GSH levels decreased during reperfusion after complete or incomplete Ischemia without any concomitant accumulation of GSSG in the brain or cerebrospinal fluid (Cooper *et al* 1980; Rehncrona *et al* 1980). The results of the present study have shown that reversible incomplete brain ischemia lead to a reduction of concentrations of GSH in cortex, cerebellum and hippocampus accompanied by small but significant increase in GSSG. The alteration in the ratio of GSH and GSSG suggests that there is a significant amount of oxidative stress in rat brain upon 20 bilateral carotid artery occlusion. The maximum alteration was observed in brains after 1hr of reperfusion following 20min ischemia, suggesting maximum oxidative stress at that time period. These

changes were found to be normalized after 24hr of reperfusion following 20min ischemia

In our study we addressed the problem of relative contribution of ischemia and reperfusion to the free radical production estimated by the level of lipid peroxidation (LPO). The amount of MDA (malondialdehyde, a LPO intermediate) was found to be increased in all brain regions after 1hr of reperfusion following 20min ischemia. Further maximum LPO occurred in hippocampus suggesting that it is a region for the production of more free radicals and more vulnerable region for damage when compared to cortex and cerebellum.

Results of this study clearly shows that a small reduction in blood flow could lead to oxidative stress and can have deleterious effects on brain cells. This kind of small reductions in blood volume to brain can be observed in case of patients with low blood pressure, and this might lead to early onset of neurological disorders. All these results indicate oxidative stress in all the three brain regions but the amount of oxidative stress was more in 1hr reperfusion samples compared to 0hr and 24hr reperfusion. Amount of Lipid peroxidation in hippocampus was very high indicating the greater sensitivity of hippocampal neurons when compared to the neurons in cerebral cortex and cerebellum.

Title: Profiles of antioxidant enzyme activities in cerebral ischemia and varying periods of reperfusion.

Introduction

Reactive oxygen species (ROS) are continuously generated during oxidative metabolism. In order to avoid damage caused by ROS, such as lipid peroxidation, protein oxidation, and DNA strand breaks, mechanisms exists that remove ROS or prevent generation of ROS (Sies 1991, Halliwell and Gutteridge 1999). The cellular antioxidant status determines the susceptibility to oxidative damage and is usually altered in response to oxidative stress (Halliwell and Gutteridge 1999). The major defense against toxicity of superoxide radicals is conferred to superoxide dismutase (SOD) (Fig 2A). SOD catalysis the dismutation of superoxide radicals forming hydrogen peroxide. Glutathione peroxidase (GPx) and Catalase (Cat) are the unique enzymes scavenging hydroperoxides and therefore act in concert with SOD (Guner *et al* 1996, McCord 1993, Rice-Evans 1994). Glutathione reductase transfers electrons from NADPH to GSSG, there by regenerating GSH. GST acts as a non-selenium dependent GPx.

It is generally accepted that the main factors involved in cerebral ischemia and reperfusion injury are the oxygen derived free radicals generating mostly intracellular (Ho *et al* 1998; Reilly *et al* 1991). The half-lives of these free radicals are extremely short. Therefore, it is technically difficult to measure their concentrations in the brain during an ischemic

insult and subsequent reperfusion periods (Chan 1996; Michowiz *et a*l 1990). One way to overcome this problem is to measure the antioxidant levels of the tissue as an indicator of oxidative stress. However, the changes in levels of antioxidants like **α** tocopherol and glutathione alone in the brain would not explain the oxidative damage induced by superoxide radicals and hydrogen peroxide. Since these reactive oxygen radicals are likely controlled by SOD, catalase, GPx, GR, and GST, which constitute the primary line of defense against the intracellularly generating free radicals (Chan *et al* 1983). Therefore, in the present study Cat, GR, GPx and GST activities were investigated.

Catalase (Cat)

Thernard, discoverer of H_2O_2, first noted in 1818 that animal tissues could decompose H_2O_2. Loew in 1901 introduced the name catalase for the natural compound that decomposes H_2O_2. Wolft and de Stoecklin achieved first hemoglobin-free purification of catalase in 1910.

Catalase has mainly 2 functions i.e. enzymatic and biological functions. Enzymatic Functions includes the decomposition of

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2.$$

Biological Functions are Removal of H_2O_2, addition of O_2, protection against lipid peroxidation, May participate in alcohol metabolism.

There are many forms of catalase. Most contain Fe - heme, but some contain Mn. Most anaerobic bacteria do not contain catalase, most aerobic bacteria contain catalase. In Maize - 3 Cat found in different cells

and expressed differentially during development. All are tetramers of MW 240,000 with each one or two amino acids different. Human contains at least 2 forms, found in cytoplasm and peroxisomes. Mostly catalase is present in liver (hepatocyte, peroxisomes) and erythrocyte (cytoplasm), some found in brain, heart, skeletal muscle, and kidney.

Glutathione Reductase (GR)

Glutathione reductase has two functions, one is enzymatic where it takes parts in reducing glutathione.

GSSG + NADPH + H+ $\xrightarrow{\text{GR}}$ NADP+ + 2GSH

Biological functions of GR include removal of GSSG, which is toxic. GR keeps GSH in reduced form so it can be used in detoxification reactions. There are families with low levels of GR in red cells, such kinds of people are normal under normal circumstances, but under oxidative stress, red cells hemolyze leading to anemia. GR is located in eukaryotic cell cytoplasm and mitochondria. GR is present in human RBC, its molecular weight is 104,800Da, it contains 2 identical subunits. Cells need to be supplied with riboflavin in diet to make GR.

Glutathione Peroxidase (GPx)

GPx discovered by Mills in 1957, functions include both enzymatic and biological. GPx acts on hydroperoxides and reduces them to nontoxic alcohols, it oxidizes glutathione molecule in this reaction.

ROOH + 2GSH ® ROH + H2O + GSSG

GPx is unspecific for hydro peroxides, it can act on anything from H_2O_2 to peroxidized membranes and DNA, but it is specific for GSH. It yields a single oxidation product, in contrast to heme peroxidases. Biological functions include removal of H_2O_2. Genetic or alimentary deficiency in GPx suffers hemolytic episodes if exposed to drugs generating O_2. Removal of other hydroperoxides, protection against lipid peroxidation, protection against DNA hydroperoxides, arachidonic acid cascade catalyze formation of prostaglandins are the other functions of GPx. GPx is found in all eukaryotes. It is present in high amounts in liver, moderate amounts in heart, lung and brain, low amounts in muscle. There are five known forms of GPx: Cytosolic GPx (GPX-1) (Examples : Bovine erythrocyte of MW = 85,000 Da, Rat liver MW = 75,000 Da, Human erythrocyte = 95,000 Da, Human placenta = 85,500 Da). Active site contains a selenocysteine. Mitochondrial GPx has never been isolated, but mitochondria have no Cat, so GPx must be important for the removal of peroxide. Selenium is essential for protein synthesis and enzymatic activity of GPx. Humans need 60 mg/day minimum selenium in diet. Selenite, selenomethionine, and selenocysteine can be used as drugs for selenium deficiency. Se deficiency signs include liver necrosis, exudative diathesis, failure to grow and reproduce, degenerative heart disease (Keshan disease) found in China.

Glutathione-S-transferases (GSTs)

GST is a Non-Se containing GPx discovered in 1976 by Lawrence and Burk. The enzymatic function of GST is the same as glutathione peroxidase, i.e. rids cells of hydroperoxides. GST does not act on H_2O_2.

ROOH + GSH ® GSOH + ROH

GSOH + GSH ® GSSG + H2O

Biological functions of GSTs are GST may function as GPx when Se is low, GST can Detoxify the foreign compounds, conjugate with GSH (catalytic), binds with ligands which are not substrates, covalently binds with very reactive compounds leading to their inactivation, Conjugation reactions involving endogenous compounds, *i.e.,* make steroids, prostaglandins, etc. GST is located in the cytoplasm, nucleus and cell surface of eukaryotic cells. GST is present in liver, red cell and intestine. It Accounts for 10% of soluble protein in liver. Liver GST is a dimer with 4 possible subunits Ya (22,000 Da); Yb (23,500 Da); Yb¢ (23,500 Da); Yc (25,000 Da). Subunits combine to form 6 isozymes YaYa, YaYc, YcYc, YbYb, YbYb¢, Yb¢b¢. Only proteins with Ya or Yc exhibit high GPx activity.

There have been unclear results about the antioxidant enzyme activities in different studies performed on various rat brain ischemia reperfusion models (Liu *et al* 1994, Michowiz *et al* 1990, Stanimirovic *et al* 1994, Tokuda *et al* 1993). However there have been less reports on the responses of endogenous antioxidant enzymes in a moderate ischemic

tissue which can survive for hours before it is recruited in the infarction process and can be salvaged by pharmacological treatment. The present study was undertaken to study the antioxidant enzymes in bilateral carotid artery occlusion inducing moderate ischemia in rats.

The antioxidant enzymes are regional specific and cell type specific in brain. The expression and the activity of these enzymes vary largely depending on the cell type and region of there presence. The vulnerability to oxidative stress in the brain is region-specific (Baek *et al* 1999). The cortex and hippocampus both contain relatively high densities of N – methyl – D – aspartate receptors when compared to cerebellum and so cortex and hippocampus are prone to glutamate- induced elevations in intraneuronal Ca^{2+} and the subsequent increased production of ROS and thereby alterations in antioxidant enzyme activities (Maile R Brown *et al* 2004).

Objective: The aim of this study was to induce incomplete global moderate ischemia in rat brain and to determine Cat, GR, GPx, and GST activities in different brain regions (Cerebral cortex, Cerebellum and Hippocampus) in order to investigate the antioxidant enzymatic changes in brain ischemia with varying periods of reperfusions.

Results

Antioxidant enzyme activities in cerebral cortex (Table 2.1)

Catalase activity increased significantly in 0hr (61%) reperfusion samples when compared to sham operated animals (Fig 2.1a). Glutathione

reductase activity decreased in 0hr (13.8%) and 1hr (17%) samples when compared to sham operated animals (Fig 2.1b). There was a decrease in glutathione peroxidase activity in 0hr (18.9%) and 1hr (207.3%) reperfusion samples when compared to sham operated (Fig 2.1c). Glutathione S-transferase activity decreased by 24.3% in 0hr reperfusion samples but there was 9.6% increase in 1hr reperfusion when compared to sham operated animals (Fig 2.1d).

Catalase, glutathione reductase and glutathione peroxidase activities decreased whereas glutathione S-transferase activity increased in cerebral cortex upon ischemia and 1hr reperfusion when compared to sham operated animals.

Antioxidant enzyme activities in cerebellum (Table 2.2)

A significant increase in catalase activity was observed in 0hr, 1hr and 24hr reperfusion samples when compared to sham operated. The increase was 74.7%, 90.4% and 84.2% in 0hr, 1hr and 24hr reperfusion samples respectively (Fig 2.2a). Glutathione reductase activity decreased significantly in 0hr reperfusion samples (23.1%), but it was increased in 1hr (116.6%) and 24hr (24.34%) reperfusion samples when compared to sham operated animals (Fig 2.2b). Glutathione peroxidase activity decreased by 42.38 % in 1hr reperfusion samples and was 38.1% increased in 24hr reperfusion samples when compared to sham operated animals (Fig 2.2c). Glutathione S transferase activity decreased in 0hr (249.8%) whereas it

was increased in 1hr (469.5%) and 24hr (848.7%) reperfusion samples when compared to sham operated (Fig 2.2d).

Catalase, glutathione reductase and glutathione S-transferase activities increased whereas and glutathione peroxidase activity decreased upon ischemia 1hr reperfusion in cerebellum when compared to sham operated.

Antioxidant enzyme activities in Hippocampus (Table 2.3)

Catalase activity decreased in all the three samples. The decrease was 32.2%, 32.7% and 21.7% in 0hr, 1hr and 24hr reperfusion samples respectively when compared to sham operated (Fig 2.3a). There was a decrease in glutathione reductase activity in 0hr (30.1%) while 1hr (109.4%) reperfusion samples and 24hr reperfusion (94.68%) samples showed an increase in glutathione reductase activity when compared to sham operated (Fig 2.3b). A small decrease of glutathione peroxidase activity was observed in 0hr (3.2%) reperfusion samples. 1hr and 24hr reperfusion samples showed a very high increase when compared to sham operated (Fig 2.3c). Glutathione S transferase activity decreased in 0hr (27.5%) samples, where as the same was found to be increased in 1hr and 24hr reperfusion samples when compared to sham operated (Fig 2.3d).

Catalase activity decreased whereas GR, GPx and GST activities increased upon ischemia and 1hr reperfusion in Hippocampus.

Discussion

Ischemia reperfusion alters the antioxidant enzyme activities; the main reasons for the changes in the antioxidant enzymatic activities can be explained as follows.

Ohtsuki *et al* (1993) explained the decrease in the antioxidant enzyme activities during ischemia - hypoxia and reperfusion as being due to the attack of sulphydril (-SH) groups of enzymes by oxygen free radicals and interaction of enzymes with peroxidation products, which can affect the active site of the enzyme (Mishra *et al* 1990; Ohtsuki *et al* 1993). Another reason for reduction of enzyme activities can be attributed to the reduction in pH i.e acidosis (Weihai Ying *et al* 1999). Ischemia renders the cells to undergo anaerobic metabolism there by producing lactic acid and acidosis. Enzymes that are pH sensitive will be therefore easily affected. Stanimirovic *et al* (1994) explained the stimulation of the antioxidant enzymes in brain ischemia reperfusion as the transient substrate induction.

The present study was carried out in three different brain regions, which have different antioxidant profiles, each region is dealt separately here.

Cerebral cortex: Our results indicate that catalase and GR activities increased after ischemia, which can be correlated with pilocarpine administration and resulting Status epilepticus produced a significant increase in the catalase activity in the hippocampus (36%), striatum (31%) and frontal cortex (15%) of treated adult rats (Freitas *et al* 2004). The

activity of the antioxidant enzymes glutathione peroxidase, glutathione reductase, and Mn superoxide dismutase were significantly increased by 54%, 73% and 32%, respectively, in neuronal cultures subjected to hypoxic preconditioning (Arthur *et al* 2004). But in our experiments GPx and GST activities reduced after ischemia. Reperfusion has reduced the activities of catalase and GPx where as GR and GST activities have increased.

Cerebellum: Catalase and GPx activities increased after ischemia whereas in the adult rat cerebellum after Status Epilepticus induced by pilocarpine no change was observed in the catalase activity (Freitas *et al* 2004). GR and GST activities were found to be reduced after ischemia. Reperfusion increased the activities of catalase, GR and GST activities, and GPx activity was found to be lowered after reperfusion.

Hippocampus: Catalase, GR, GPx and GST activities reduced during ischemia. The reduction of antioxidant enzymes can be attributed to the higher sensitivity of hippocampus when compared to cerebral cortex and cerebellum It has been widely recognized that the hippocampus and striatum are clearly more susceptible to oxidative stress than the remaining brain regions. (Hercília *et al* 2002). There is a decline in the activity of antioxidant enzymes with age (Aspberg *et al* 1992)

Ischemia reperfusion increased catalase activity in intestinal tissue (Paiva *et al* 2004). Ischemia alone in rat kidney did not alter either the activities or protein levels of superoxide dismutase and glutathione peroxidase. However, catalase activity was found to be inhibited to 82% of

control. The inhibition of catalase was due to the inactivation of the enzyme as there was no significant change in enzyme protein level. Reperfusion following ischemia, however, led to a significant decrease in both the activities as well as the protein levels of all the antioxidant enzymes (Singh *et al* 2004). Hepatocyte peroxisomal catalase enzyme activity decreased during 60min of ischemia and declined further during reperfusion. mitochondrial and microsomal glutathione peroxidase (GPx) activity increased significantly ($p < 0.001$) after 60min of ischemia and was sustained during 24hr of reperfusion (Ram gupta *et al* 2003). The timing of catalase over-expression relative to ischemia is a critical variable determining its potential therapeutic value (Gu *et al* 2004)

In conclusion, the generally observed reduction in the antioxidant enzyme activities during brain ischemia may compromise the antioxidative capacity of the tissue to defend itself adequately against oxygen free radicals generated during ischemia. Reoxygenation renders the cerebral tissue more susceptible to cellular damage.

Title: Activation of signaling cascades upon cerebral ischemia and varying periods of reperfusion.

Introduction:

Recent studies have suggested the early involvement of oxidative DNA damage in ischemic brain injury (Nagayama et al, 2000; Liu *et al*., 1996; Chen *et al*., 1997). Oxidative damage may consist of several highly specific chemical events, such as DNA strand breaks or modified bases. Single-strand DNA breaks as a result of attack by reactive oxygen species, expressed through positive DNA polymerase I-mediated biotin-dATP nick-translation (PANT) staining, have been detected as early as 1 min following transient focal ischemia (Chen et al., 1997). Incompletely repaired or unrepaired oxidative DNA damage is an important trigger of apoptotic cell death under many conditions and may contribute to ischemic brain injury. Thus, as in other types of cells, self-defense systems in neurons rely on an efficient repair system that can quickly reconstruct damaged DNA. Cellular responses to oxidative DNA damage involve gene expression and functional activation of DNA repair enzymes. Among the numerous putative DNA repair proteins studied, poly (ADP-ribose) polymerase (PARP) has attracted much attention.

PARP:

The DNA repair enzyme poly (ADP-ribose) polymerase (PARP) (EC 2.4.2.30), also known as poly (ADP-ribose) synthetase or poly (ADP-ribose) transferase (PADRT), has emerged as a major player along the continuum of cell death. Poly (ADP – ribosylation) is involved in a variety of physiological

events such as chromatin decondensation, DNA replication, DNA repair, gene expression, malignant transformation, cellular differentiation and apoptosis (Lagueux *et al* 1994; De Murcia. and Menissier de Murcia. 1994; Lindahl *et al* 1995). Nuclear PARP activity is abundant through out the body, particularly in the immune system and germ cell lines. PARP enzyme can be grouped in to three major domains (Kameshita 1984). PARP is an abundant nuclear protein with two distinct regions: an amino-terminal DNA-binding domain and a carboxyl-terminal catalytic domain. *In vitro*, PARP is activated by binding to DNA strand breaks. In the presence of NAD^+, PARP strongly stimulates DNA excision repair, the predominant restoration mechanism for oxidative DNA damage (Satoh *et al.,* 1994). The function of PARP depends largely on the availability of NAD^+ in the nuclei, along with the completion of auto modification, enabling the release of PARP from strand breaks. In the absence of NAD^+, PARP is unable to release from DNA breaks, thus inhibiting DNA polymerase and other repair enzymes and blocking the reconstruction of DNA. Although PARP may not be essential for DNA repair to occur, its functional status could affect DNA repair activities and the subsequent recovery of a cell from injury (Satoh *et al*., 1994; Sternster *et al.,* 1994; Lindahl *et al*., 1995). This result is dependent on cellular levels of NAD^+. For example, in neuronal cultures, inhibition of PARP activity can save neurons from glutamate exitotoxicity or nitric oxide toxicity, most likely by preventing NAD^+ depletion.

PARP is efficiently cleaved and inactivated in programmed cell death into a 24*kDa* fragment containing the N-terminal DNA binding domain and an

89*kDa* peptide comprising the central auto modification domain and the C-terminal NAD binding and catalytic domains. Activation of cytosolic proteases and a fairly specific degradation of proteins, including PARP, are important for apoptosis (Pettmann and Henderson, 1998). Proteolytic cleavage of PARP into characteristic fragments was first recognized as a hallmark feature of apoptosis by Kaufmann and colleagues in chemotherapeutically treated human acute myelogenous leukaemia cells (Kaufmann 1989). Although Kaufmann originally identified cleavage of PARP into 85*kDa* and 25*kDa* fragments, these fragments are now designated as 89*kDa* and 24*kDa* on the basis of sequence data (Duriez and Shah 1997). To date, PARP is among the first set of proteins known to be degraded in apoptosis (Casiano *et al* 1996; Eric *et al* 1996).

PARP cleaving protease in apoptosis is granzyme B, a serine protease that is involved in cytotoxic T-lymphocyte granule-mediated apoptosis of target cells. This enzyme acts through caspase-3 (Darmon, Nicholson and Bleackley, 1995; Quan, *et al* 1996) via upstream activation of caspase-8 (Fernandes-Alnemri *et al* 1996; Muzio et al 1997), the convergence signal for many apoptotic stimuli. Granzyme B also directly cleaves PARP within its catalytic domain at a later time point after caspase activation into a 64*kDa* N-terminal fragment and a 54*kDa* C-terminal fragment (Froelich et al 1996). Cleavage occurs at D535 in human PARP and D537 in bovine PARP (Froelich *et al.* 1996). Additional cleavage within the C-terminal fragment

yields a catalytically active 42*kDa* fragment (Froelich *et al.* 1996). This pattern of cleavage by granzyme B suggests both early activation of proteases and later direct cleavage of target proteins perhaps upon entry of granzyme B into the nucleus. The existence of additional potential cleavage sites in PARP suggests the potential for additional PARP fragmentation patterns in different models of apoptosis by both these and perhaps other proteases (Duriez and Shah 1997). Susceptibility of these other sites to proteolytic cleavage could depend on the conformation of intact or cleaved PARP during various stages of cell death. Necrotic cell death has also been associated with a unique cleavage of PARP, which produces a major 50*kDa* fragment and minor 40*kDa* and 35*kDa* fragments, (Shah and Poirier 1996).

Understanding proteolytic events underlying apoptosis, such as those leading to PARP cleavage should prove useful for developing neuroprotective agents and therapeutic interventions for neurodegenerative disorders. The role of PARP in the brain under ischemic conditions is incompletely known (Eliasson et al., 1997; Takahashi et al., 1997; Lo et al., 1998). We have therefore investigated the potential role of PARP activation in cell survival using a rat model of transient global ischemia.

Caspases:

Caspases (Cysteine aspartate specific proteases) are the principle effectors of apoptosis (Muzio *et al* 1997). These cysteine proteases reside in the cytosol of all animal cells as inactive zymogens. Proteolytic processing of these zymogens generate active enzymes and trigger apoptosis. Induction of

apoptosis via death receptors results in the activation of an initiator caspase such as caspase 8 or caspase 10. The initiator caspases can then activate other caspases in a cascade, which can eventually lead to the activation of the effector caspases, such as caspase 3 and caspase 6. Effector caspases are responsible for the cleavage of the key cellular proteins that leads to the typical morphological changes observed in cells undergoing apoptosis.

There are a number of other mechanisms through which the caspase cascade can be activated. Granzyme B can be delivered into cells by cytotoxic T lymphocytes and is able to directly activate caspases 3, 7, 8 and 10. The mitochondria are also key regulators of the caspases cascade and apoptosis. Release of cytochrome *c* from mitochondria can lead to the activation of caspases-9, and then caspases-3. This effect is mediated through the formation of an apoptosome, a multi-protein complex consisting of cytochrome *c*, Apaf-1, pro-caspase-9 and ATP.

One of the major pathways of caspase activation involves the participation of mitochondria (Green and Reed 1998). Release of cytochrome *c* (cyt-*c*) from intermembrane space (IMS) of these organelles occurs on treatment of cells with many apoptotic stimuli. Cytochrome *c* released from the mitochondria binds to the cytosolic protein Apaf-1, this interaction results in a conformational change in Apaf-1 which, when stabilized by the binding of ATP, allows molecules of Apaf-1 to associate with each other (Li *et al* 1997). This results in the formation of a wheel-like structure that contains 7 molecules each of Apaf-1, cytochrome *c* and ATP. The wheel-like structure, known as the

apoptosome, permits the recruitment of 7 molecules of procaspase-9 to the complex. The exact mechanism of caspase activation is still uncertain although two possibilities have been proposed. In one case the Apaf-1, cytochrome *c* and procaspase-9 complex can act as a stage to activate cytosolic procaspase-9 as it is recruited to the apoptosome. In the other scenario two apoptosome have been proposed to interact with each other and to activate the caspase-9 located on the other apoptosome. One role of caspases is to inactivate proteins that protect living cells from apoptosis.

Cytochrome c

Cytochrome *c* is a small heme protein found loosely associated with the inner membrane of the mitochondrion. It is a soluble protein, unlike other cytochromes and is an essential component of the electron transfer chain. It is capable of undergoing oxidation and reduction, and does not bind oxygen. It transfers electrons between Complexes III and IV. Cytochrome *c* is a highly conserved protein across the spectrum of species, found in plants, animals, and many unicellular organisms. This, along with its small size (molecular weight about 12,000 Daltons), makes it useful in studies of evolutionary divergence. Cytochrome *c* is an essential component of the mitochondrial respiratory chain. It is a soluble protein, localized in the intermembrane space and loosely attached to the surface of the inner mitochondrial membrane. In response to a variety of apoptosis-inducing agents, cytochrome *c* is released from mitochondria to the cytosol (Kluck *et al*., 1997). The release of

cytochrome *c* from the mitochondria during apoptosis precedes the mitochondrial delta PSI disruption. In cells over expressing Bcl-2 (or Bcl-XL) the release of cytochrome *c* is blocked, aborting the apoptotic response. Cytochrome *c* was found to be one essential component of the complex that induces Caspase-3 activation and apoptotic activity in cytoplasmic HeLa extracts upon dATP stimulation. Cytochrome *c* was suggested to mediate (together with dATP) the formation of the Apaf-1/Caspase-9 complex, which results in, activated Caspase-9. Caspase-9 is thought to trigger a caspase-cascade leading to apoptosis (Li et al., 1997). Therefore, in the present study we have investigated the role of caspase-3 activation and cytochrome *c* release in cell survival using a rat model of transient global ischemia.

Results:

The proteins from cerebral cortex, cerebellum and hippocampus regions of ischemia and varying periods of reperfusion were isolated separated on SDS PAGE, transferred on to nitrocellulose membrane and then probed with respective antibodies. In the present study PARP (anti rabbit), caspase-3 (anti mouse) and cytochrome *c* (anti mouse) protein levels were analyzed.

Cerebral cortex:

PARP antibody used for the present study recognized both the full length PARP (116KDa) and 2 cleaved fragments at 89*kDa* and 64*kDa*. Sham operated cerebral cortex showed the basal level of PARP cleavage

represented in lane 1 (CC) (Fig 3.1). PARP levels upon 20 min ischemia without any reperfusion are shown in lane 2 (IC) (Fig 3.1). Full length PARP (116*kDa*) level is more in lane 2 compared to lane 1 suggesting the increased expression of PARP upon 20 min ischemia. Lane 3 (1C) (Fig 3.1) represents cerebral cortex after 20 min ischemia followed by 1hr reperfusion. More amount of PARP cleavage was been observed in lane 3 compared to lane 1, indicating increased amount of PARP cleavage and activation of apoptotic cascades upon ischemia and 1hr reperfusion. Lane 4 represents PARP levels in cerebral cortex after 20 min ischemia and 24hr reperfusion. The band levels in lane 4 are same as that of lane 1. PARP expression increased in 20 min ischemia, PARP cleavage increased in 1hr reperfusion samples and it was restored back to normal after 24hr of reperfusion.

Caspase-3 antibody used fro the present study is specific for the active form of caspases-3 at a molecular weight of 17KDa. There was not much difference in the caspases-3 levels in lane 2 (IC)(Fig 3.2) and lane 3 (1C)(Fig 3.2) compared to lane 1 (CC)(Fig 3.2). But lane 4 (Fig 3.2) showed an increased level of caspases-3. Caspase-3 was found to be activated in cerebral cortex region after 24 hours of reperfusion.

Cytochrome *c* antibody used for the present study is mouse monoclonal antibody, which recognizes a band at 14.5*kDa*. Lane 1 (CC)(Fig 3.3) represents the cytochrome *c* levels in cytosol fraction of sham-operated cerebral cortex. Lane 2 (IC) (Fig 3.3) represents the cytochrome *c* level in cytosol fraction of 20 min ischemia without any reperfusion cerebral cortex.

Lane 2 shows an increased level of cytochrome *c* compared to sham operated. 20 min ischemia followed by 1hr reperfusion is represented in lane 3 (1C)(Fig 3.3). Cytochrome *c* level was maximum in lane 3 compared to lane 1, lane 2 and lane 4. Lane 4 (24C)(Fig 3.3) also shows an increased amount of cytochrome *c* on 20 min ischemia and 24hr reperfusion but maximum increase was in samples after 1hr reperfusion in cerebral cortex.

Cerebellum:

Sham operated cerebellum showed the basal level of PARP cleavage along with full length PARP represented in lane 1 (CB) (Fig 3.4). PARP levels upon 20 min ischemia without any reperfusion are shown in lane 2 (IB) (Fig 3.4). It was completed cleaved as suggested by absence of full length PARP (116KDa) in lane 2 compared to lane 1 and the cleaved protein band intensity increased in lane 2. The band at 64*kDa* was at a high level when compared to lane 1 suggesting increased cleavage of PARP upon 20 min ischemia in cerebellum. Lane 3 (1B) (Fig 3.4) represents cerebellum after 20 min ischemia followed by 1hr reperfusion. More amount of PARP cleavage and absence of full length PARP is been observed in lane 3 compared to lane 1, indicating increased amount of PARP cleavage and activation of apoptotic cascades upon ischemia and 1hr reperfusion. Lane 4 (24B)(Fig 3.4) represents PARP levels in cerebellum after 20 min ischemia and 24hr reperfusion. Even in lane 4 full length PARP was absent when compared to sham operated but cleaved fragments were less compared to lane 2 and lane 3. There was no change in PARP expression upon ischemia and reperfusion

but PARP cleavage increased in all ischemia reperfusion samples suggesting the activation of apoptotic cascades in cerebellum.

Caspase-3 active form was seen in all the three ischemia reperfusion periods in cerebellum when compared to sham operated. Lane 1 (CB)(Fig 3.5) represents sham operated caspase-3 level in cerebellum. Lane 2 (IB) (Fig 3.5) represents caspase-3 levels in 20 min ischemia without any reperfusion. There was an increased band intensity in lane 2 compared to lane 1. Caspase-3 levels in 1hr reperfusion cerebellum are represented in lane 3 (1B)(Fig 3.5). Lane 4 (24B)(Fig 3.5) represents caspase-3 in 24hr reperfusion cerebellum. Caspase-3 activation increased in all ischemia reperfusion cerebellum samples.

Lane 1 (CB)(Fig 3.6) represents the Cytochrome *c* levels in cytosolic fraction of sham-operated cerebellum. Lane 2 (IB)(Fig 3.6) represents the cytochrome *c* level in cytosol fraction of 20 min ischemia without any reperfusion cerebellum. Lane 2 shows an increased level of cytochrome *c* compared to sham operated. 20 min ischemia followed by 1hr reperfusion is represented in lane 3 (1B)(Fig 3.6). Cytochrome *c* level was more in lane 3 compared to lane 1. Lane 4 (24B)(Fig 3.6) also shows an increased amount of cytochrome *c* upon 20 min ischemia and 24hr reperfusion. Cytochrome *c* level was found to be increased in cytosolic fractions of ischemia reperfusion samples in cerebellum in all the three time periods.

PARP cleavage, caspase-3 levels and cytochrome *c* levels increased in all the three periods of ischemia reperfusion in cerebellum region.

Hippocampus:

Sham operated hippocampus showed the basal level of PARP cleavage represented in lane 1 (CH) (Fig 3.7). PARP levels upon 20 min ischemia without any reperfusion are shown in lane 2 (IH) (Fig 3.7). Lane 3 (1H) (Fig 3.7) represents hippocampus after 20 min ischemia followed by 1hr reperfusion. Lane 4 represents PARP levels in hippocampus after 20 min ischemia and 24hr reperfusion. There was faint signal at a molecular weight of 116*kDa* full length PARP. But the cleaved fragment at 64KDa showed an increased band intensity upon ischemia and 3 periods of reperfusion when compared to sham operated. PARP cleavage increased in 20 min ischemia, 1hr reperfusion and 24hr reperfusion samples in hippocampus.

Caspase-3 active form was seen in all the three ischemia reperfusion periods in hippocampus when compared to sham operated. Lane 1 (CH)(Fig 3.8) represents sham operated caspase-3 level in hippocampus. Lane 2 (IH) (Fig 3.8) represents caspase-3 levels in 20 min ischemia without any reperfusion. Caspase-3 levels in 1hr reperfusion hippocampus are represented in lane 3 (1H)(Fig 3.8). Lane 4 (24H)(Fig 3.8) represents caspase-3 in 24hr reperfusion hippocampus. Caspase-3 activation increased in all ischemia reperfusion hippocampus samples and a more prominent band in 24 hr reperfusion samples when compared to other 3 samples.

Lane 1 (CH)(Fig 3.9) represents the cytochrome *c* levels in cytosol fraction of sham operated hippocampus. Lane 2 (IH)(Fig 3.9) represents the cytochrome *c* level in cytosol fraction of 20 min ischemia without any

reperfusion hippocampus. Lane 2 shows an increased level of cytochrome *c* compared to sham operated. 20 min ischemia followed by 1hr reperfusion in represented in lane 3 (1H)(Fig 3.9). Cytochrome *c* level was more in lane 3 compared to lane 1. Lane 4 (24H)(Fig 3.9) also shows an increased amount of cytochrome *c* on 20 min ischemia and 24hr reperfusion Cytochrome *c* level was found to be increased in cytosolic fractions of ischemia reperfusion samples in hippocampus in all the three time periods when compared to sham operated.

Discussion

Emerging evidence has suggested that induction of oxidative DNA damage plays an important role in mediating neuronal death in cerebral ischemia (Chen *et al* 1997; Nagayama *et al* 2000; Lan.J *et al* 2003). However, little is known about how the endogenous DNA repair mechanisms in the brain respond to ischemia and oxidative DNA damage. In the present study the role of PARP a DNA repair enzyme was investigated in a rat model of transient incomplete global ischemia. In parallel with the measurement of endogenous oxidative DNA damage, mechanisms responsible for the cleavage of PARP like caspase 3 activation and cytochrome c release were also examined.

It has been shown that PARP is activated following DNA strand breaks under many oxidative stress-related conditions and that the inhibition of PARP activity in certain circumstances may have a detrimental, or alternatively beneficial, effect on cell survival (Ding *et al.,* 1992; Zhang *et al.,* 1995; Thiemermann *et al* 1997). Cleavage of PARP might occur when the cell is no

longer able to repair its DNA, and could play a role in cellular disassembly that might ensure commitment to apoptosis. In the present study PARP cleavage was observed in cerebral cortex, cerebellum and hippocampus during periods of ischemia and reperfusion indicating blockage of DNA repair mechanism and placing the cells morein a more vulnerable condition. Cleavage of PARP by caspase-3 is a defining characteristic of apoptosis, and PARP also plays a pivotal role in classical necrosis (Andrew *et al* 1999). PARP activation and subsequent cleavage have active and complex roles in apoptosis (Hamid Boulares 1999). PARP cleavage prevents induction of necrosis during apoptosis and ensures appropriate execution of caspase-mediated programmed cell death (Zdenko Herceg *et al* 1999). The cleavage pattern which we observed yielded 2 fragments corresponding to 89*kDa* and 64*kDa*; the first fragment produced as a result of cleavage by caspase 3 and the second fragment produced as a result of later cleavage by granzyme B. A dual pattern of 89*kDa* and 64*kDa* fragmentation of PARP has also been noted in γ-irradiation induced apoptosis in mouse lung cells (Paris *et al.* 1997).

Further, cerebral cortex region showed an increased expression of PARP with a concomitant increase in cleavage. PARP was cleaved completely in cerebellum region after ischemia reperfusion without any increase in expression. In the present study PARP cleavage increased with a concomitant decrease of full length PARP in hippocampus region in correlation with increase in PARP activity and level in hippocampus after ischemia (Nagayama et al 2000). Cleavage of PARP was found to be maximum at 1hr

reperfusion in contrast with PARP-1 immunoreactivity and PARP-1 cleavage reached peak levels 2 to 3 days after delivering the excitotoxin in to brain (Gilliams-Francis *et al* 1999)

. Caspase-3 was activated in our study in correlation with clear induction of caspase-3 upon hypoxia in neonatal rat brain (Gill *et al* 2002). Caspase-3 is activated extensively in the immature brain after hypoxic ischemia (Wang *et al* 2001). The subsequent cleavage of proteins involved in cellular homeostasis and repair may contribute to the process of brain injury (Wang *et al* 2001). Induction of the MTP by TNF-α causes a release of cytochrome *c*, caspase-3 activation with PARP cleavage and DNA fragmentation (Marco Tafani *et al* 2000). In the neonate, caspase-3 activation is likely to contribute substantially to cell death not only in the penumbra but also in the core after ischemia with reperfusion (Manabat *et al* 2003).

Analysis of cytochrome c distribution did not provide definitive evidence for selective cytochrome c release in the permanent focal ischemia model, whereas in the transient model a small but consistent amount of cytochrome c was found in the cytosolic fraction (Gill *et al* 2002). This is in correlation with our studies where we found release of cytochrome c upon ischemia reperfusion. The mitochondrial inner membrane permeability transition occurs during ischemia and/or after reperfusion, resulting in translocation of cytochrome c and activation of caspases (Hirakawa *et al* 2003). Cytochrome c release is prevented in CA1 neurons in gerbils in which ischemia-tolerance had been induced (Nakatsuka *et al* 2000). Cytochrome *c*

was released and there was a loss in mitochondrial respiratory activity during cardiac ischemia/reperfusion (Lech *et al* 2003). There was a Significant release of cyt c at very early reperfusion times (2 and 5 min) in the CA1 but not the DG area of the hippocampus (Kristian *et al* 2002) in correlation with our studies where cytochrome C release was more in samples immediately after reperfusion.

In the present study activation of caspase-3 and cytochrome *c* levels varied with different periods of reperfusion. Though caspase-3 activation is via cytochrome *c* pathway, there are several other mechanisms, which might be responsible for its activation without participation of cytochrome *c*. D-GalN induced apoptosis through caspase-3 activation but without modification of the activity of caspase-6, -8, -9, SMases or cytochrome c release (Siendones *et al* 2005). The studies regarding the involvement of other pathways in ischemia induced cell death are under progress.

In conclusion, DNA damage was observed and DNA repair enzymes were cleaved by caspases resulting in obstruction to DNA repair placing the cells more under a risk. Oxidative stress is the may be the major culprit in inducing DNA damage and antioxidants may be beneficial in reducing stress to cells under ischemia reperfusion.

Title: Role of antioxidants Vitamin C, Vitamin E and aqueous extract of *Phyllanthus* in cerebral ischemia and varying periods of reperfusion.

Introduction:

Many laboratory and animal studies suggest that vitamins C and E help reduce the risk of coronary artery disease in a number of ways. First, they inhibit the oxidation of LDL ("bad") cholesterol, both individually and through their interaction. Oxidation makes LDL more likely to promote the buildup of fatty plaque in coronary artery walls (atherosclerosis). Vitamin E may also reduce the blood's ability to clot, thus lowering the risk of heart attacks. Finally, E may help reduce inflammatory processes (inflammation has been linked with coronary artery disease). Therefore for the present study the antioxidative abilities of vitamin C and E were studied.

Vitamin C:

Vitamin C protects the DNA of the cells from the damage caused by free radicals and mutagens. As Gaby and Singh report, it prevents harmful genetic alterations within cells and protects lymphocytes from mutations to the chromosomes. In several studies, vitamin C reduced chromosome abnormalities in workers exposed to pollutants such as coal tar, styrene, methyl methacrylate and halogenated ethers. Another way in which vitamin C protects us is by preventing the development of nitrosamines, the cancer-causing chemicals that stem from the nitrates contained in many foods (Singh and Gaby 1991).

Vitamin C prevents free radical damage in the lungs and may even help to protect the central nervous system from such damage. Ascorbic acid also was tested as an antioxidant to inflammatory reaction in mice. As an antioxidant, vitamin C's primary role is to neutralize free radicals. Since ascorbic acid is water soluble, it can work both inside and outside the cells to combat free radical damage. As explained earlier, free radicals will seek out an electron to regain their stability. Vitamin C is an excellent source of electrons; therefore, it "can donate electrons to free radicals such as hydroxyl and superoxide radicals and quench their reactivity," (Bendich 1990). The versatile vitamin C also works along with glutathione peroxidase (a major free radical-fighting enzyme) to revitalize vitamin E, a fat-soluble antioxidant. In addition to its work as a direct scavenger of free radicals in fluids, then, vitamin C also contributes to the antioxidant activity in the lipids.

Vitamin E: Vitamin E is a fat-soluble vitamin; it is one of the vitamins that act as antioxidants. Vitamin E is found in wheat germ, corn, nuts, seeds, olives, spinach, asparagus, and other green leafy vegetables, vegetable oils (corn, sunflower, soybean, and cottonseed), and products made from them such as margarine. Vitamin E (alpha tocopherol) is an anti-oxidant that assists in maintaining cell integrity. It is important in the formation of red blood cells..

Only a small amount of vitamin E is needed to meet normal daily requirements. However, research using vitamin E at far higher doses than the daily requirement has provided preliminary evidence that it may be helpful for

preventing or treating various medical conditions. These uses include treating menstrual pain, cardiac autonomic neuropathy (a complication of diabetes), low sperm count, restless leg syndrome, inflammation of eye tissues, Alzheimer's, Parkinson's and rheumatoid arthritis.

Topical formulations of vitamin E are used for a variety of purposes. Weak preliminary evidence hints that topical vitamin E might benefit blood circulation in people with diabetes and vitamin E may be of some benefit in preventing and treating sunburn. Vitamin E cream does not appear to help prevent surgical scarring.

Side effects: There are no known toxic effects to megadoses of vitamin E. However, occasional side effects such as headache have been reported.

Scientists identified vitamin E about 80 years ago, but only in the past few decades has its power as an antioxidant been revealed and fully appreciated. Initially, there was great interest and excitement regarding what this vitamin could do for a number of ailments, particularly heart disease. Well-designed studies completed recently, however, have found that the effect may be a less beneficial than once thought. When summoned from the body's fatty tissue where it's stored, vitamin E--and it's antioxidant powers--go into action, protecting cells by deactivating or destroying the potentially damaging oxygen molecules called free radicals. Vitamin E also helps in the formation of red blood cells and facilitates the use of the trace mineral selenium and vitamins A and K.. Vitamin E is actually an umbrella term for a group of compounds called

tocopherols and tocotrienols. Until recently, most vitamin E products contained only tocopherols (alpha-, beta-, delta-, and gamma-tocopherols), with alpha-tocopherol recognized as the body's predominant and most potent form.

As a key antioxidant, vitamin E appears to play a modest but notable role in protecting the body from many chronic disorders. It may even slow the aging process and guard against damage from second hand smoke and other pollutants. According to test-tube studies, the tocotrienols (alpha-tocotrienol, specifically) appear to be the most powerful of the vitamin E antioxidants. Circulatory disorders, skin and joint problems, diabetes-related nerve complications, high cholesterol, endometriosis, immune-system function, and memory are also believed to benefit from vitamin. A combination of vitamin C and vitamin E holds some promise for preventing and possibly easing complications of such disorders as congestive heart failure, alcoholism, cancer, HIV infection, lupus, multiple sclerosis and nail problems.

Phyllanthus

Several Indian medicinal plants have been extensively used in the Indian traditional (ayurveda) system of medicine for the management of various pathological conditions (Scartezzini *et al* 2000 and Auddy *et al* 2003)). Since ancient times, plants of the genus *Phyllanthus* (family: Euphorbiaceae) have commonly been used in the treatment of liver diseases (Thyagarajan *et al* 1992). Reports about jaundice and other liver abnormalities describe

Phyllanthus administration in India, China, Burma, Pakistan, Philippines, Guam, West Indies, South America, East and West Africa, and else where in tropic and sub-tropical areas (Blumberg. *et al*; 1989). *Phyllanthus tenellus* has been used in Brazilian traditional medicine for the treatment of kidney and bladder calculi, diabetes, hepatitis, jaundice and asthma (Martins *et al* 1995 and Menezas *et al* 1997). Crude extracts of *Phyllanthus amarus* were found to inhibit hepatitis B virus (HBV) polymerase activity and hepatitis B antigen (HBS Ag) in chronic HBV carriers (Venkateswaran *et al* 1987 and Thyagarajan *et at;* 1988)

P. urinaria has been proven to be effective in protecting CCl4-induced injuries of liver cells (Zhou *et al* 1997), relaxing the histamine-induced contraction of trachea (Paulino *et al* 1996a,b), producing pronounced systemic, spinal and supraspinal antinociception (Santos *et al* 1995, 1999), inducing the contractile response in the urinary bladder (Dias *et al* 1995) and decreasing the blood glucose level in streptozotocin-induced diabetic rats (Higashino *et al* 1992). Clinically, *P. urinaria* has been shown to convert hepatitis B antibody status in the patients from negative to positive (Wang *et al* 1995). *P. urinaria* has also been demonstrated to exhibit the inhibitory effect on the intracellular HbsAg formation in hepatoma cells (Ji *et al* 1993) and the activity of retroviral reverse transcriptase (Suthienkul *et al* 1993). More importantly, no side effect or toxicity has been reported in any of the above clinical studies. The anticancer effect of the genus *Phyllanthus* has been reported in a few papers. *Phyllanthus amarus* protected the liver from

hepatocarcinogenesis induced by N-nitrosodiethylamine in animal models (Jeena *et al* 1999). Root of *Phyllanthus acuminatus* has been shown to inhibit the growth of murine P-388 lymphocytic leukemia and B-16 melanoma cell lines (Powis and Moore, 1985; Pettit *et al* 1990). Recently, 7V-hydroxy-3V,4V,5,9,9V pentamethoxy-3,4-methylene dioxy lignan isolated from the ethylacetate extract of P. urinaria was shown to exhibit anticancer activity by inducing apoptosis through the inhibition of telomerase activity and Bcl-2 expression (Giridharan *et al*., 2002).

the aqueous extract of *P. urinaria* is substantially useful in treating various kinds of human cancer cells without toxic side effect on normal cells (Huang et al 2004 b). The *P. urinaria*-induced apoptosis in HL-60 cells is mediated through a ceramide-related pathway (Huang *et al* 2004 a). chronic Emblica officinalis administration causes myocardial adaptation by augmenting endogenous antioxidants and protects rat hearts from oxidative stress associated with ischemic-reperfusion injury (Rajak *et al* 2004). *P. amarus* extract could increase the antioxidant defense mechanism in mice and there by protect the animals from radiation-induced cellular damage (Kumar and Kuttan 2004). Antihepatotoxic activity of *Phyllanthus fraternus* (Ahmed *et al* 2002). A standardized extract of *Phyllanthus emblica* (trade named Emblica) was found to have a long-lasting and broad-spectrum antioxidant activity (Chaudary 2002).

Results:

Inhibitory effect of *Phyllanthus* on lipid peroxidation in Rat liver homogenate: (Table 4.1 Fig 4.1a, Fig 4.1b)

Different concentrations (10 -100 µg) of the aqueous extracts were tested for the inhibition of lipid peroxidation induced by Fe2+/ascorbate system. Dose dependent inhibition was observed. The maximum inhibition (40%) was found at a dose of 40 µg in the total reaction mixture. The data was tabulated and calibration curves were constructed.

Oxidative stress and alterations in the antioxidant enzyme activities have been studied in ischemia and varying periods of reperfusion. According to our study 20 minutes of bilateral carotid artery occlusion and followed by 1 hour of reperfusion induced maximum oxidative stress and maximum alterations in the antioxidant enzymes activities, hence this time period was chosen to test the role of antioxidants.

Cerebral cortex: (Table 4.2) Reduced glutathione levels decreased by 6.2%; oxidized glutathione levels increased by 26% and LPO levels increased by 2% in 1hr reperfusion samples compared to control. Treatment with antioxidants increased the GSH levels (Fig 4.2a) by 3.9%, 276%, 116% in *Phyllanthus*, vitamin C and Vitamin E treatment respectively when compared to 1hr reperfusion samples without any treatment. There was a concomitant increase in the GSSG levels (Fig 4.2b). The increase was 1.49% and 16.79% in *Phyllanthus* and Vitamin C treatments respectively when compared to 1hr reperfusion without any treatment. Vitamin E treatment decreased GSSG level

by 5.3%. *Phyllanthus* treatment increased LPO levels (Fig 4.2c) 62.7%, while vitamin C and vitamin E treatments decreased LPO levels by 61.39% and 52.79%respectively when compared to 1 hr reperfusion samples without any treatment.

Cerebellum: (Table 4.3) Reduced glutathione levels decreased by 6.12%; oxidized glutathione levels increased by 12.4% and LPO levels increased by 69.9% in 1hr reperfusion samples compared to control. Treatment with antioxidants increased the GSH levels (Fig 4.3a) by 90.6%, 39.7%, 17.7% in *Phyllanthus*, vitamin C and Vitamin E treatment respectively when compared to 1hr reperfusion samples without any treatment. There was a concomitant increase in the GSSG levels (Fig 4.3b). The increase was 49.7%, 85.5% and 43.1% in *Phyllanthus*, Vitamin C and Vitamin E treatments respectively when compared to 1hr reperfusion without any treatment. Antioxidant treatment decreased LPO levels (Fig 4.3c) by 38.7%, 17.1% and 50.3% in *Phyllanthus*, vitamin C and Vitamin E treatment respectively when compared to 1hr reperfusion samples without any treatment.

Hippocampus: (Table 4.4) Reduced glutathione levels decreased by 31.46%; oxidized glutathione levels increased by 10.75% and LPO levels increased by 29.9% in 1hr reperfusion samples compared to control. Treatment with antioxidants decreased the GSH levels (Fig 4.4a) by 25%, 60.5%, 75.5% in *Phyllanthus*, vitamin C and Vitamin E treatment respectively when compared to 1hr reperfusion samples without any treatment. There was an increase in the GSSG levels (Fig 4.4b). The increase was 5.23%, and 1.55% in

Phyllanthus and Vitamin E treatments respectively when compared to 1hr reperfusion without any treatment. Vitamin C treatment decreased GSSG levelk by 44.6%. Antioxidant treatment decreased LPO levels (Fig 4.4c) by 19.4% and 33.6% in *Phyllanthus* and Vitamin E treatment respectively when compared to 1hr reperfusion samples without any treatment. Vitamin C treatment increased LPO by 21.6%.

Discussion:

Oxidative stress is known to have major contribution to the neuronal cell death due to ischemia reperfusion; hence mechanisms, which could minimize oxidative stress, would be proved highly beneficial in ischemia reperfusion injury. Several synthetic free radical scavengers have been evaluated in animal models of cerebral ischemia and reperfusion and have been shown to be protective (Umemura *et al* 1994; Baker *et al* 1998; Kuroda *et al* 1999). An ascorbic acid pretreatment effectively diminished the acute lung damage caused by the introduction of superoxide anion free oxygen radicals to the trachea in guinea pigs (Becher and Winsel, 1989). Exogenous administration of a tocopherol has been found to be protective in middle cerebral artery occlusion in rats (Chaudhary *et al* 2003). A number of traditional herbal kampo preparation has been tested in animal models of central neuropathy, including cerebral ischemia (Kabuto *et al* 1997), epilepsy (Komatsu *et al* 1996; Hamada *et al* 1993) and ageing (Hiramatsu *et al* 1989; Inada *et al* 1996; Ueda *et al* 1996), and their effectiveness has been suggested. *Phyllanthus emblica* fruits showed antioxidant activity on

prevention from indomethacin induced gastric ulcer (Bandyopadhyay *et al* 2000). In view of these the present study is undertaken to investigate the role of antioxidants like Vitamin C and vitamin E and antioxidant potential of aqueous extract of a herb *Phyllanthus*.

In the present study aqueous extract of *Phyllanthus* showed a dose dependent inhibition of lipid peroxidation induced in rat liver homogenate. This was similar to a study *by* Jagetia *et al* 2004 where plant extracts exhibited a dose-dependent NO scavenging activity. These results indicate the protective roles of vitamin C, vitamin E and aqueous extract of *Phyllanthus* against ischemia reperfusion injury.

Although vitamin E supplements significantly reduced the skin malondialdehyde concentration, neither supplement affected other measures of UVR-induced oxidative stress in human skin, which suggested no photoprotection of supplementation. (McArdle 2004). *Phyllanthus emblica* showed the highest cardioprotective effect in doxorubicin-induced cytotoxicity when compared to ascorbic acid and trolox in a study on cardiac myoblasts H9c2 (Wattanapitayakul *et al* 2005). *Phyllanthus* has an additional medicinal quality i.e PUW has the potential of antithrombosis due to its inhibition of platelet-neutrophil adhesion. PUW (a fraction containing 60% corilagin from a Chinese herbal plant *Phyllanthus urinaria* shows the tendency to bleeding, however, it could not cause serious bleeding side effect as compared with aspirin or urokinase (Shen *et al* 2004). Thus, *Phyllanthus* can be more potent antioxidant than vitamin C and vitamin E. Moreover**,** when aqueous extracts of

Phyllanthus emblica L. fruit and an equivalent amount of vitamin C were administered orally, significantly reduced the frequency of chromosome aberrations induced by CsCl indicating that vitamin C, an essential component of *P. emblica* extract, was the effective agent (Ghosh *et al* 1992). But aqueous extract of edible dried fruits of *Phyllanthus emblica* showed a greater efficacy when compared to equivalent amount of Vitamin C against nickel clastogenicity in mice**.** The greater efficacy of the fruit extract could be due to the interaction of its various natural components rather than to any single constituent (Dhir *et al* 1991). Ingestion of *Phyllanthus emblica* fruit extract showed a greater efficacy against clastogenicity of lead and aluminium in mouse bone marrow cells of mice when compared to equivalent dose of vitamin C (Dhir *et al* 1990). Thus, different components of *Phyllanthus* could act synergistically and can be proved more beneficial than synthetic antioxidants.

The present study revealed the following salient features in bilateral carotid artery occlusion in rat brain. The cell morphology observed in paraffin embedded tissue sections stained with hematoxylin and eosin showed characteristic apoptotic features like shrinked cytoplasm, vacuolated neuropile in all the three regions of the brain i.e. cerebral cortex, cerebellum and hippocampus, upon ischemia reperfusion.

GSH/GSSG ratio was altered significantly upon ischemia and reperfusion. The alteration was more in 1hr reperfusion samples than 0hr and 24hr reperfusion samples. Hippocampal region showed higher alteration when compared to cortex and cerebellum regions. Lipid peroxidation was significantly high in samples treated with 20min ischemia followed by 1hr reperfusion. The increase was 57.55% and 69.9% in cerebral cortex and cerebellum regions respectively when compared to controls. However, hippocampal regions showed much higher increase in LPO in 20min ischemia samples (1085%) compared to control. These results revealed a higher amount of oxidative stress after reperfusion than ischemia alone and hippocampus was observed as the most sensitive region compared to cerebral cortex and cerebellum.

Cortex: The study on antioxidant enzymes revealed that ischemia alone increased catalase (Cat) activity (61%) compared to sham operated samples whereas glutathione reductase (GR) activity increased significantly (762.1%, 730.8% and 923.0% respectively) in all the three groups i.e. ischemia (20min), ischemia with reperfusion (1hr) and ischemia

with reperfusion (24hrs) when compared to sham operated (control) animals. Glutathione Peroxidase (GPx) activity decreased in 20min ischemia (18.9%) and 1 hour reperfusion samples (207.3%) when compared to control but an increase of 56.1% was observed in 24-hour reperfusion samples. Similarly the activity of Glutathione S-transferase (GST) was decreased in 20min ischemia (24.3%) but the activity remained same as that of control in 1hour and 24 hour reperfusion samples.

Cerebellum: Catalase activity increased in all the three groups (74.7%, 90.4% and 84.2% respectively) when compared to control animals. GR activity decreased (23.1%) after 20min ischemia but there was an increase in its activity after 1hour (116.6%) and 24 hour (24.34%) reperfusion compared to control samples. There was a slight increase in GPx activity in 20min ischemia (0.6%) and significant decrease in 1-hour (42.38%) reperfusion. GST activity significantly increased in all the three groups (249.8%, 469.5% and 848.7% respectively) when compared to control samples.

Hippocampus: Catalase activity decreased in all the three groups (32.2%, 32.7% and 21.7% respectively) when compared to control animals. GR activity decreased in 20 min ischemia (30.1%), 1-hour reperfusion (109.4%) but increased in 24-hour reperfusion (94.68%) samples when compared to control animals. GPx activity increased significantly after 1-hour reperfusion (7002.6%) when compared to sham.

GST activity decreased after 20min ischemia (27.51%) when compared to control animals.

In addition to the oxidative stress we have also studied the cell death events like PARP cleavage, caspase-3 activation and cytochrome c release by western blot analysis. PARP cleavage was more prominent in 1hour reperfusion cerebral cortex, cerebellum and hippocampus when compared to other two treatment samples. Caspase-3 activation, and cytochrome c release was also observed in samples after ischemia reperfusion confirming the activation of apoptotic cascades upon oxidative stress. In these samples, we have studied the DNA damage by Agarose gel electrophoresis, and it was further confirmed by terminal deoxy nick end labeling (TUNEL) technique. Most of the alterations were observed in samples after 1 hour of reperfusion. Hence this time period was fixed for studying the role of antioxidants like Vitamin C, Vitamin E and extract of the herb, *Phyllanthus.* Vitamin C administration increased GSH content and decreased LPO levels in cerebral cortex region when compared to control. *Phyllanthus* and vitamin E treatment did not have much effect when compared to vitamin C. *Phyllanthus* treatment prior to inducing ischemia was more potent in increasing the GSH levels by (90.6%) with no effect on LPO levels when compared to vitamin C and Vitamin E in cerebellum. Vitamin C and Vitamin E increased GSH content at the same time decreased LPO levels in cerebellum. The antioxidant treatments

were less effective in hippocampal tissues when compared to cerebral cortex and cerebellum.

The present study confirmed oxidative stress and cell death in different brain regions after ischemia reperfusion. Antioxidants in addition to the plant extract of *phyllanthus* were proved to be beneficial in reducing oxidative stress in brain upon ischemia reperfusion. The antioxidant properties of *Phyllanthus* have to be explored further in order to be added to the wide group of antioxidants.

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Table 1.1

Levels of Glutathione (GSH and GSSG) and Lipid peroxidation in Cerebral Cortex

Sample	GSH (ng/mg tissue)	GSH (%)	GSSG (ng/mg tissue)	GSSG (%)	MDA (nM/g tissue)	MDA (%)
Sham Operated	451.5 ± 7.6	100	51.1 ± 3.4	100	98.0 ± 2.4	100
0hr R	423.6 ± 31.8	6.18 ↓	63.3 ± 6.29	23.87 ↑	100.0 ± 9.0	2.04 ↑
1hr R.	423.8 ± 70.4	6.12 ↓	64.4 ±1.85	26.02 ↑	154.4 ± 3.9	57.55 ↑
24hr R	469.3 ± 38.3	4.03 ↑	68.5 ± 0.45	34.04 ↑	102.0 ± 5.17	4.08 ↑

Data is expressed as Mean ± SEM (n=5). Significance of the data was evaluated by ANOVA.

Table 1.2

Levels of Glutathione (GSH and GSSG) and Lipid peroxidation in Cerebellum

Sample	GSH (ng/mg tissue)	GSH (%)	GSSG (ng/mg tissue)	GSSG (%)	MDA (nM/g tissue)	MDA (%)
Sham Operated	307.8 ± 9.9	100.0	63.4 ± 3.1	100.0	130.1 ± 23.29	100.0
0hr R	339.7 ± 5.8	10.3 ↑	53.5 ± 0.28	15.7↓	73.5 ±15.6	43.6↓
1hr R.	289.0 ± 54.3	6.4 ↓	71.28 ± 0.28	12.4↑	221.1 ± 4.3	69.9↑
24hr R	423.0 ± 4.21	37.4 ↑	73.03 ± 0.29	15.1↑	220.4 ± 0.57	69.4↑

Data is expressed as Mean ± SEM (n=5). Significance of the data was evaluated by ANOVA.

Table 1.3

Levels of Glutathione (GSH and GSSG) and Lipid peroxidation in Hippocampus.

Sample	GSH (ng/mg tissue)	GSH (%)	GSSG (ng/mg tissue)	GSSG (%)	MDA (nM/g tissue)	MDA (%)
Sham Operated	409.6 ± 10.74	100.0	59.3 ± 2.07	100.0	0.54 ± 0.123	100.0
0hr R	282.4 ± 2.73	31.1↓	50.8 ± 1.65	14.4↓	6.453 ± 1.57	1085↑
1hr R.	280.8 ± 17.5	31.5↓	52.9 ±2.73	10.8↓	0.711 ± 0.118	88.4↑
24hr R	389.5 ± 2.3	5.0↓	59.5 ± 4.69	0.3↑	0.839 ± 0.09	55.3↑

Data is expressed as Mean ± SEM (n=5). Significance of the data was evaluated by ANOVA.

Table 2.1

Antioxidant enzyme activities in Cerebral cortex.

Sample	Catalase *U/mg	Catalase %change	GSH Reductase **U/mg	GSH Reductase %change	GSH Peroxidase ***U/mg	GSH Peroxidase %change	GSH s-Transferase ****U/mg	GSH s-Transferase %change
Sham Operated	4.900 ± 0.3464	0	0.04333 ± 0.002603	100	0.14533 ± 0.002603	0	0.3367 ± 0.00726	0
0hr R	7.900 ± 0.5196	61↑	0.03733 ± 0.002028	13.8 ↓	0.118 ± 0.021385	18.9↓	0.2550 ± 0.00520	24.3 ↓
1Hr R.	4.233 ± 0.1453	13.6↓	0.03600 ± 0.01732	17 ↓	0.04467 ± 0.004842	207.3 ↓	0.3693 ± 0.02742	9.6 ↑
24Hr R	6.433 ± 1.0105	31.28↑	0.04433 ± 0.003180	2.3 ↑	0.22700 ± 0.004041	56.1↑	0.3393 ± 0.00088	0.7 ↑

Data is expressed as Mean ± SEM (n=5). Significance of the data was evaluated by ANOVA.

*1 unit of enzyme is defined as 1 Micro mole of H_2O_2 metabolized per min per mg protein
** 1 unit of enzyme is defined as 1 Micro moles of NADPH oxidized per min per mg protein
*** 1 unit of enzyme is defined as 1 nanomole of NADPH oxidized per minute per mg protein
****1 unit of enzyme is defined as 1 Micro mole of CDNB consumed per min per mg protein

Table 2.2

Antioxidant enzyme activities in Cerebellum.

Sample	Catalase *U/mg	Catalase %change	GSH Reductase **U/mg	GSH Reductase %change	GSH Peroxidase ***U/mg	GSH Peroxidase %change	GSH s-Transferase ****U/mg	GSH s-Transferase %change
Sham Operated	3.833 ± 0.3756	0	0.2600 ± 0.000577	0	0.21867 ± 0.012719	0	0.3160 ± 0.00289	0
0hr R	6.700 ± 0.1528	74.7↑	0.2000 ± 0.001732	23.1 ↓	0.22000 ± 0.021939	0.6↑	0.2323 ± 0.01011	249.8↓
1hr R.	7.300 ± 1.0970	90.4↑	0.5633 ± 0.000333	116.6↑	0.12600 ± 0.015011	42.38↓	0.3823 ± 0.01646	469.5↑
24hr R	7.000 ± 0.0000	84.2↑	0.3233 ± 0.004178	24.34↑	0.30200 ± 0.007506	38.1↑	0.2843 ± 0.02742	848.7↑

Data is expressed as Mean ± SEM (n=5). Significance of the data was evaluated by ANOVA.

*1 unit of enzyme is defined as 1 Micro mole of H_2O_2 metabolized per min per mg protein
** 1 unit of enzyme is defined as 1 Micro moles of NADPH oxidized per min per mg protein
***1 unit of enzyme is defined as 1 nanomole of NADPH oxidized per minute per mg protein
****1 unit of enzyme is defined as 1 Micro mole of CDNB consumed per min per mg protein

Table 2.3

Antioxidant enzyme activities in Hippocampus.

Sample	Catalase *U/mg	Catalase %change	GSH Reductase **U/mg	GSH Reductase %change	GSH Peroxidase ***U/mg	GSH Peroxidase %change	GSH s-Transferase ****U/mg	GSH s-Transferase %change
Sham Operated	15.197 ± 0.4694	0	0.00433 ± 0.001453	0	0.01033 ± 0.000333	0	0.1963 ± 0.02058	0
0hr R	10.317 ± 0.6146	32.2↓	0.00303 ± 0.001639	30.1 ↓	0.01000 ± 0.000577	3.2↓	0.1423 ± 0.00260	27.51↓
1hr R.	10.237 ± 0.6918	32.7↓	0.00907 ± 0.001467	109.4 ↑	0.7337 ± 0.036633	7002.6↑	0.2163 ± 0.02050	10.18 ↑
24hr R	11.913 ± 2.7182	21.7↓	0.00843 ± 0.001598	94.68 ↑	0.01300 ± 0.000577	25.8 ↑	0.2013 ± 0.00924	2.5 ↑

Data is expressed as Mean ± SEM (n=5). Significance of the data was evaluated by ANOVA.

*1 unit of enzyme is defined as 1 Micro mole of H_2O_2 metabolized per min per mg protein
** 1 unit of enzyme is defined as 1 Micro moles of NADPH oxidized per min per mg protein
***1 unit of enzyme is defined as 1 nanomole of NADPH oxidized per minute per mg protein
****1 unit of enzyme is defined as 1 Micro mole of CDNB consumed per min per mg protein

Table 4.2

Antioxidant treatment: Levels of Glutathione (GSH and GSSG) and Lipid peroxidation in Cerebral Cortex

Sample	GSH (ng/mg)	GSH (%) change	GSH (%) change	GSSG (ng/mg)	GSSG (%) change	GSSG (%) change	MDA (nM/g)	MDA (%) change	MDA (%) change
Control	451.533 ± 7.6	0		51.100 ± 1.96	0		98.0 ± 1.38	0	
1hr R	423.8 ± 1.189	6.2 ↓	0	64.433 ± 30.5	26.0 ↑	0	100.05 ± 0.3	2 ↑	0
1hr R *Phyllanthus*	440.7± 1.586	2.4 ↓	3.9 ↑	65.36 ± 40.5	27.7 ↑	1.49 ↑	162.70 ± 1.70	65.3 ↑	62.7 ↑
1hr R *Vitamin C*	1594.0 ± 0.4236	253 ↑	276 ↑	75.17 ± 3.619	47.1 ↑	16.7 ↑	61.39 ± 0.68	37.4 ↓	61.39 ↓
1hr R Vitamin E	919.5 ± 1.3847	103.6 ↑	116 ↑	60.97 ± 11.820	19.3 ↑	5.3 ↓	52.79 ± 0.827	46.2 ↓	52.79 ↓

Data is expressed as Mean ± SEM (n=5). Significance of the data was evaluated by ANOVA. R=Reperfusion

Column 4, 7 and 10 represent the percentage changes compared to the 1hr reperfusion samples.

Table 4.3

Antioxidant treatment: Levels of Glutathione (GSH and GSSG) and Lipid peroxidation in Cerebellum.

Sample	GSH (ng/mg)	GSH (%) change	GSH (%) change	GSSG (ng/mg)	GSSG (%) change	GSSG (%) change	MDA (nM/g)	MDA (%) change	MDA (%) change
Control	307.833 ± 9.97	0		63.4 ± 3.1005	0		130.150 ± 23.2	0	
1hr R	289.0 ± 3.744	6.12 ↓	0	71.283 ± 43.250	12.4 ↑	0	221.150 ± 17.05	69.9 ↑	0
1hr R *Phyllanthus*	551.4± 9.5636	79.1 ↑	90.6↑	106.68 ± 110.55	68.2 ↑	49.7↑	135.30 ± 4.337	3.9 ↑	38.7 ↓
1hr R *Vitamin C*	404.0 ± 3.6324	31.23 ↑	39.7↑	132.18 ± 41.98	108.2↑	85.5↑	183.0 ± 4.466	40.6 ↓	17.1 ↓
1hr R Vitamin E	341.0 ± 2.6909	10.77 ↑	17.7↑	101.96 ± 31.14	60.82↑	43.1↑	109.8 ± 8.05	15.7 ↓	50.3 ↓

Data is expressed as Mean ± SEM (n=5). Significance of the data was evaluated by ANOVA. R=Reperfusion

Column 4, 7 and 10 represent the percentage changes compared to the 1hr reperfusion samples.

Table 4.4

Antioxidant treatment: Levels of Glutathione (GSH and GSSG) and Lipid peroxidation in Hippocampus.

Sample	GSH (ng/mg)	GSH (%) change	GSH (%) change	GSSG (ng/mg)	GSSG (%) change	GSSG (%) change	MDA (nM/g)	MDA (%) change	MDA (%) change
Control	409.633 ± 107416	0		59.3 ± 2.0744	0		0.547 ± 0.1235	0	
1hr R	280.8 ± 0.6839	31.46 ↓	0	52.927 ± 7.5160	10.75 ↓	0	0.711 ± 2.14	29.9 ↑	0
1hr R *Phyllanthus*	210.5± 0.3636	48.62 ↓	25.0 ↓	55.698 ± 3.9405	6.10 ↓	5.23 ↑	0.5730 ± 0.537	4.7 ↑	19.4 ↓
1hr R *Vitamin C*	110.79± 0.5041	72.96 ↓	60.5 ↓	29.27 ± 5.533	50.65 ↓	44.6 ↓	0.865 ± 0.286	58.1 ↓	21.6 ↑
1hr R Vitamin E	68.70 ± 0.4047	83.23 ↓	75.5 ↓	53.75 ± 4.444	9.362 ↓	1.55 ↑	0.472 ± 0.00	13.8 ↓	33.6 ↓

Data is expressed as Mean ± SEM (n=5). Significance of the data was evaluated by ANOVA. R=Reperfusion

Column 4, 7 and 10 represent the percentage changes compared to the 1hr reperfusion samples

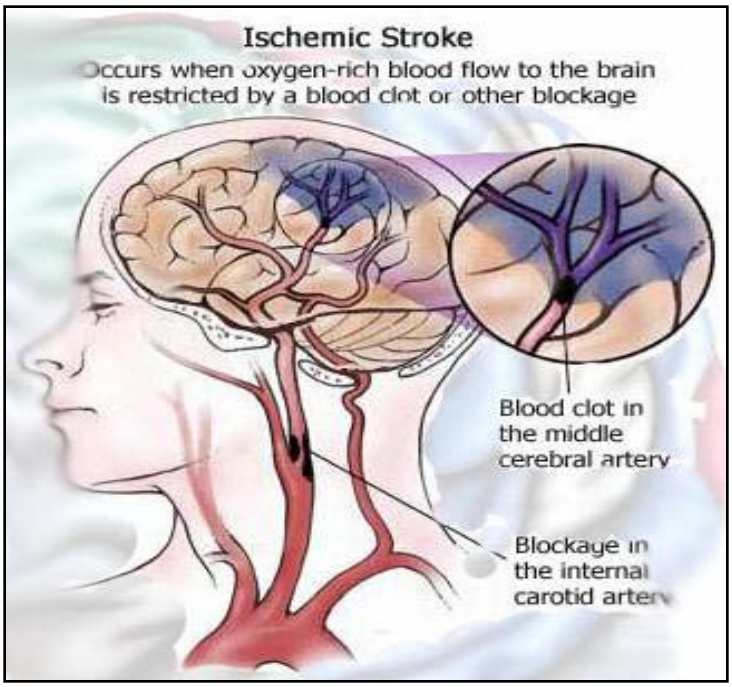
Table 4.1 Percent inhibition of lipid peroxidation by aqueous extract of Phyllanthus

Concentration of the extract μg of the total reaction mixture.	Lipid peroxidation nM/g tissue	Percent Inhibition of lipid peroxidation. %
0	360	0
5	255	29.1
10	248	31.1
15	245	31.9
20	242	235
25	235	34.7
30	221	38.6
35	216	40
40	212	40
45	232	35.6
50	256	28.8
55	256	28.8
60	268	25.5
65	268	25.5
80	300	16.6
100	353	1.9

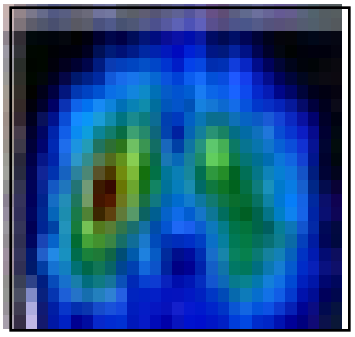
Inhibitory effects of *Phyllanthus fraternus* extract on lipidperoxidation. Rat liver homogenate was stimulated with Fe^{2+}/ascorbate as described under Materials and Methods. Values are expressed as percentage inhibition of peroxidation, values of 0% being the extent of peroxidation in the presence of extract vehicle only.

Fig In 1 : The developing infarct

a



b



c

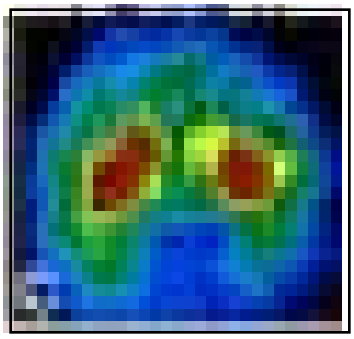
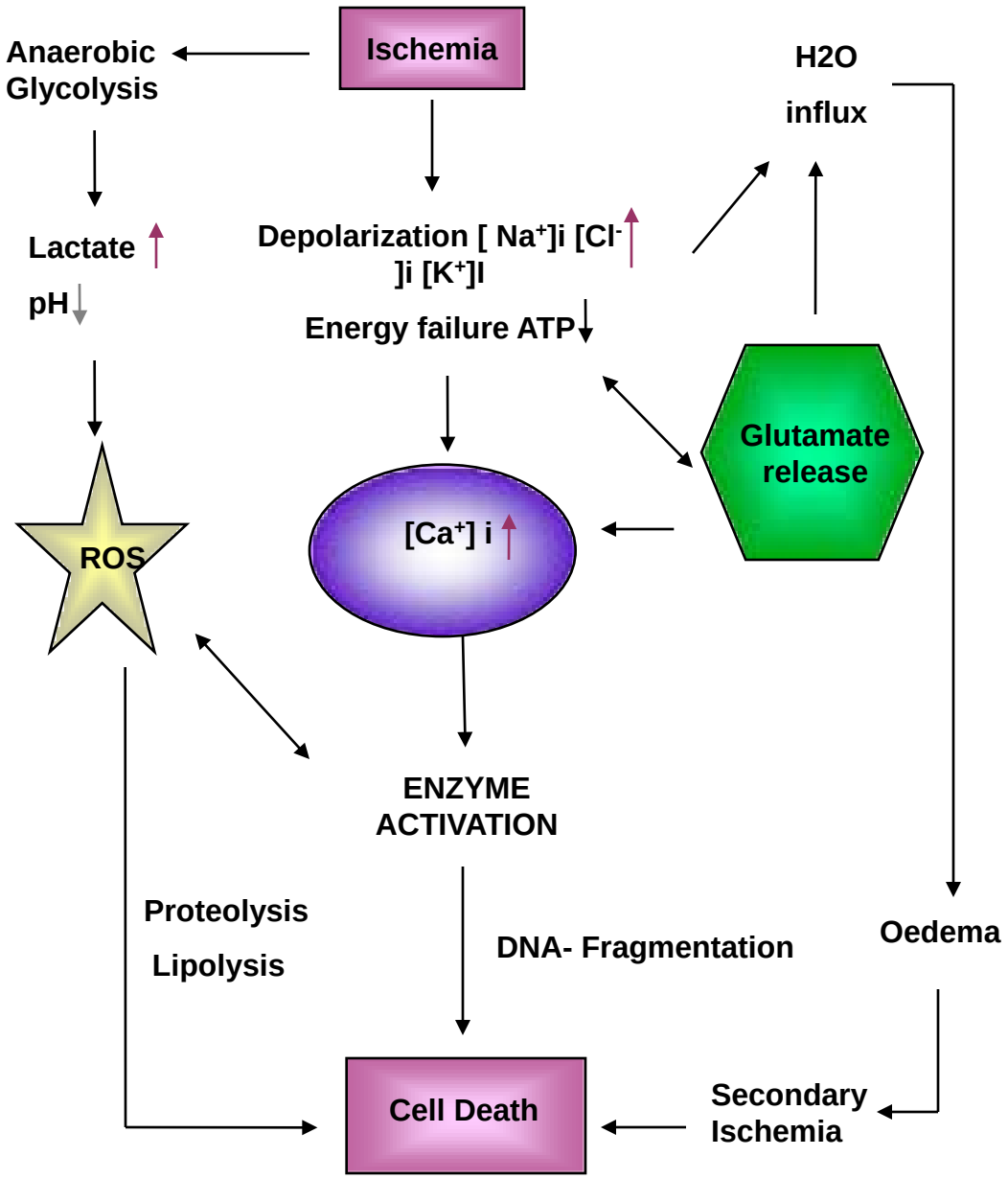


Fig In 1a represents the ischemic stroke.

b and c - A brain section depicting the increase in infarct size with increase in time.

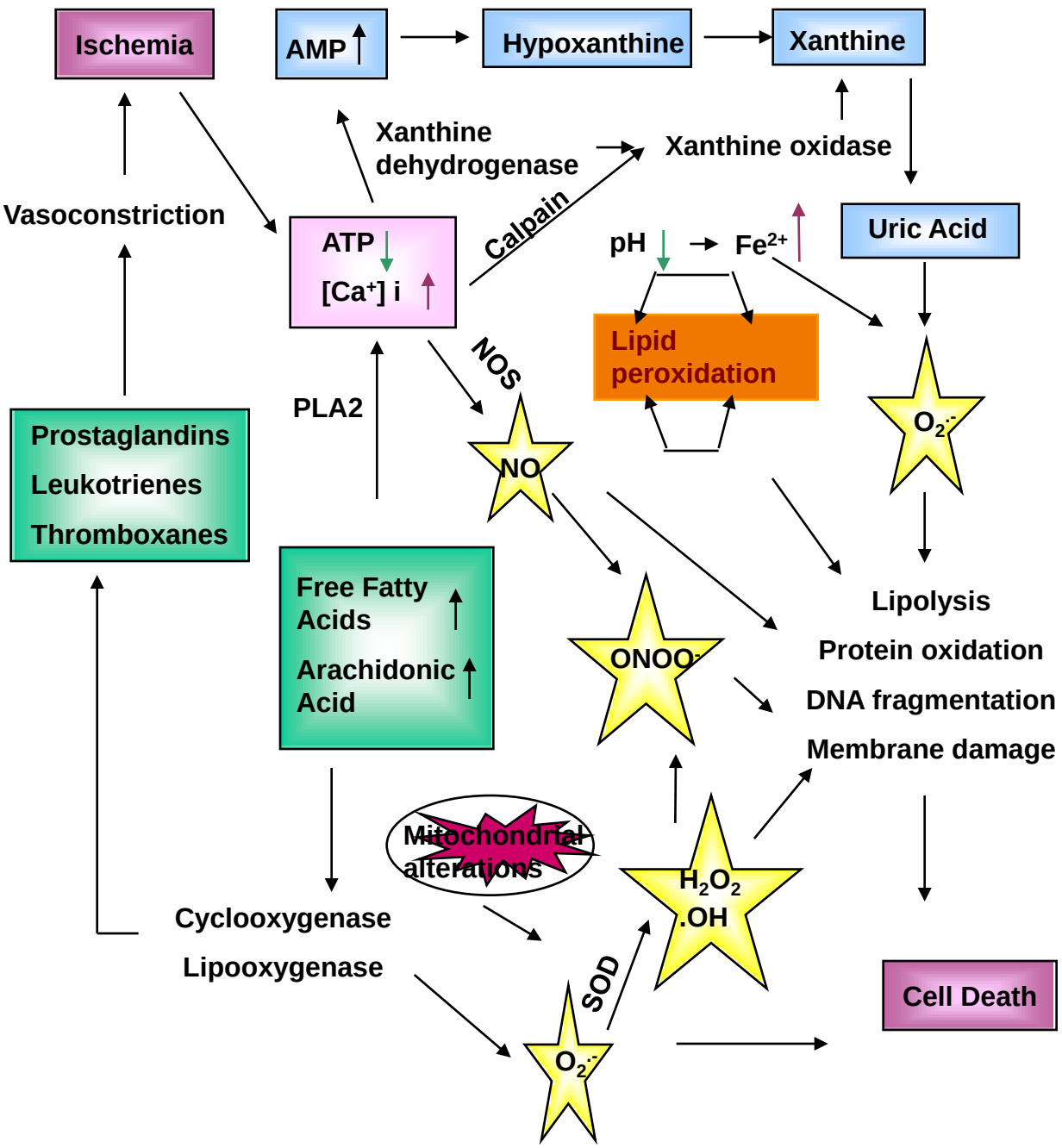
Core
Penumbra

Fig In 2 : Molecular mechanisms of ischemia



Pathophysiological processes leading to cell death in cerebral ischemia

Fig In 3 : Cerebral ischemia : Cell Death



Mechanisms contributing to the formation of free radicals in cerebral ischemia

Fig In 4 : Animal Models

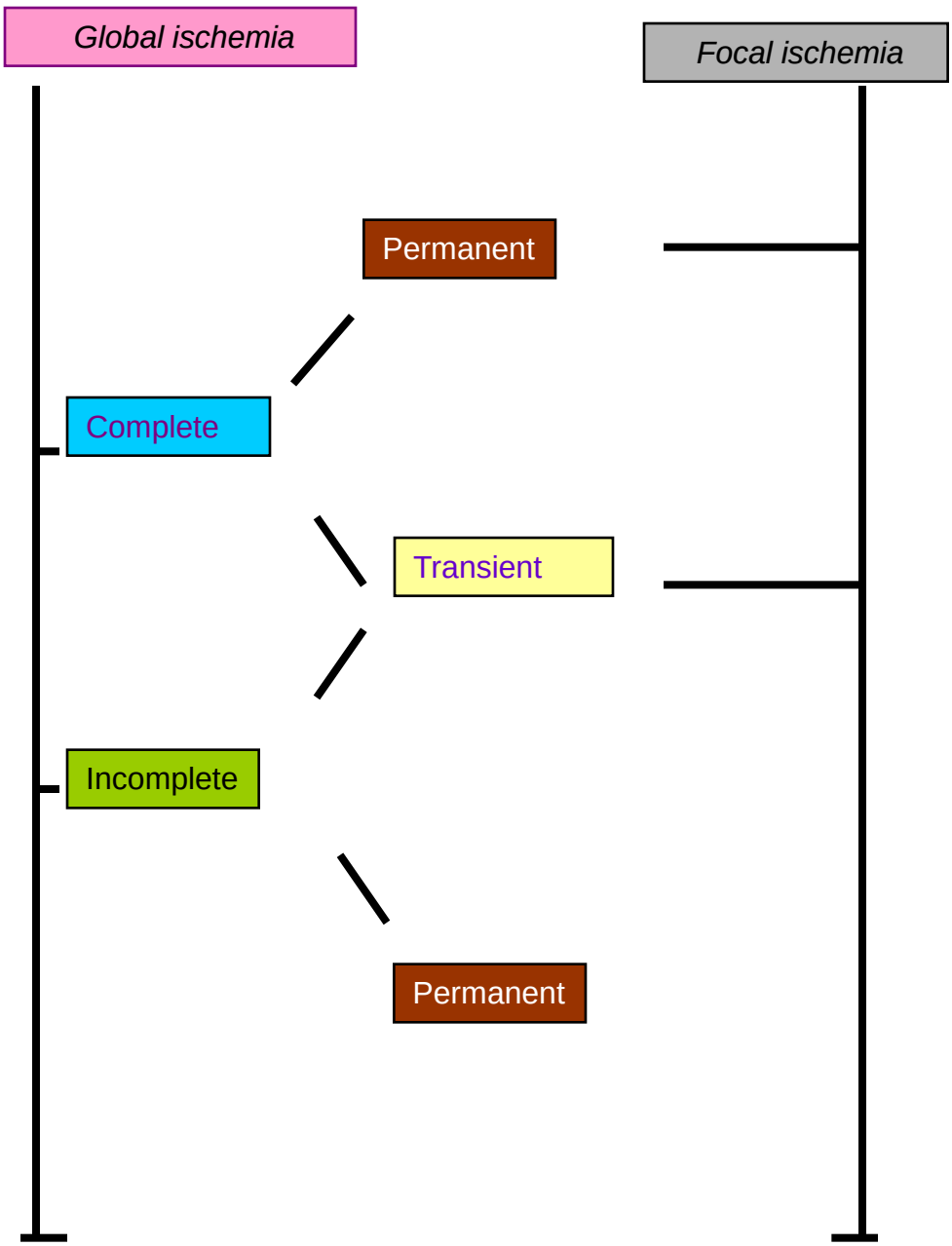


Fig M1 Rat anatomy showing the exposed carotid arteries.

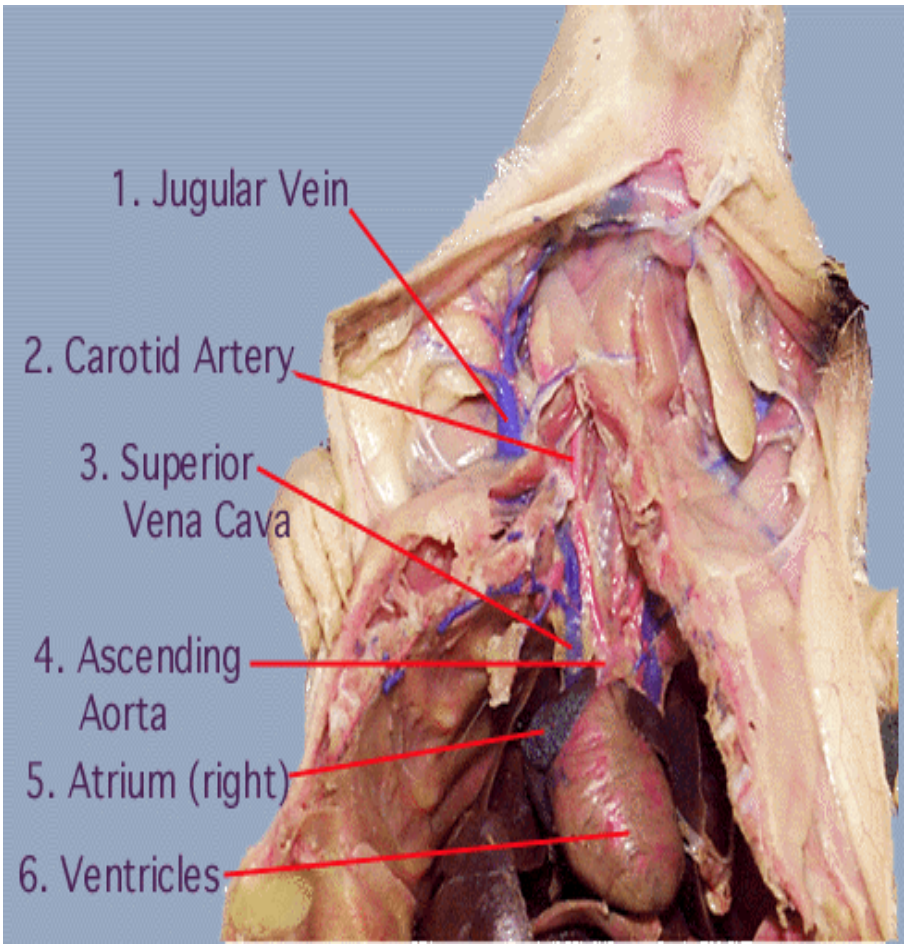
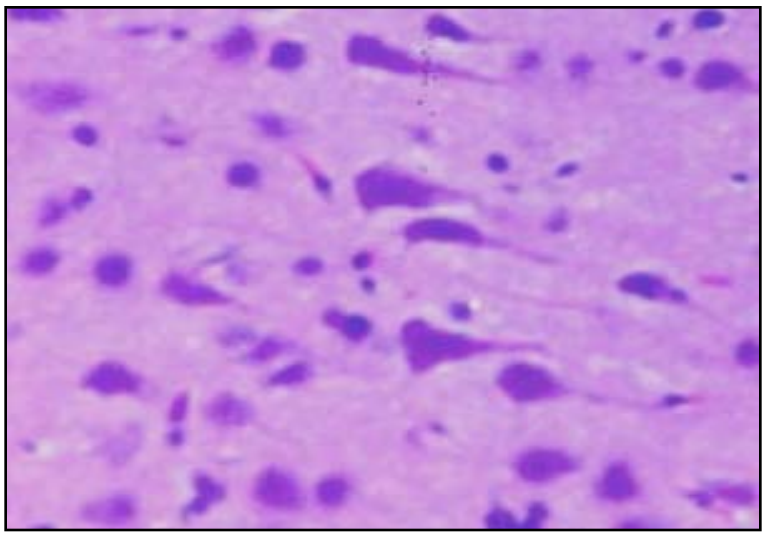
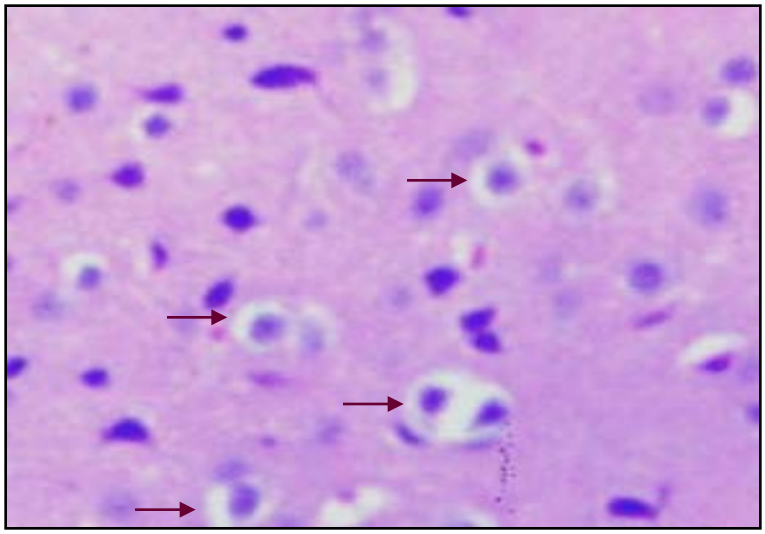


Fig 1.1 Histopathological sections of control and ischemic cerebral cortex.



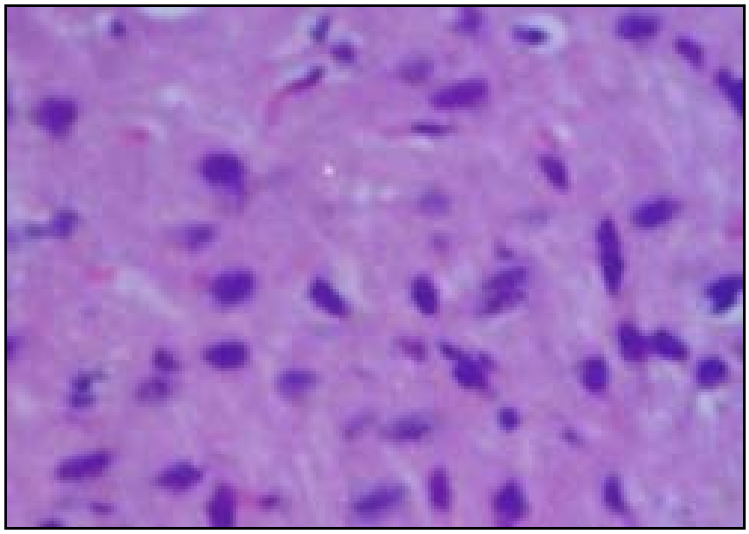
a) Control Cerebral cortex



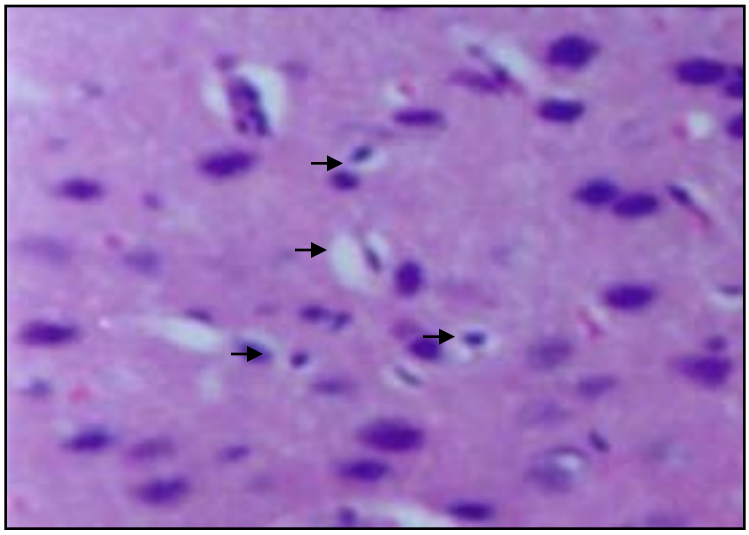
b) Ischemic Cerebral cortex

Control section shows uniformly distributed cells in the normal neuropile. Cells are of normal size with clear neuronal projections. Ischemic section shows cells with reduced size in the vacuolated neuropile. x400

Fig 1.2 Histopathological sections of control and ischemic cerebellum.



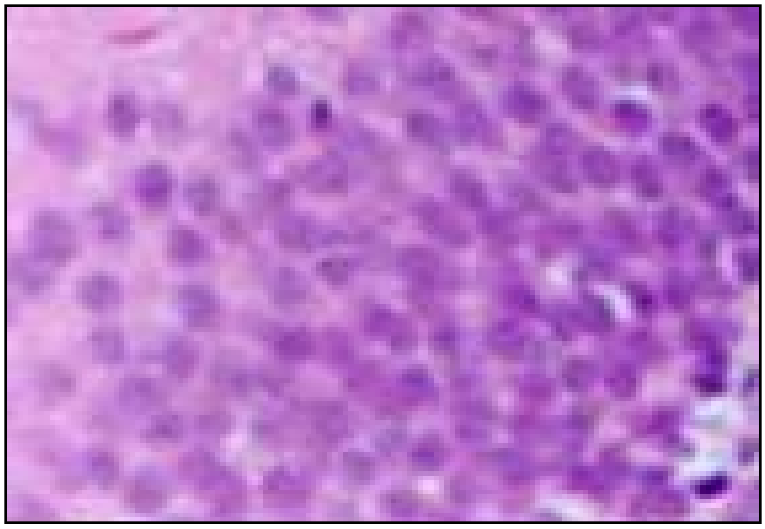
a) Control Cerebellum



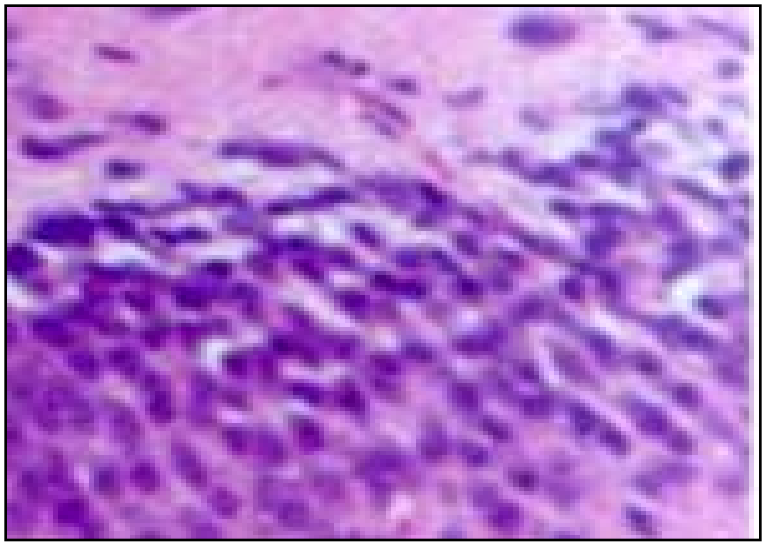
b) Ischemic Cerebellum

Control section shows uniformly distributed cells in the normal neuropile. Cells are of normal size. Ischemic section shows cells with reduced size in the vacuolated neuropile. x400

Fig 1.3 Histopathological sections of control and ischemic hippocampus



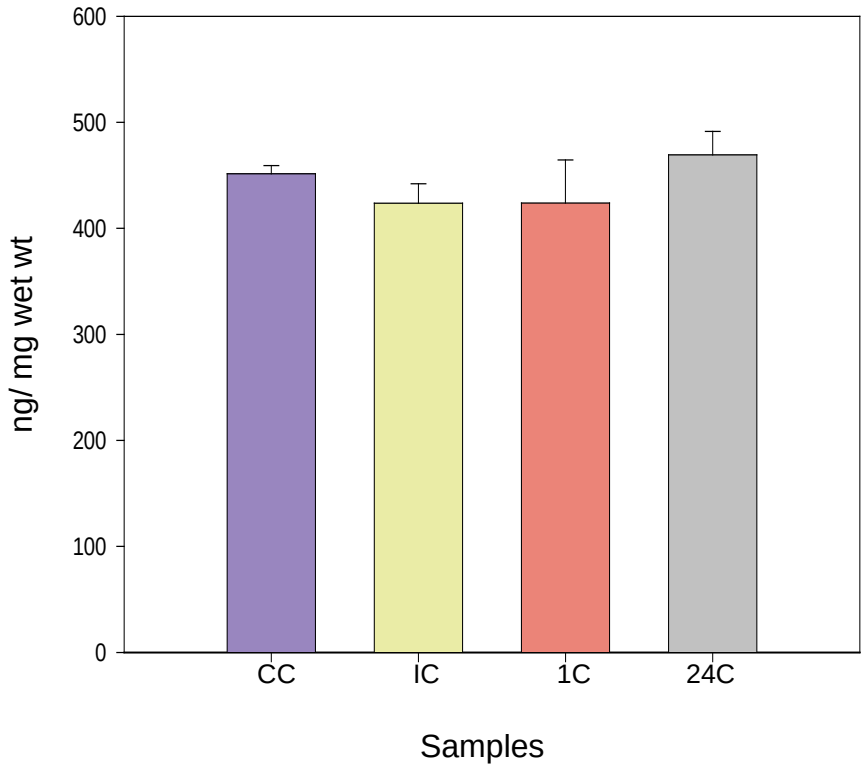
a) Control Hippocampus



b) Ischemic Hippocampus

Control section shows uniformly distributed cells in the normal neuropile. Cells are of normal size. Ischemic section shows cells with reduced size in the vacuolated neuropile. x400

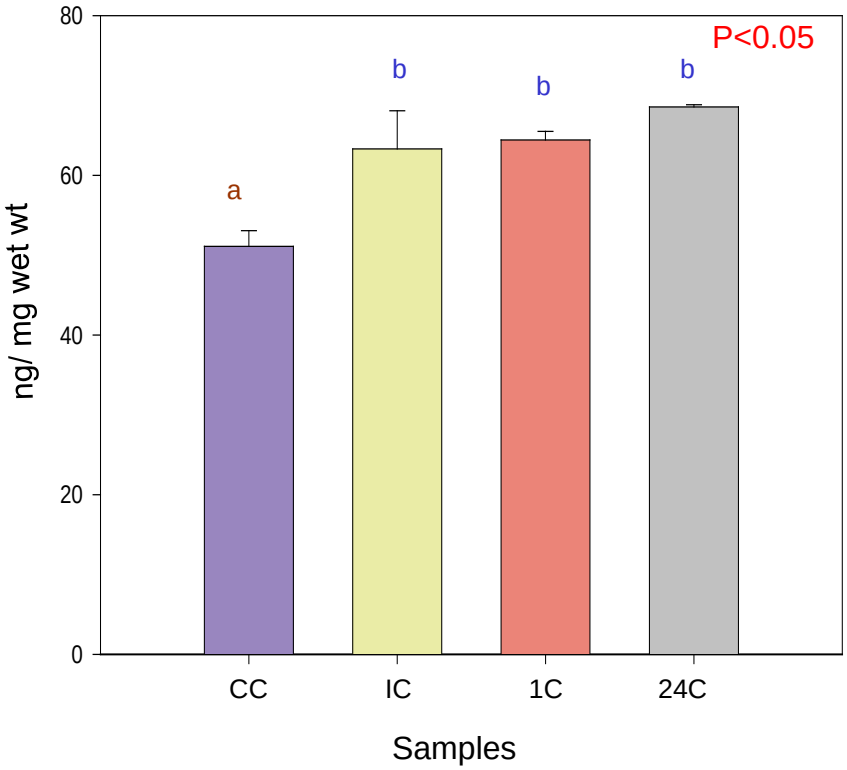
Fig 1.6a Levels of Reduced Glutathione (GSH) in Control and Ischemic Cerebral Cortex



Reduced glutathione levels decreased in cerebral cortex after ischemia reperfusion but it was not significant. GSH levels were estimated in cerebral cortex after ischemia and varying periods of reperfusion using the method of Hissin and Hilf. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and Post hoc multiple comparisons with significance level set at $P<0.05$.

- CC - Control (Sham operated) cerebral cortex.
- IC - Ischemic (20 min occlusion no reperfusion) cerebral cortex.
- 1C -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) cerebral cortex.
- 24C -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) cerebral cortex.

Fig 1.6b Levels of Oxidized Glutathione (GSSG) in Control and Ischemic Cerebral Cortex



Oxidized glutathione level was increased significantly after ischemia reperfusion in cerebral cortex. GSSG levels were estimated in cerebral cortex after ischemia and varying periods of reperfusion by the method of Hissin and Hilf. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with significance set at P< 0.05. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

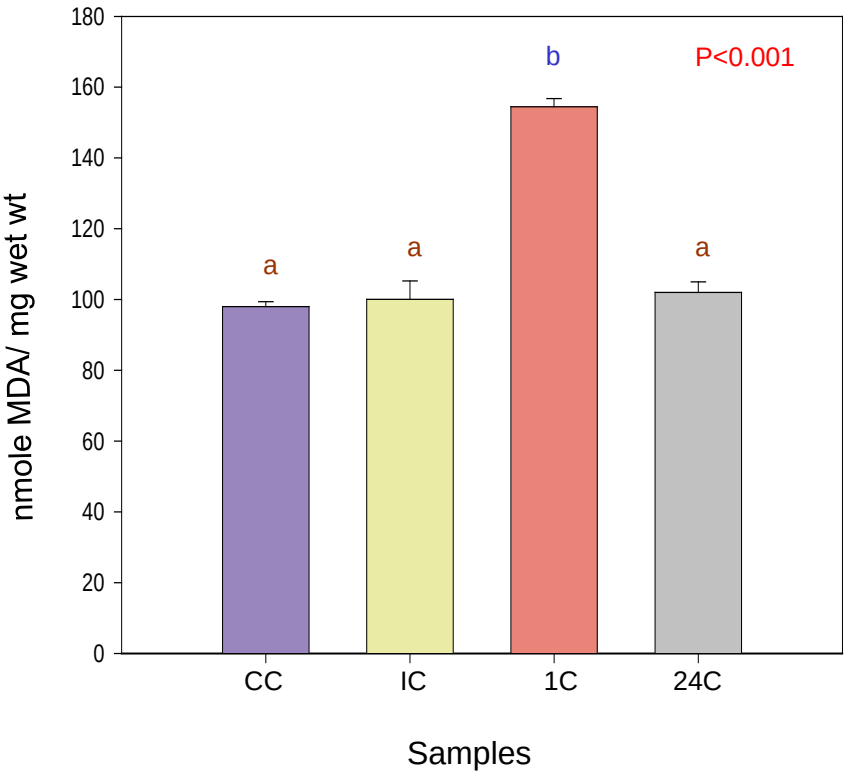
CC - Control (Sham operated) cerebral cortex.

IC - Ischemic (20 min occlusion no reperfusion) cerebral cortex.

1C -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) cerebral cortex.

24C -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) cerebral cortex.

Fig 1.6c Lipid peroxide levels in Control and Ischemic Cerebral Cortex



A significant increase in lipid peroxidation was observed after ischemia reperfusion. Lipid peroxide levels were estimated in cerebral cortex after ischemia and varying periods of reperfusion. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with significance level set at P<0.001. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

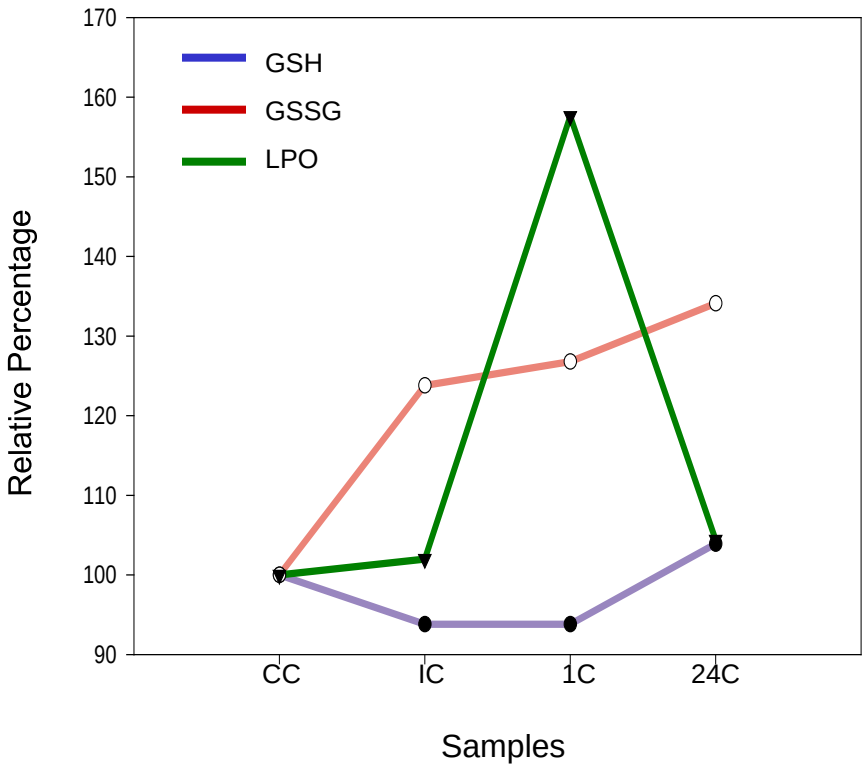
CC - Control (Sham operated) cerebral cortex.

IC - Ischemic (20 min occlusion no reperfusion) cerebral cortex.

1C -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) cerebral cortex.

24C -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) cerebral cortex.

Fig 1.6 Comparative graphs of Glutathione (GSH & GSSG) and Lipid peroxide levels in Cerebral Cortex



A decrease in GSH, an increase in GSSG and increase in lipid peroxidation indicates the induction of oxidative stress in cerebral cortex after ischemia and reperfusion.

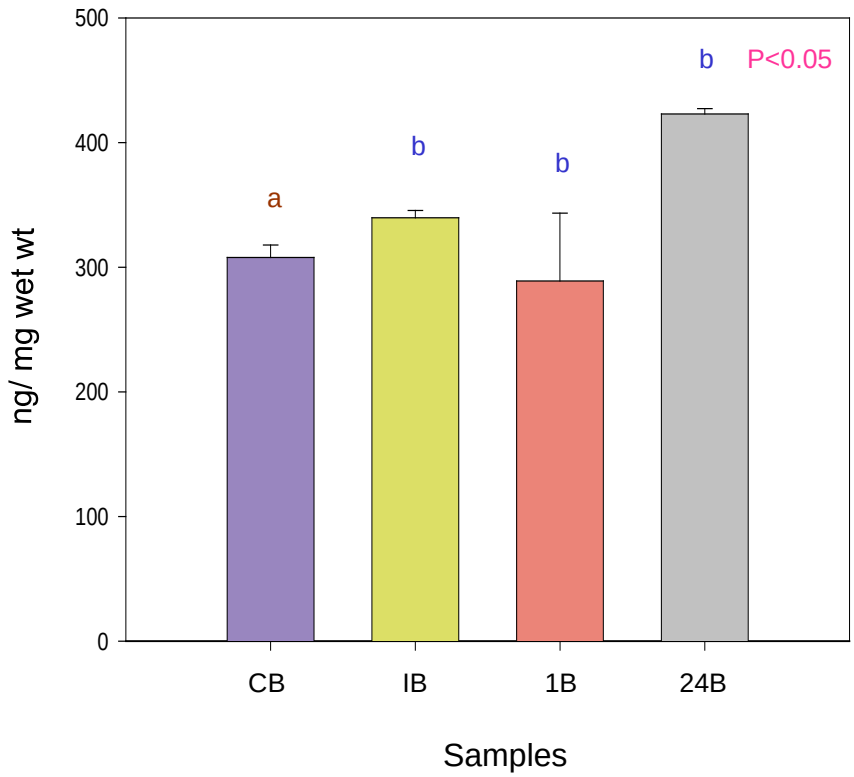
CC - Control (Sham operated) Cerebral cortex.

IC - Ischemic (20 min occlusion no reperfusion) Cerebral cortex.

1C -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) Cerebral cortex.

24C -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) Cerebral cortex.

Fig 1.7a Levels of Reduced Glutathione (GSH) in Control and Ischemic Cerebellum



Reduced glutathione level increased significantly after ischemia reperfusion in cerebellum. GSH levels were estimated in cerebellum after ischemia and varying periods of reperfusion. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with significance level set at P<0.05. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

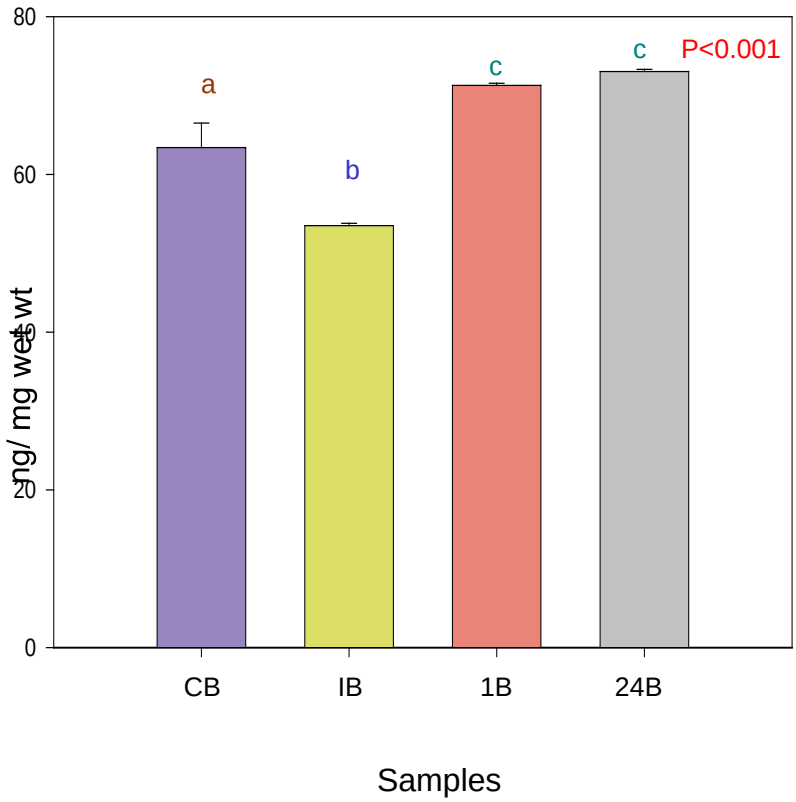
CB - Control (Sham operated) cerebellum.

IB - Ischemic (20 min occlusion no reperfusion) cerebellum.

1B -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) cerebellum.

24B -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) cerebellum.

Fig 1.7b Levels of Oxidized Glutathione (GSSG) in Control and Ischemic Cerebellum



There was a significant increase in GSSG after ischemia reperfusion in cerebellum. Oxidized Glutathione levels were estimated in cerebellum after ischemia and varying periods of reperfusion by the method of Hissin and Hilf. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with significance level set at P<0.001. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

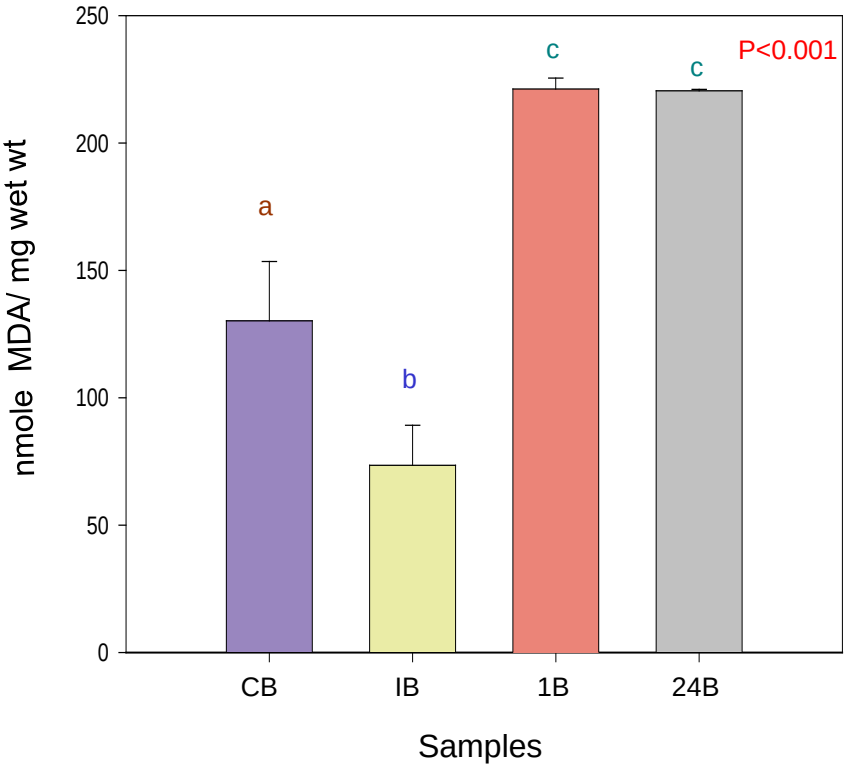
CB - Control (Sham operated) cerebellum.

IB - Ischemic (20 min occlusion no reperfusion) cerebellum.

1B -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) cerebellum.

24B -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) cerebellum.

Fig 1.7c Lipid peroxide levels in Control and Ischemic Cerebellum



There was a significant change in lipid peroxidation after ischemia reperfusion in cerebellum. Lipid peroxide levels were estimated in cerebellum after ischemia and varying periods of reperfusion as described in methodology. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with significance level set at P<0.001. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

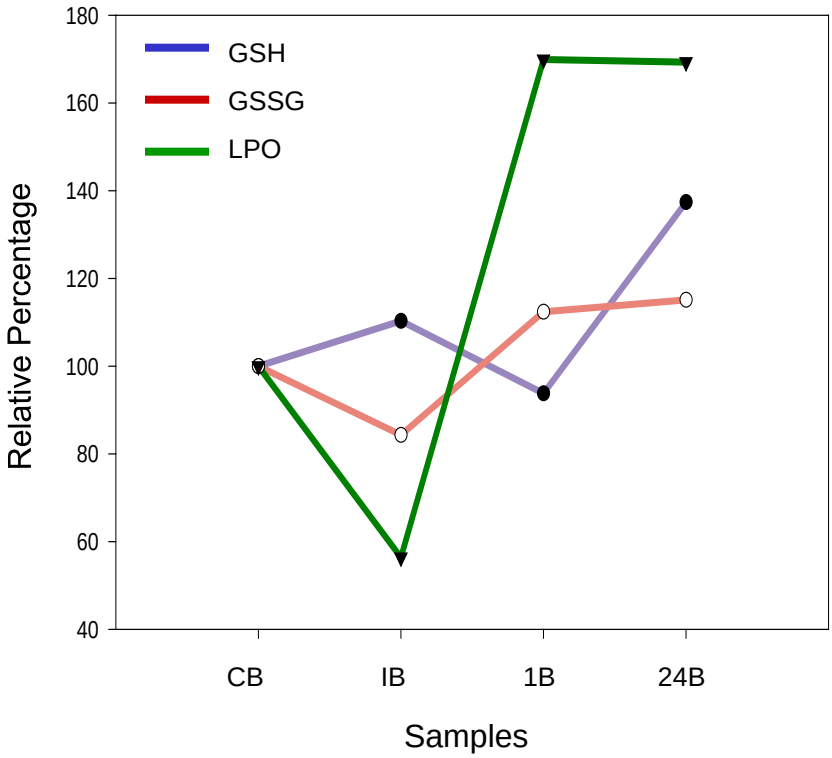
CB - Control (Sham operated) cerebellum.

IB - Ischemic (20 min occlusion no reperfusion) cerebellum.

1B -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) cerebellum.

24B -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) cerebellum.

Fig 1.7 Comparative graph of Glutathione (GSH & GSSG) and Lipid peroxide levels in Cerebellum



An increase in GSSG and lipid peroxidation indicates the induction of oxidative stress in cerebellum after ischemia and reperfusion

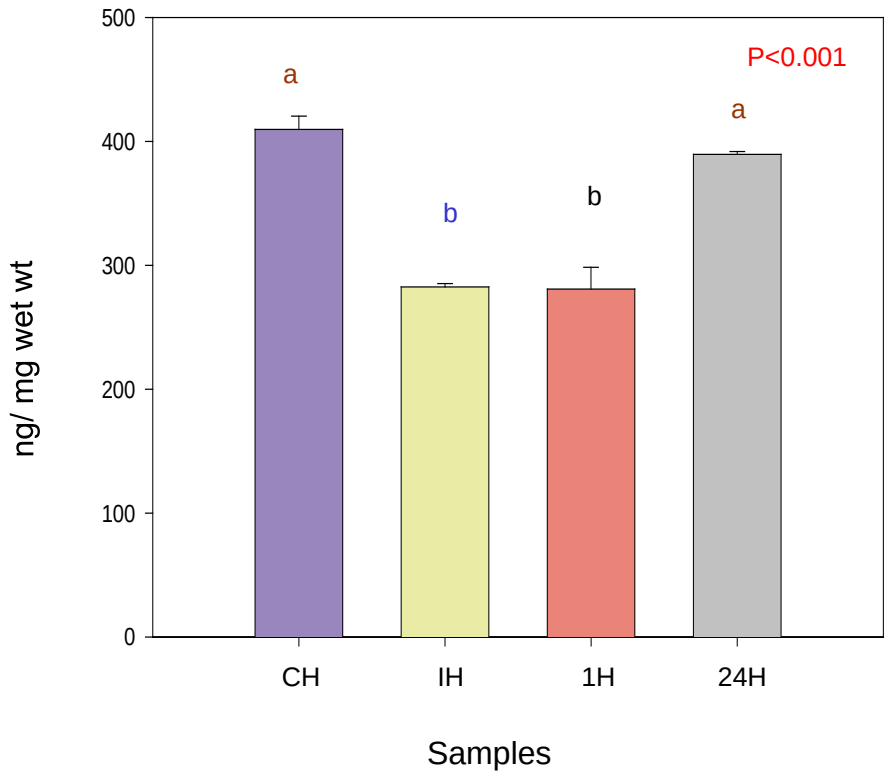
CB - Control (Sham operated) cerebellum.

IB - Ischemic (20 min occlusion no reperfusion) cerebellum.

1B -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) cerebellum.

24B -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) cerebellum.

Fig 1.8a Levels of Reduced Glutathione (GSH) in Control and Ischemic Hippocampus



There was a significant decrease in reduced glutathione level after ischemia reperfusion. Reduced Glutathione levels were estimated in Hippocampus after ischemia and varying periods of reperfusion. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with significance level set at P<0.001. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

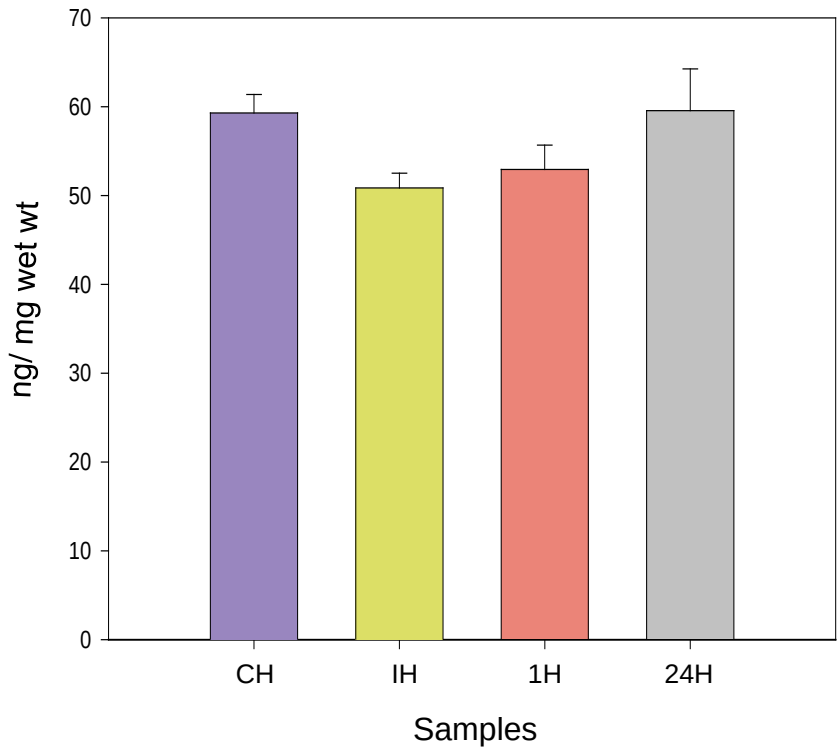
CH - Control (Sham operated) hippocampus.

IH - Ischemic (20 min occlusion no reperfusion) hippocampus .

1H -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) hippocampus.

24H -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) hippocampus.

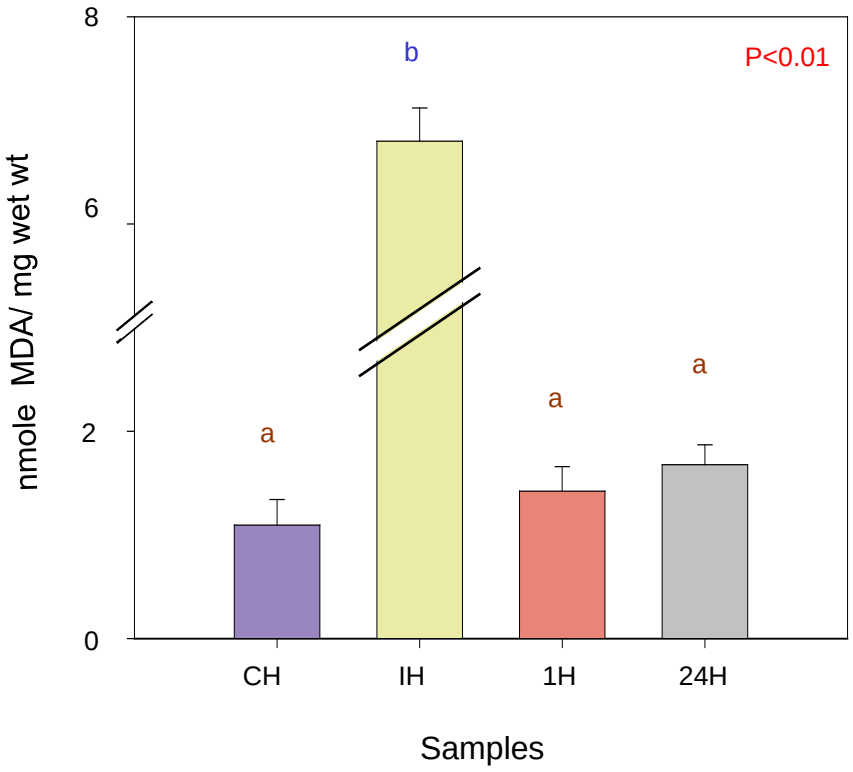
Fig 1.8b Levels of Oxidized Glutathione (GSSG) in Control and Ischemic Hippocampus



There was a decrease in GSSG after ischemia reperfusion but the decrease was not significant. Oxidized glutathione levels were estimated in hippocampus after ischemia and varying periods of reperfusion. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with significance level set at $P<0.05$..

- CH - Control (Sham operated) hippocampus.
- IH - Ischemic (20 min occlusion no reperfusion) hippocampus .
- 1H -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) hippocampus.
- 24H -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) hippocampus.

Fig 1.8c Lipid peroxide levels in Control and Ischemic Hippocampus



There was a significant increase in lipid peroxidation after ischemia reperfusion. Lipid peroxide levels were estimated in hippocampus after ischemia and varying periods of reperfusion. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with significance level set at P<0.01. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

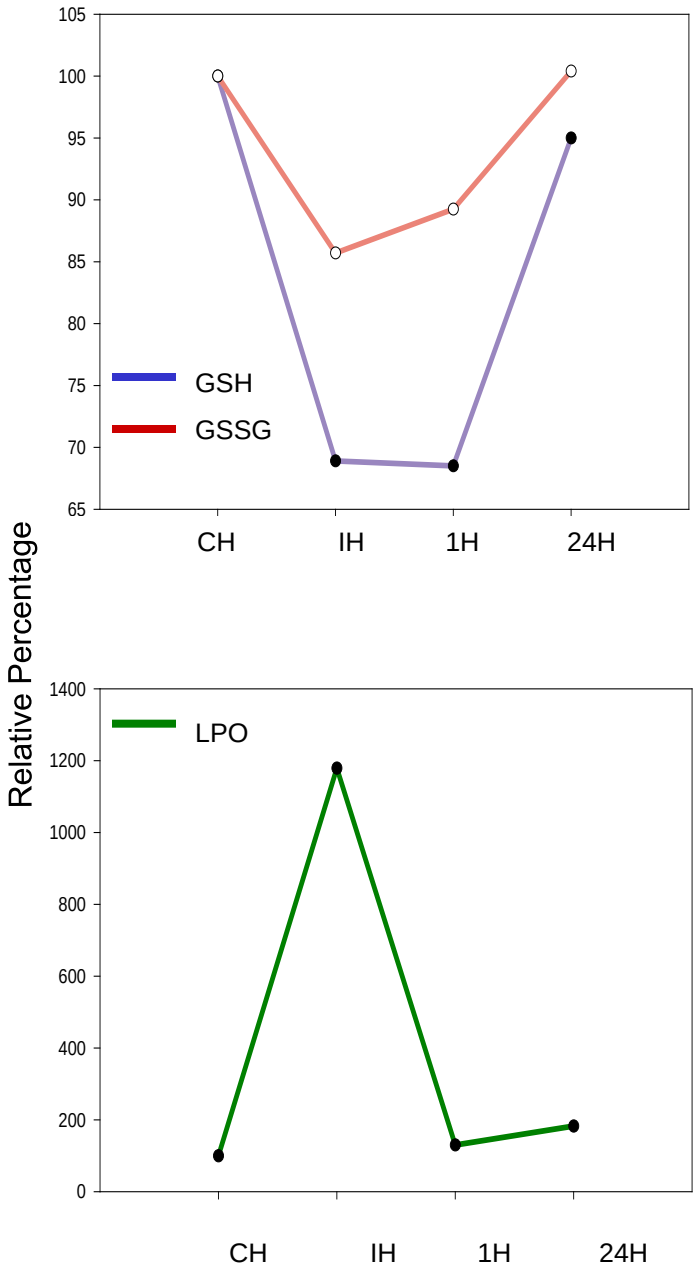
CH - Control (Sham operated) hippocampus.

IH - Ischemic (20 min occlusion no reperfusion) hippocampus .

1H -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) hippocampus.

24H -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) hippocampus.

Fig 1.8 Comparative graphs of Glutathione (GSH & GSSG) and lipid peroxide levels in Hippocampus



A decrease in GSH and Increase in lipid peroxidation indicates the induction of oxidative stress in Hippocampus after ischemia and reperfusion

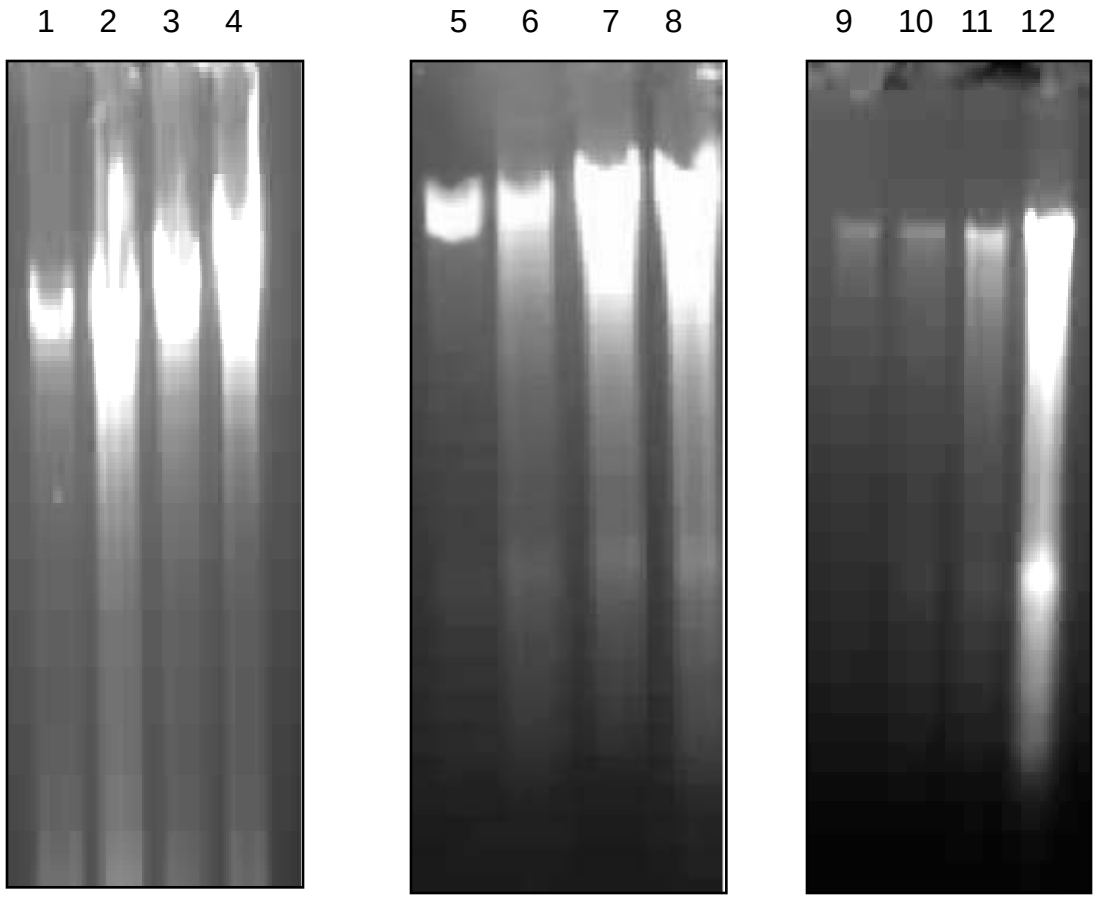
CH - Control (Sham operated) hippocampus.

IH - Ischemic (20 min occlusion no reperfusion) hippocampus .

1H -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) hippocampus.

24H -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) hippocampus.

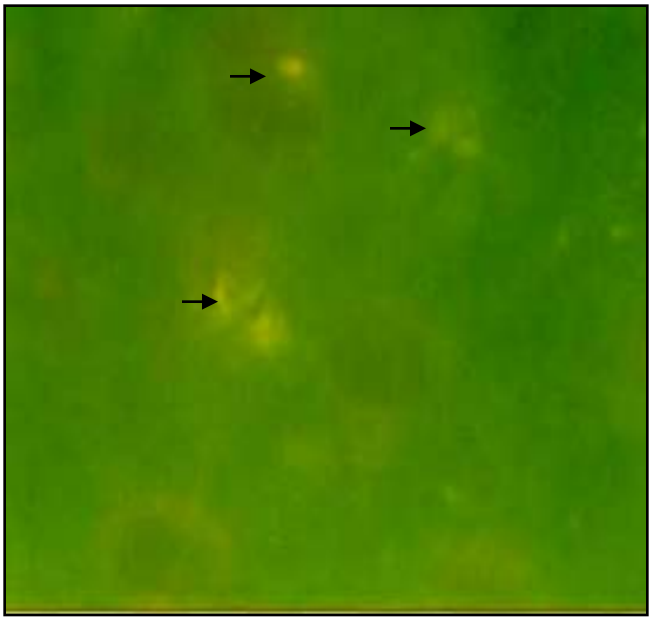
Fig 1.5 Agarose Gel electrophoresis of DNA isolated from cerebral cortex, cerebellum and hippocampus of ischemia and reperfusion.



DNA Agarose gel electrophoresis was done in cerebral cortex, cerebellum and hippocampus in ischemia and varying periods of reperfusion. DNA damage is observed in samples of ischemia when compared to control.

Fig 1.4 TUNEL assay of control and ischemic rat brains

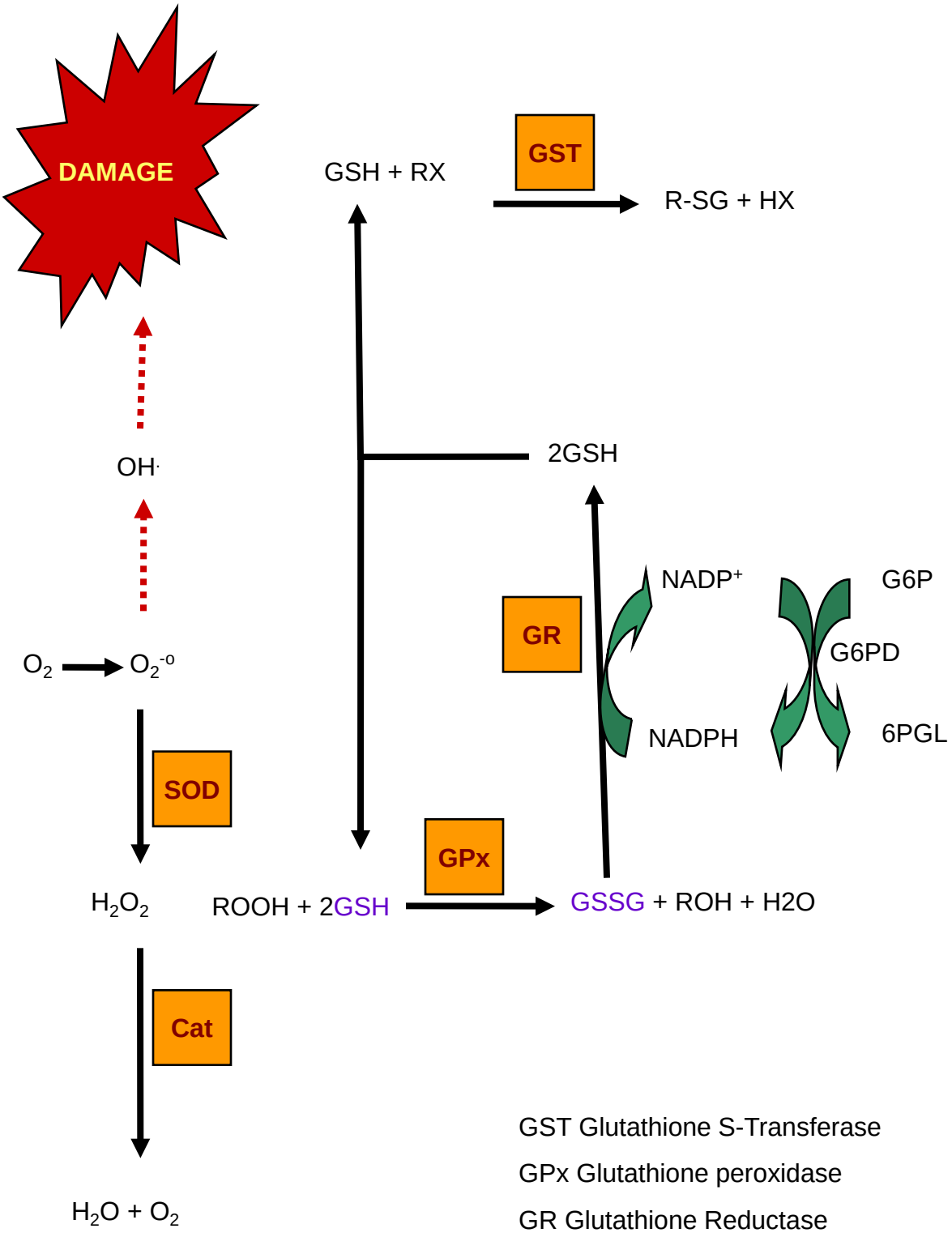
a) Control



b) Ischemic

Insitu DNA nick end labeling was done in control and ischemic brain sections. Cells with DNA Damage fluoresce green.

Fig 2A Antioxidant Defenses



GST Glutathione S-Transferase

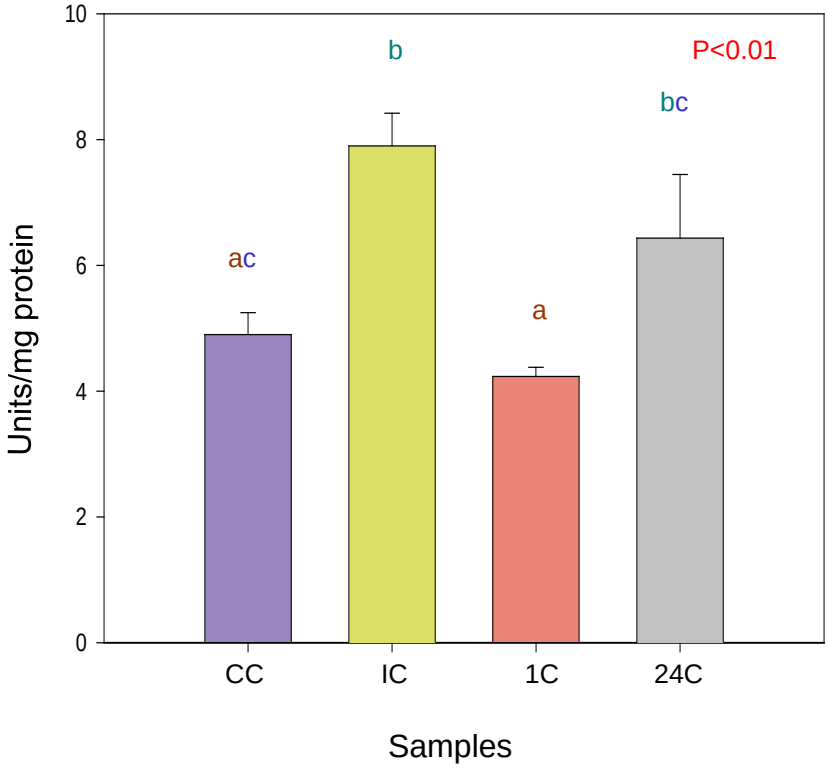
GPx Glutathione peroxidase

GR Glutathione Reductase

SOD Super oxide dismutase

Cat Catalase

Fig 2.1a Catalase activity in Control and Ischemic Cerebral cortex



Catalase activity was increased in cerebral cortex after ischemia when compared to control. Catalase activity was estimated in cerebral cortex after ischemia and varying periods of reperfusion as described in methodology.
1 unit of enzyme is defined as 1 micromole of H_2O_2 metabolized per min per mg protein. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with level of significance set at P<0.01. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

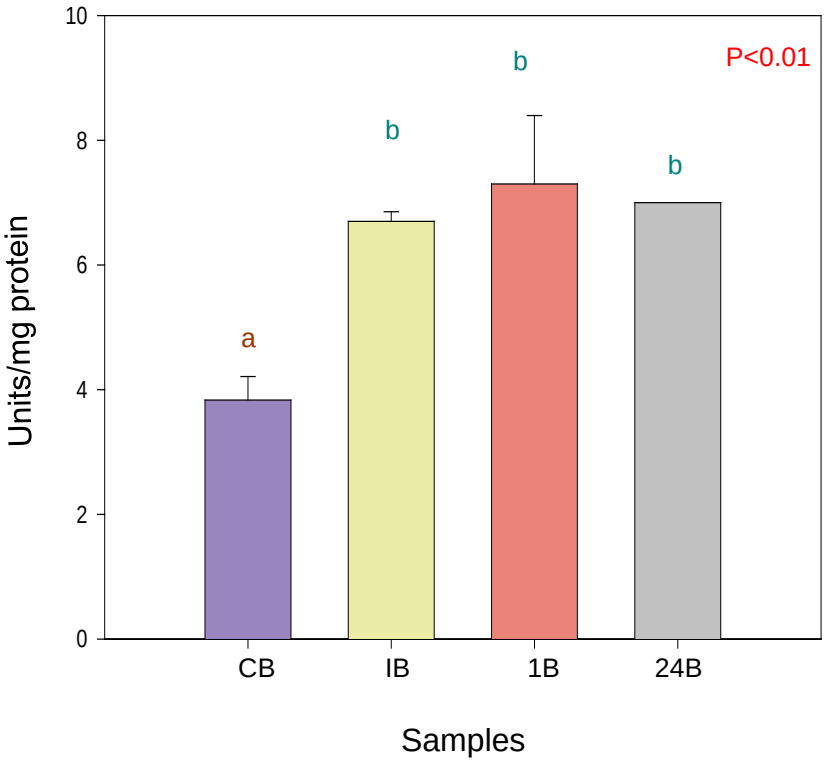
CC - Control (Sham operated) cerebral cortex.

IC - Ischemic (20 min occlusion no reperfusion) cerebral cortex.

1C -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) cerebral cortex.

24C -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) cerebral cortex.

Fig 2.2a Catalase activity in Control and Ischemic Cerebellum



Catalase activity was increased in cerebellum in all the three periods of ischemia and reperfusion when compared to control. Catalase activity was estimated in cerebellum after ischemia and varying periods of reperfusion as described in methodology. 1 unit of enzyme is defined as 1 micromole of H_2O_2 metabolized per min per mg protein. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with level of significance set at P<0.01. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

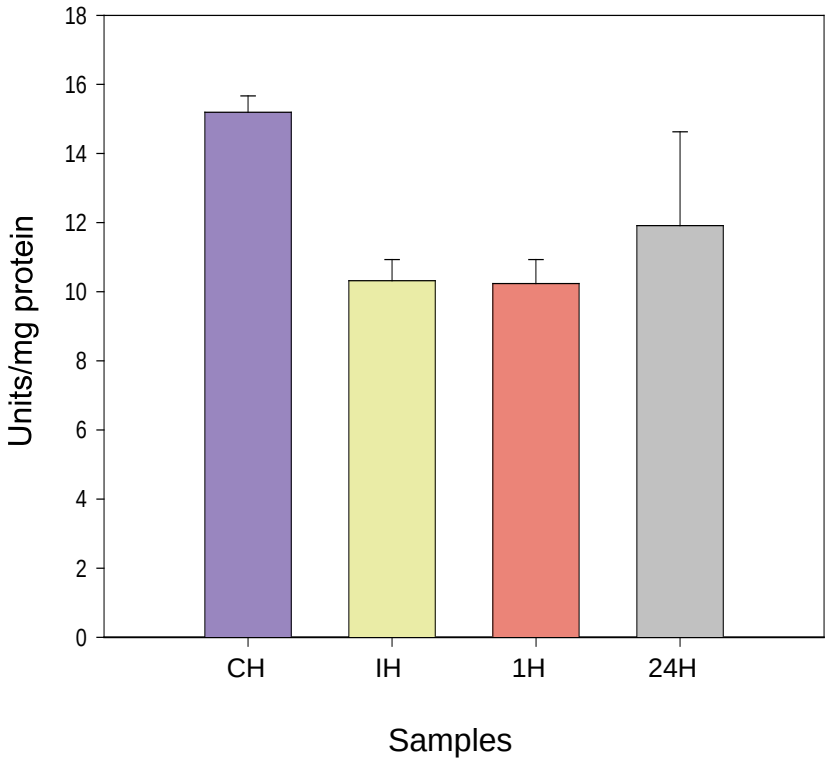
CB - Control (Sham operated) cerebellum.

IB - Ischemic (20 min occlusion no reperfusion) cerebellum.

1B -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) cerebellum.

24B -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) cerebellum.

Fig 2.3a Catalase activity in Control and Ischemic Hippocampus



Catalase enzyme activity did not show significant variation upon ischemia and reperfusion in hippocampus. The activity was estimated in hippocampus after ischemia and varying periods of reperfusion as described in methodology. 1 unit of enzyme is defined as 1 micromole of H_2O_2 metabolized per min per mg protein. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with level of significance set at $P<0.05$. Data was not significant

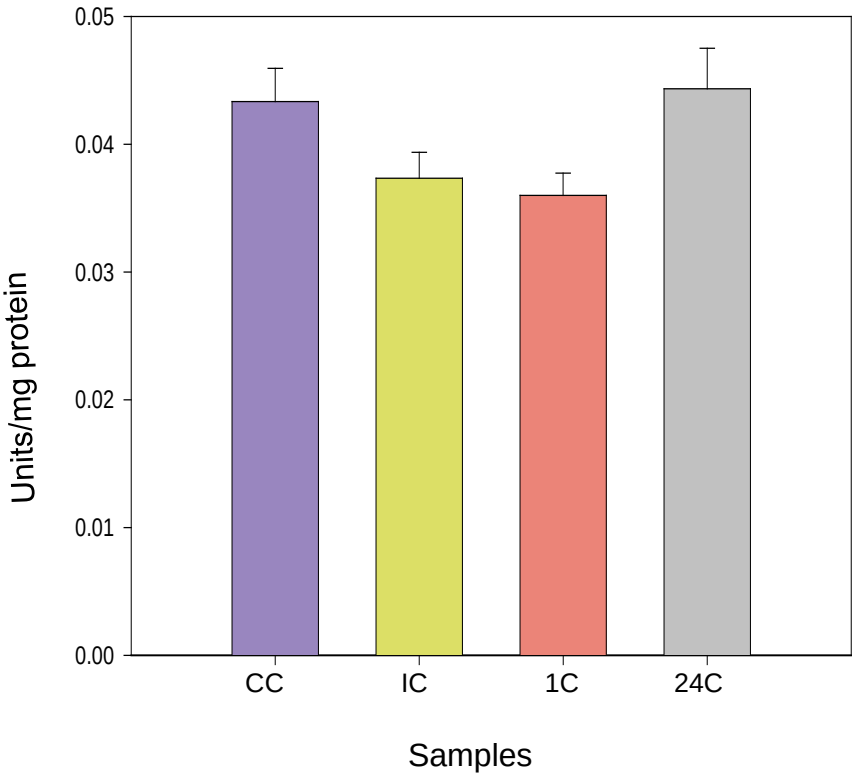
CH - Control (Sham operated) hippocampus.

IH - Ischemic (20 min occlusion no reperfusion) hippocampus .

1H -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) hippocampus.

24H -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) hippocampus.

Fig 2.1b Glutathione Reductase activity in Control and Ischemic Cerebral cortex.



Glutathione reductase activity did not show any significant change in cerebral cortex after ischemia and reperfusion. Glutathione reductase activity was estimated in cerebral cortex after ischemia and varying periods of reperfusion as described in methodology. 1 unit of enzyme is defined as 1 nanomole of NADPH oxidized per min per mg protein. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with significance level set at $P<0.05$. Data was not significant.

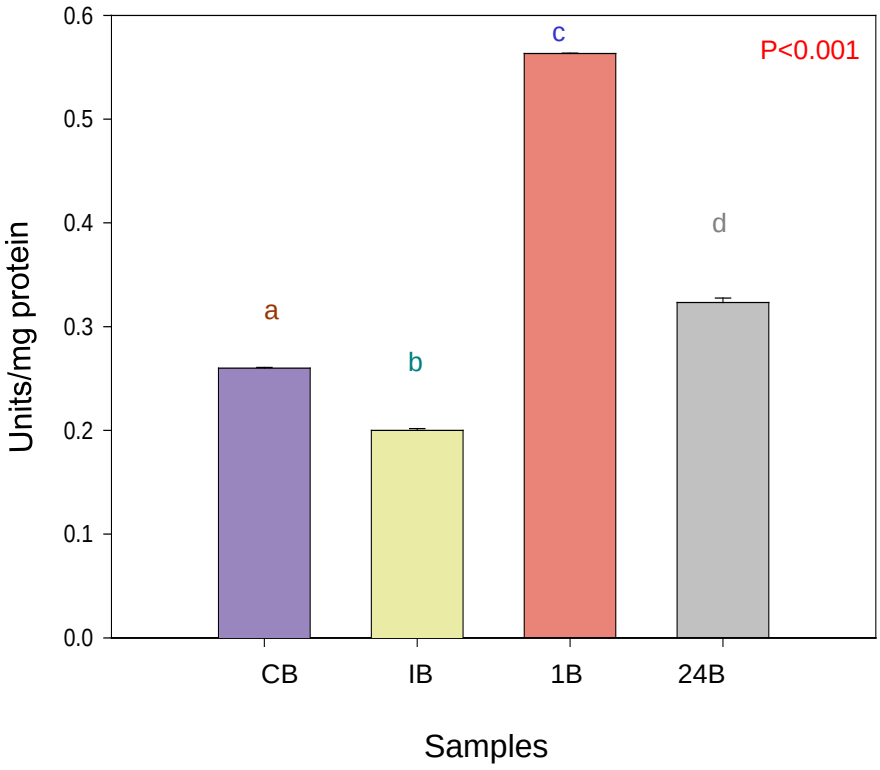
CC - Control (Sham operated) cerebral cortex.

IC - Ischemic (20 min occlusion no reperfusion) cerebral cortex.

1C -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) cerebral cortex.

24C -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) cerebral cortex.

Fig 2.2b Glutathione reductase activity in Control and Ischemic Cerebellum



Glutathione reductase activity was increased in cerebellum after 1hr reperfusion when compared to that of control. GR activity was estimated in cerebellum after ischemia and varying periods of reperfusion as described in methodology. 1 unit of enzyme is defined as 1 nanomole of NADPH oxidized per min per mg protein. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with level of significance set at P<0.001. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

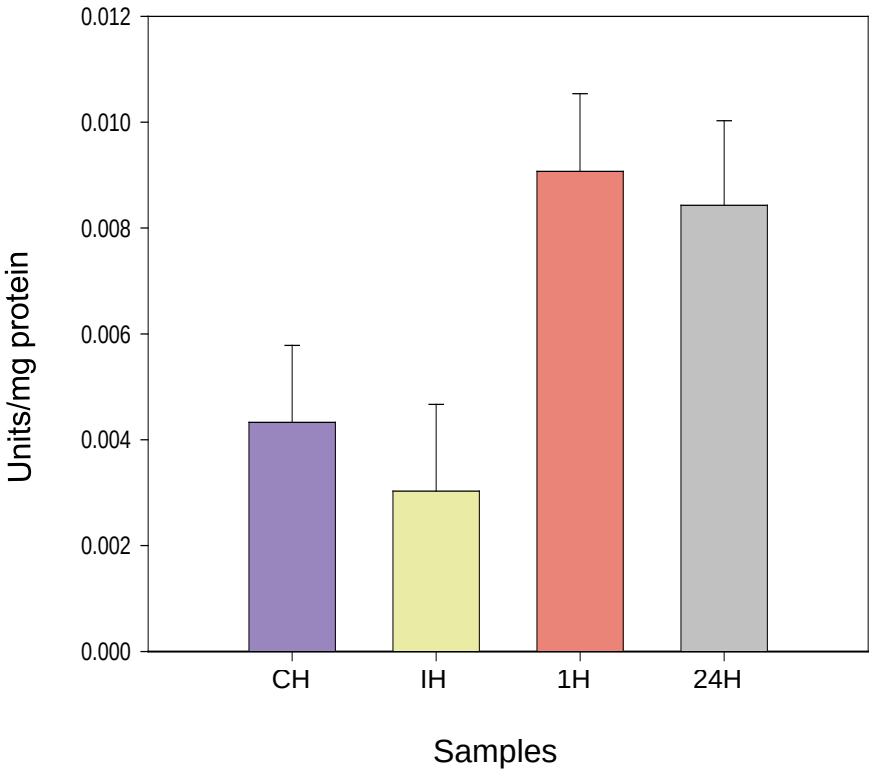
CB - Control (Sham operated) cerebellum.

IB - Ischemic (20 min occlusion no reperfusion) cerebellum.

1B -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) cerebellum.

24B -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) cerebellum.

Fig 2.3b Glutathione Reductase activity in Control and Ischemic Hippocampus



Glutathione reductase activity was increased in hippocampus after ischemia reperfusion but was not significant. GR activity was estimated in hippocampus after ischemia and varying periods of reperfusion as described in methodology. 1 unit of enzyme is defined as 1 nanomole of NADPH oxidized per min per mg protein. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with level of significance set at $P<0.05$. Data was found not to be significant.

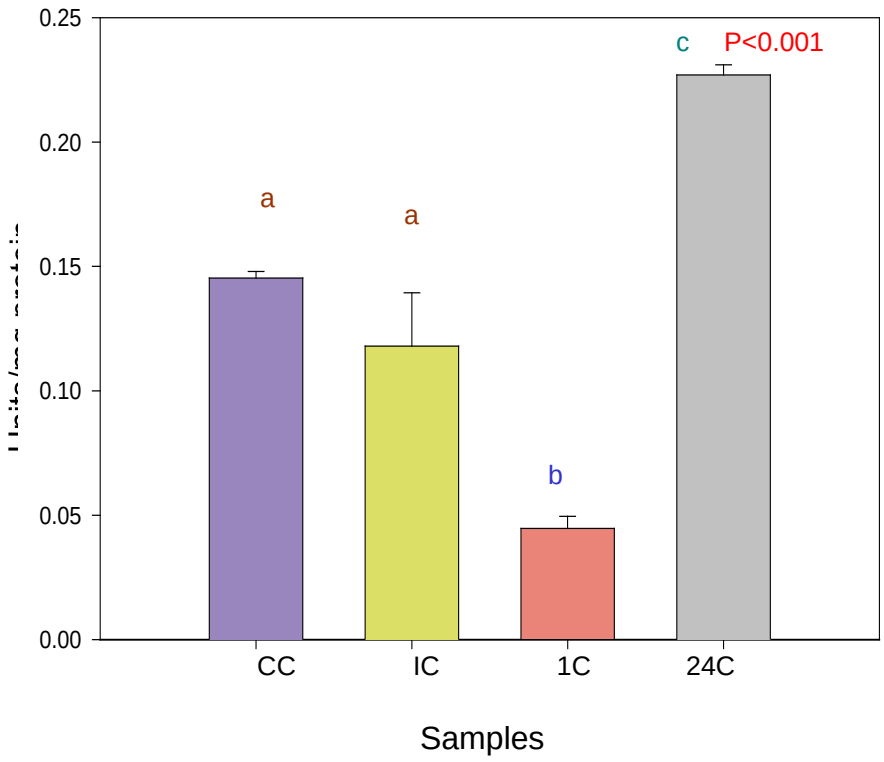
CH - Control (Sham operated) hippocampus.

IH - Ischemic (20 min occlusion no reperfusion) hippocampus .

1H -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) hippocampus.

24H -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) hippocampus.

Fig 2.1c Glutathione Peroxidase activity in Control and Ischemic Cerebral cortex.



Glutathione peroxidase activity was decreased significantly upon ischemia and 1hr reperfusion in cerebral cortex. GPx activity was estimated in cerebral cortex after ischemia and varying periods of reperfusion. 1 unit of enzyme is defined as 1 nanomole of NADPH oxidized per min per mg protein. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with level of significance set at P<0.001. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

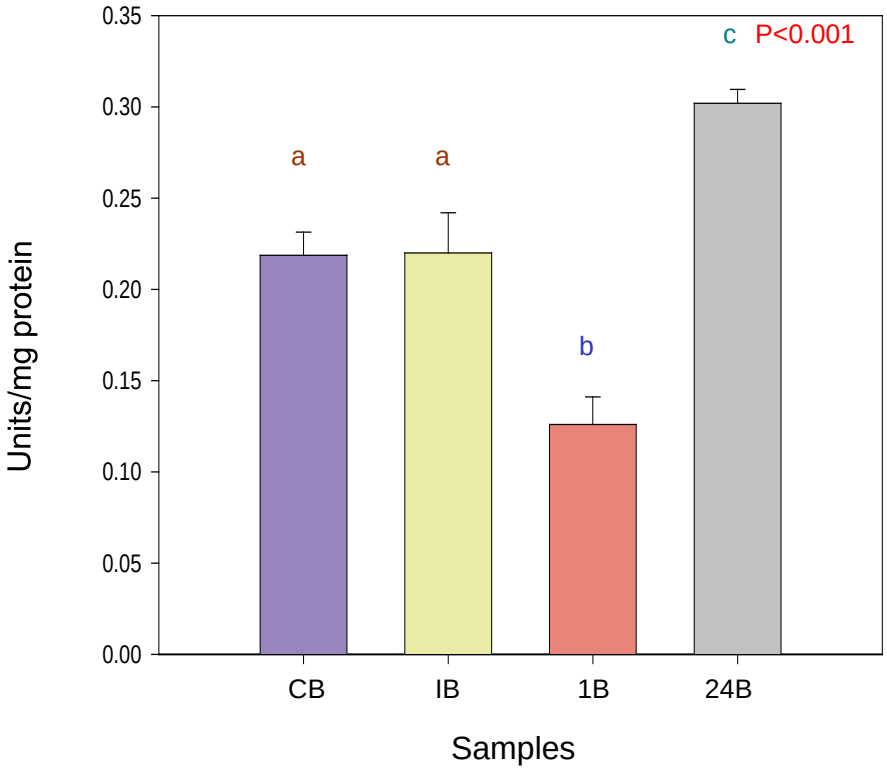
CC - Control (Sham operated) cerebral cortex.

IC - Ischemic (20 min occlusion no reperfusion) cerebral cortex.

1C -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) cerebral cortex.

24C -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) cerebral cortex.

Fig 2.2c Glutathione Peroxidase activity in Control and Ischemic Cerebellum



Glutathione peroxidase activity decreased in cerebellum after ischemia and 1hr reperfusion. GPx was estimated in cerebellum after ischemia and varying periods of reperfusion as described in methodology. 1 unit of enzyme is defined as 1 nanomole of NADPH oxidized per min per mg protein. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with level of significance set at P<0.001. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

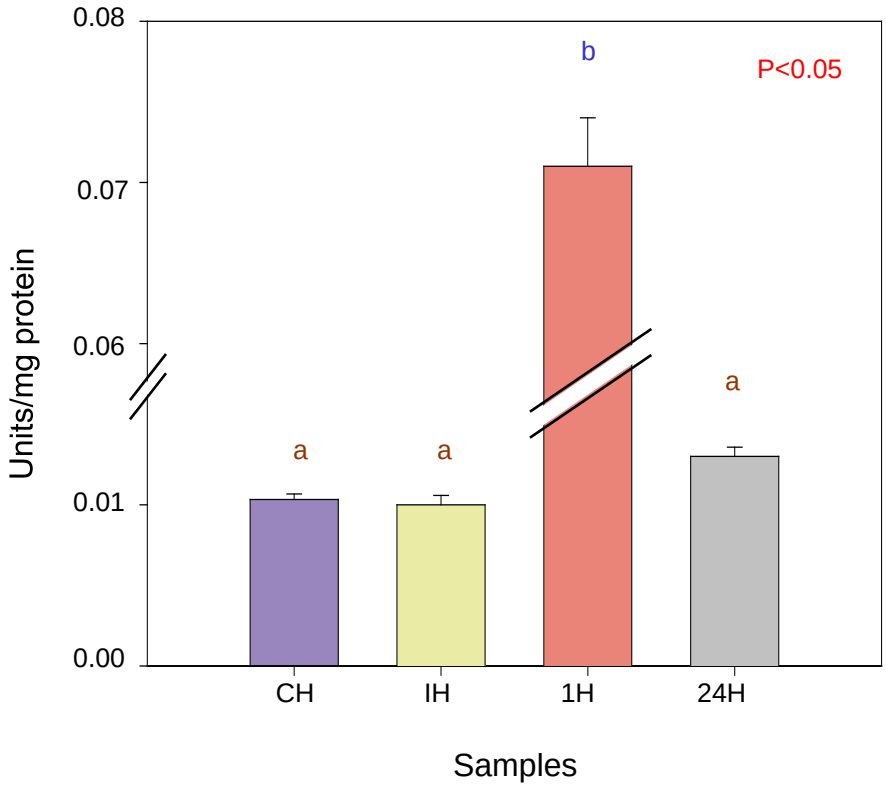
CB - Control (Sham operated) cerebellum.

IB - Ischemic (20 min occlusion no reperfusion) cerebellum.

1B -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) cerebellum.

24B -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) cerebellum.

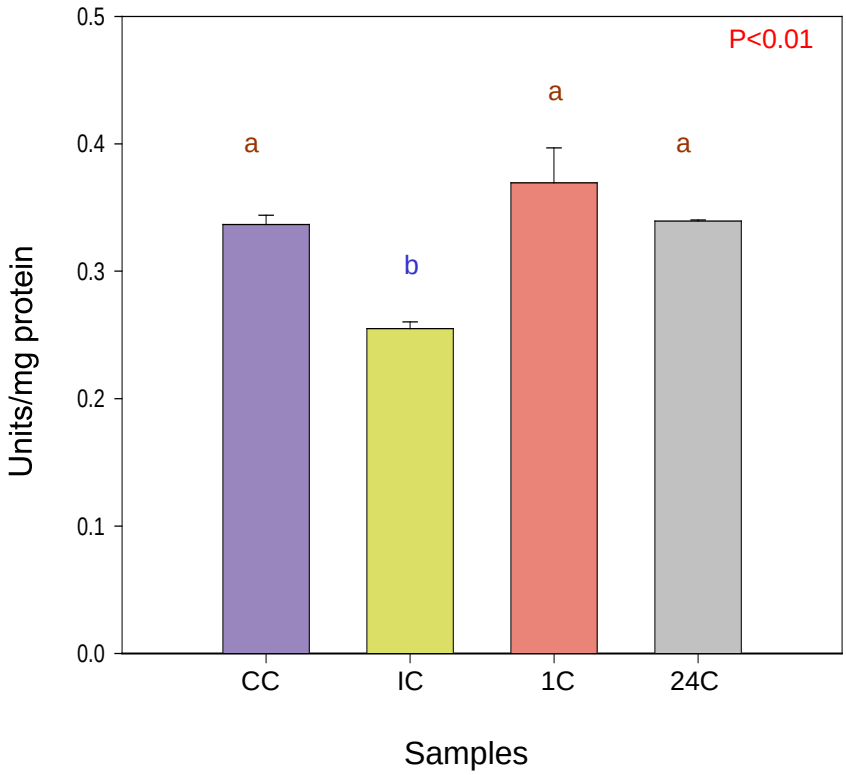
Fig 2.3c Glutathione Peroxidase activity in Control and Ischemic Hippocampus



Glutathione peroxidase activity was increased significantly in hippocampus after 20 min ischemia and 1hr reperfusion. GPx was estimated in hippocampus after ischemia and varying periods of reperfusion as described in methodology. 1 unit of enzyme is defined as 1 nanomole of NADPH oxidized per min per mg protein. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with level of significance set at P<0.05. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

- CH - Control (Sham operated) hippocampus.
- IH - Ischemic (20 min occlusion no reperfusion) hippocampus .
- 1H -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) hippocampus.
- 24H -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) hippocampus.

Fig 2.1d Glutathione S-transferase activity in Control and Ischemic Cerebral cortex



GST activity was increased in cerebral cortex after ischemia and 1hr reperfusion. GST was estimated in cerebral cortex after ischemia and varying periods of reperfusion as described in methodology. 1 unit of enzyme is defined as 1 micromole of thioether formed per min per mg protein. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with level of significance set at P<0.01. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

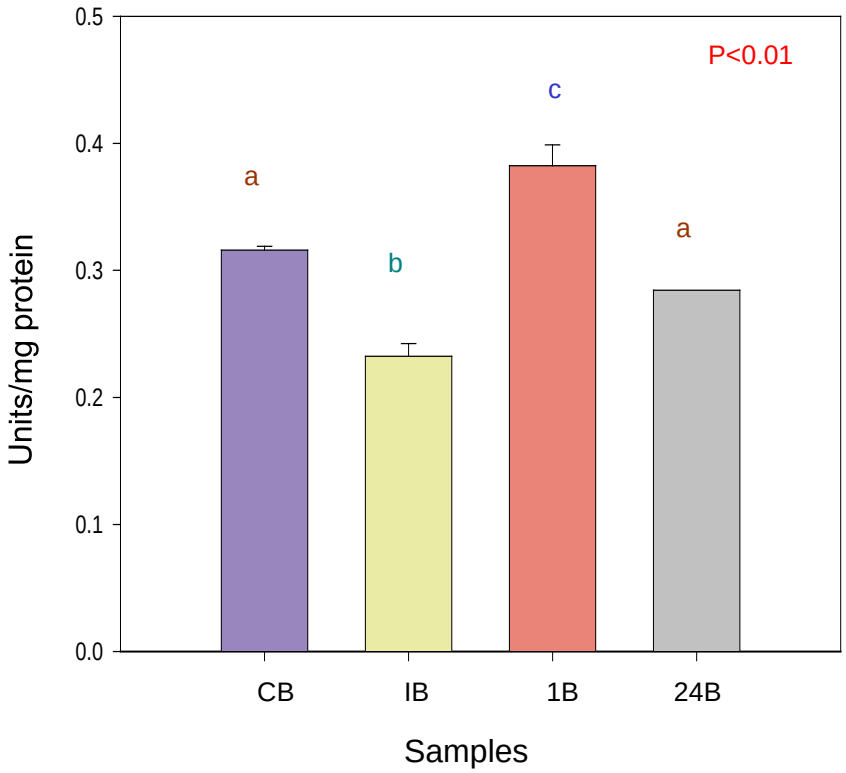
CC - Control (Sham operated) cerebral cortex.

IC - Ischemic (20 min occlusion no reperfusion) cerebral cortex.

1C -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) cerebral cortex.

24C -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) cerebral cortex.

Fig 2.2d Glutathione S-transferase activity in Control and Ischemic Cerebellum



GST activity was increased in cerebellum after ischemia and 1hr reperfusion. GST activity was estimated in cerebellum after ischemia and varying periods of reperfusion as described in methodology. 1 unit of enzyme is defined as 1 micromole of thioether formed per min per mg protein. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with level of significance set at P<0.01. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

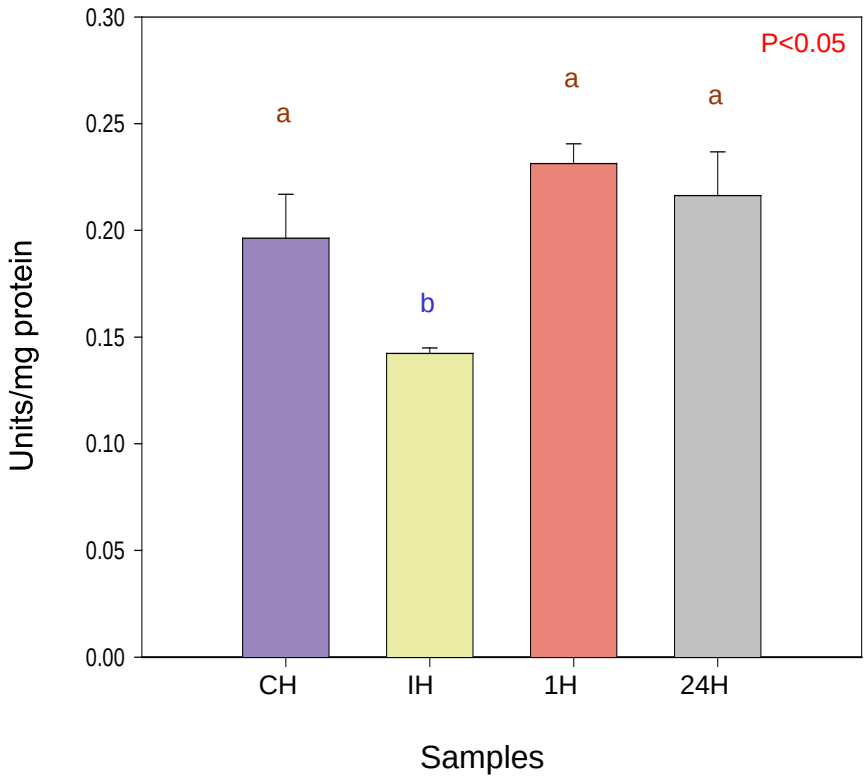
CB - Control (Sham operated) cerebellum.

IB - Ischemic (20 min occlusion no reperfusion) cerebellum.

1B -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) cerebellum.

24B -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) cerebellum.

Fig 2.3d Glutathione S-transferase activity in control and ischemic Hippocampus



GST activity was decreased in hippocampus after ischemia and was increased above normal after 1hr reperfusion. GST activity was estimated in hippocampus after ischemia and varying periods of reperfusion as described in methodology. 1 unit of enzyme is defined as 1 micromole of thioether formed per min per mg protein. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with significance level set at P<0.05. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

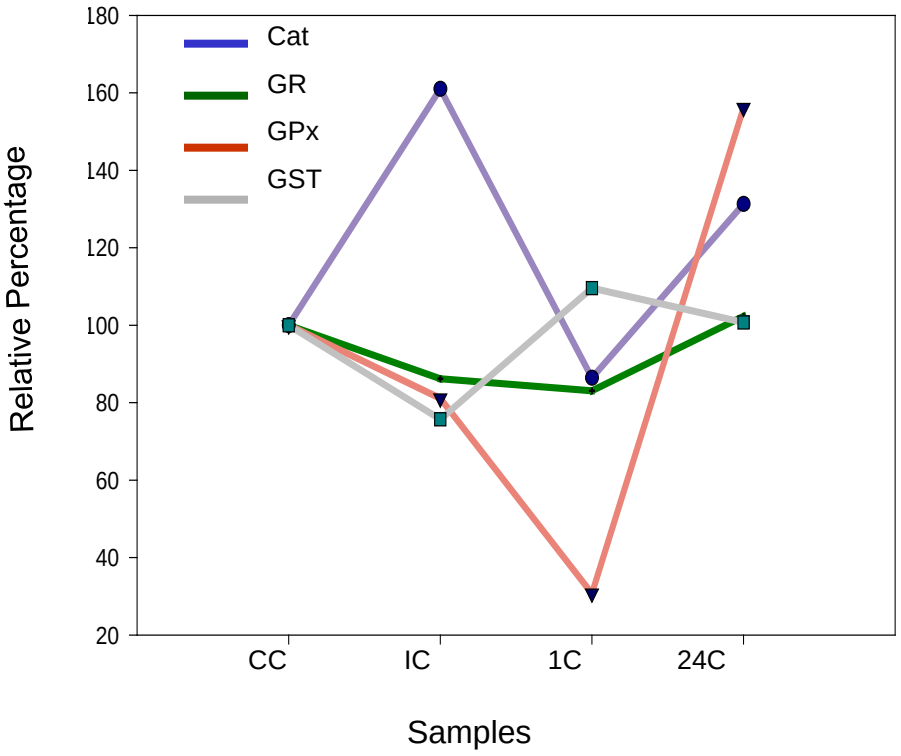
CH - Control (Sham operated) hippocampus.

IH - Ischemic (20 min occlusion no reperfusion) hippocampus .

1H -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) hippocampus.

24H -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) hippocampus.

Fig 2.1 Catalase, Glutathione reductase, Glutathione Peroxidase, Glutathione S-transferase activities in relative percentages of Control and Ischemia Cerebral cortex



Antioxidant enzyme activities altered significantly in cerebral cortex upon ischemia and varying periods of reperfusion. GPx activity was much altered after 1hr reperfusion when compared to Cat, GR and GST activites. GPx was decreased at 1hr reperfusion, there by reducing the antioxidant capacity of cells in cerebral cortex and predisposing more free radical damage.

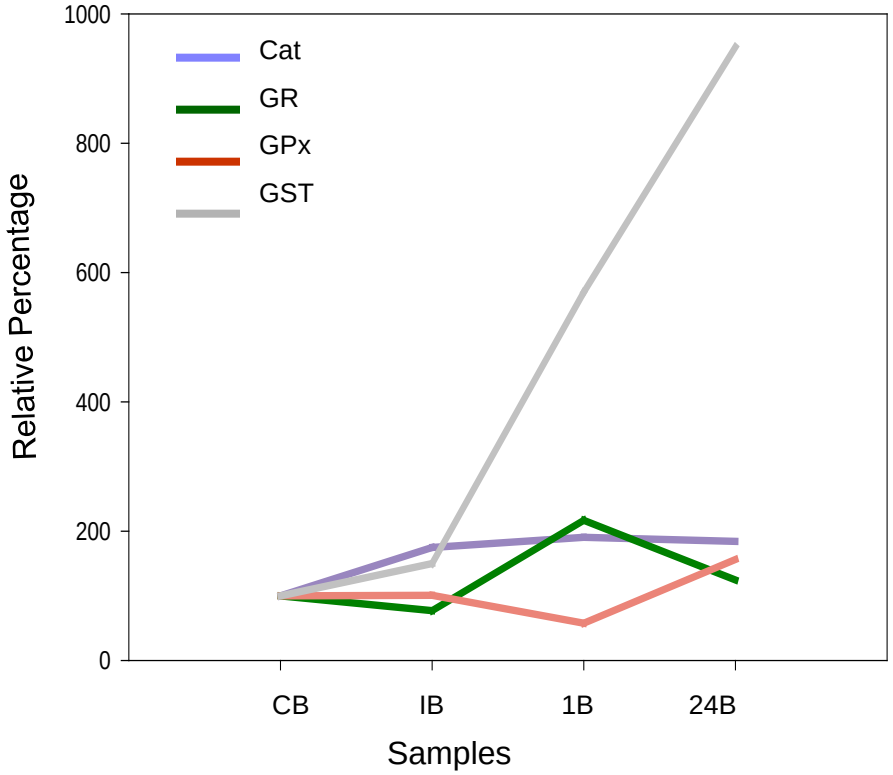
CC - Control (Sham operated) cerebral cortex.

IC - Ischemic (20 min occlusion no reperfusion) cerebral cortex.

1C -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) cerebral cortex.

24C -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) cerebral cortex.

Fig 2.2 Catalase, Glutathione reductase, Glutathione peroxidase, Glutathione S-transferase activities in relative percentages of Control and Ischemia Cerebellum



Antioxidant enzyme activities varied significantly in cerebellum upon ischemia and varying periods of reperfusion. GST activity was much altered after 1hr reperfusion when compared to Cat, GR and GST activites. GST activity was increased at 1hr reperfusion, suggesting its major antioxidant role of it in cerebellum when compared to other antioxidant enzymes.

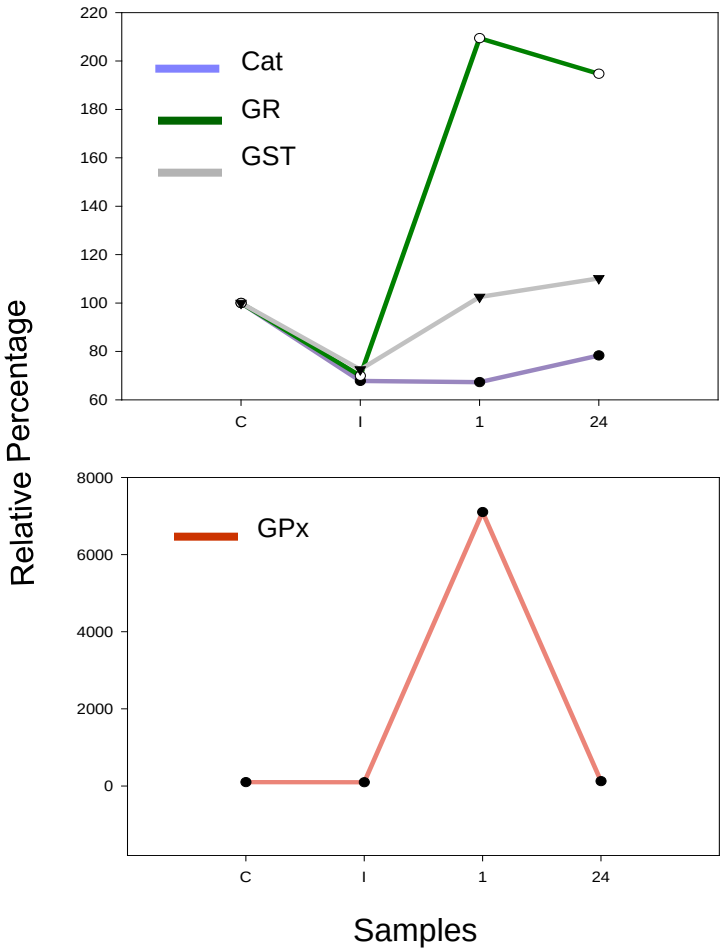
CB - Control (Sham operated) cerebellum.

IB - Ischemic (20 min occlusion no reperfusion) cerebellum.

1B -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) cerebellum.

24B -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) cerebellum.

Fig 2.3 Catalase, Glutathione reductase, Glutathione peroxidase, Glutathione S-transferase activities in relative percentages of Control and Ischemia Hippocampus



Antioxidant enzyme activities varied significantly in hippocampus upon ischemia and varying periods of reperfusion. GR and GPx activities were altered much after 1hr reperfusion when compared to Cat and GST activites. GR and GPx activities increased at 1hr reperfusion, suggesting the major role of glutathione dependent enzymes in hippocampus when compared to other antioxidant enzymes.

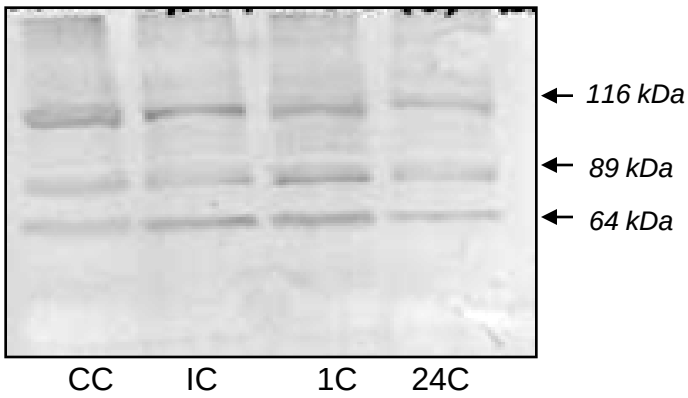
CH - Control (Sham operated) hippocampus.

IH - Ischemic (20 min occlusion no reperfusion) hippocampus .

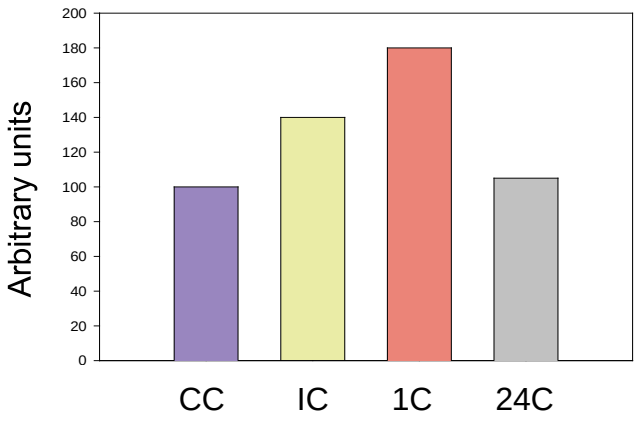
1H -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) hippocampus.

24H -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) hippocampus.

Fig 3.1 Western blot analysis of Control and Ischemic Cerebral cortex probed with PARP antibody



a) Western Blot



b) Densitometric scan

PARP cleavage varied with varying periods of ischemia and reperfusion in cerebral cortex. Proteins of cerebral cortex of ischemia and varying periods of reperfusion were separated on 10% SDS-PAGE and blotted on to nitrocellulose membrane. The blot was probed with anti rabbit PARP antibody. The antibody recognized both the full length (116kDa) and cleaved fragments of PARP. The intensity of 64kDa cleaved fragment in the blot was high in 1hr reperfusion sample which was further confirmed by densitometry.

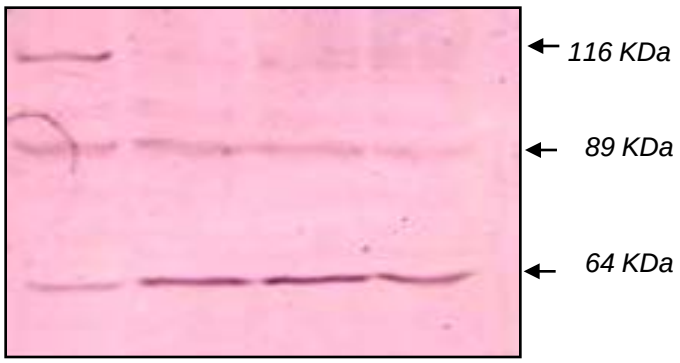
CC - Control (Sham operated) cerebral cortex.

IC - Ischemic (20 min occlusion no reperfusion) cerebral cortex.

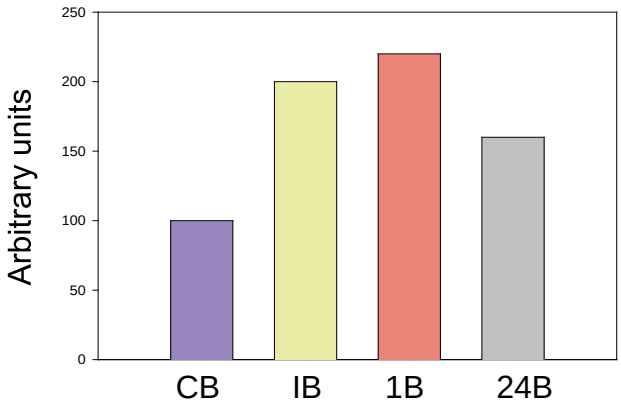
1C -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) cerebral cortex.

24C -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) cerebral cortex.

Fig 3.4 Western blot analysis of Control and Ischemic Cerebellum probed with PARP antibody



a) Western Blot



b) Densitometric scan

PARP cleavage varied with varying periods of ischemia and reperfusion in cerebellum. Proteins of cerebellum of ischemia and varying periods of reperfusion were separated on 10% SDS-PAGE and blotted on to nitrocellulose membrane. The blot was probed with anti rabbit PARP antibody. The antibody recognized both the full length (116kDa) and cleaved fragments of PARP. The intensity of 64kDa cleaved fragment in the blot was high in 1hr reperfusion sample which was further confirmed by densitometry.

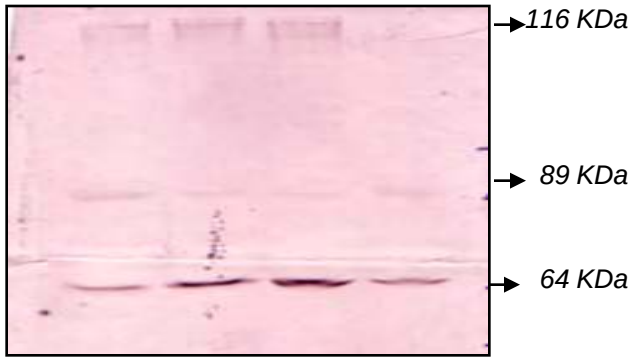
CB - Control (Sham operated) cerebellum.

IB - Ischemic (20 min occlusion no reperfusion) cerebellum.

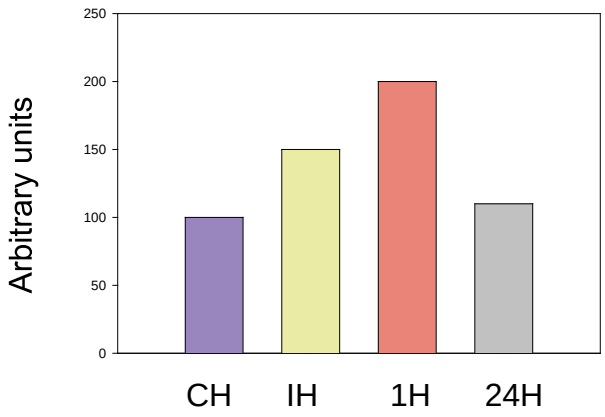
1B -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) cerebellum.

24B -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) cerebellum.

Fig 3.7 Western blot analysis of Control and Ischemic Hippocampus probed with PARP antibody



a) Western Blot



b) Densitometric scan

PARP cleavage varied with varying periods of ischemia and reperfusion in hippocampus. Proteins of hippocampus of ischemia and varying periods of reperfusion were separated on 10% SDS-PAGE and blotted on to nitrocellulose membrane. The blot was probed with anti rabbit PARP antibody. The antibody recognized both the full length (116kDa) and cleaved fragments of PARP. The intensity of 64kDa cleaved fragment in the blot was high in 1hr reperfusion sample which was further confirmed by densitometry.

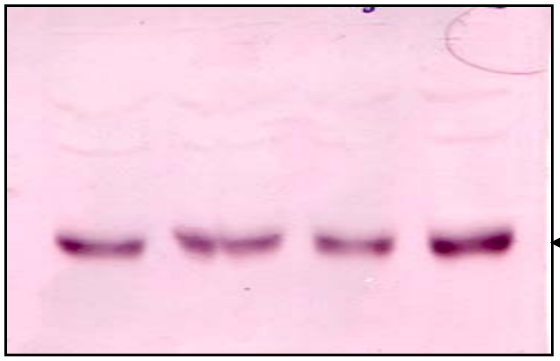
CH - Control (Sham operated) hippocampus.

IH - Ischemic (20 min occlusion no reperfusion) hippocampus.

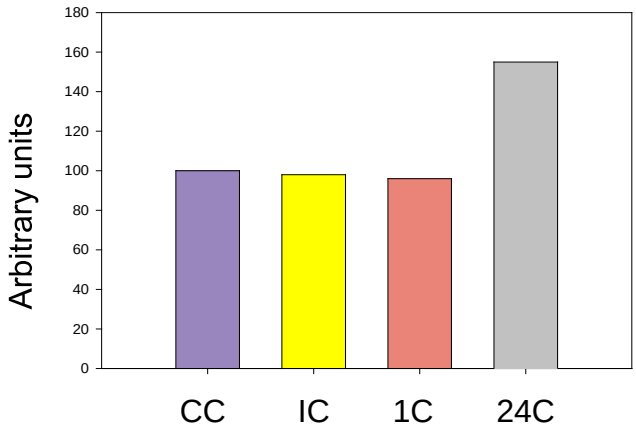
1H -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) hippocampus.

24H -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) hippocampus.

Fig 3.2 Western blot analysis of Control and Ischemic cerebral cortex probed with Caspase-3 antibody.



a) Western Blot

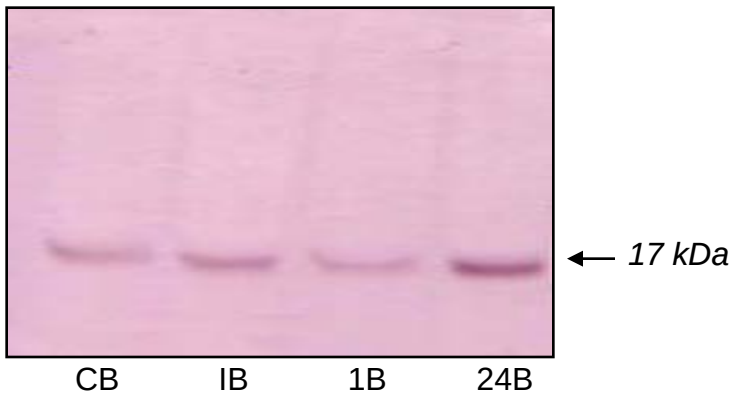


b) Densitometric scan

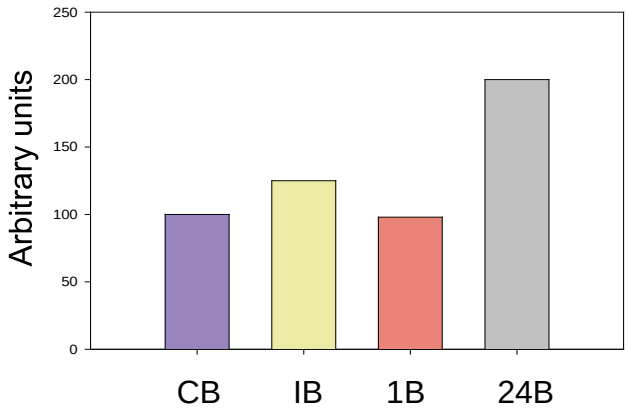
Caspase-3 activation varied with varying periods of ischemia and reperfusion in cerebral cortex. Proteins from cytosolic fraction of cerebral cortex of ischemia and varying periods of reperfusion were separated on 12.5% SDS-PAGE and blotted on to nitrocellulose membrane. The blot was probed with anti mouse caspase-3 antibody. The antibody recognized a band at 17kDa corresponding to the cleaved fragment of caspase-3. The band intensity was more in 24hr reperfusion period which was further confirmed by densitometry.

- CC - Control (Sham operated) Cerebral cortex.
- IC - Ischemic (20 min occlusion no reperfusion) Cerebral cortex.
- 1C -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) Cerebral cortex.
- 24C -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) Cerebral cortex.

Fig 3.5 Western blot analysis of Control and Ischemic Cerebellum probed with Caspase-3 antibody



a) Western Blot



b) Densitometric scan

Caspase-3 activation altered with varying periods of ischemia and reperfusion in cerebellum. Proteins from cytosolic fraction of cerebellum of ischemia and varying periods of reperfusion were separated on 12.5% SDS-PAGE and blotted on to nitrocellulose membrane. The blot was probed with anti mouse caspase-3 antibody. The antibody recognized a band at 17kDa corresponding to the cleaved fragment of caspase-3. The band intensity was more in 24 hr reperfusion period which was further confirmed by densitometry.

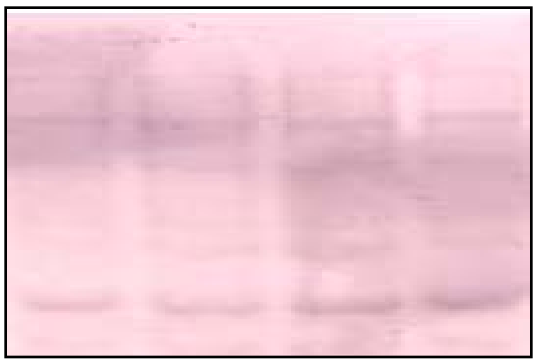
CB - Control (Sham operated) Cerebellum.

IB - Ischemic (20 min occlusion no reperfusion) Cerebellum.

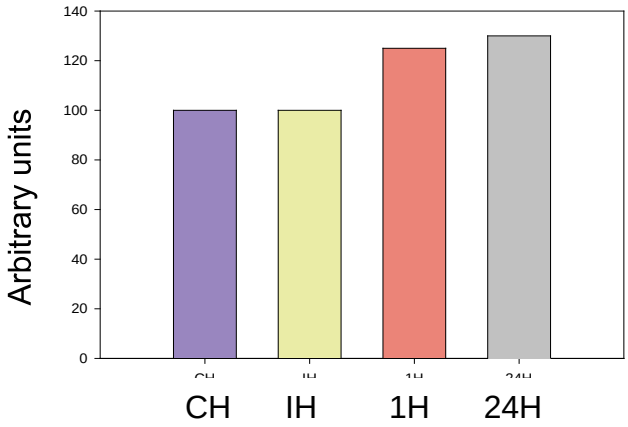
1B -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) Cerebellum.

24B -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) Cerebellum.

Fig 3.8 Western blot analysis of Control and Ischemic Hippocampus probed with Caspase-3 antibody



a) Western Blot



b) Densitometric scan

Caspase-3 activation varied with varying periods of ischemia and reperfusion in hippocampus. Proteins from cytosolic fraction of hippocampus of ischemia and varying periods of reperfusion were separated on 12.5% SDS-PAGE and blotted on to nitrocellulose membrane. The blot was probed with anti mouse caspase-3 antibody. The antibody recognized a band at 17kDa corresponding to the cleaved fragment of caspase-3. The band intensity was more in 1hr and 24hr reperfusion period which was further confirmed by densitometry.

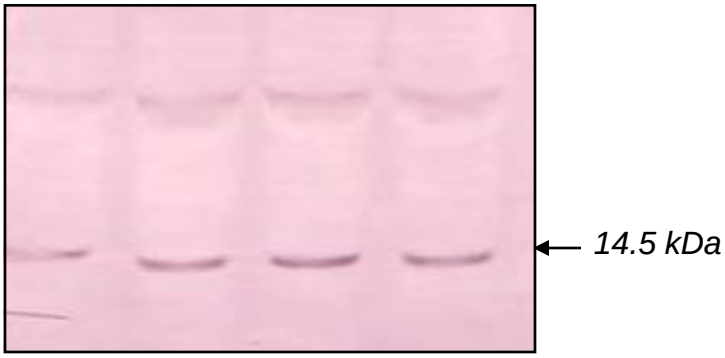
CH - Control (Sham operated) Hippocampus.

IH - Ischemic (20 min occlusion no reperfusion) Hippocampus.

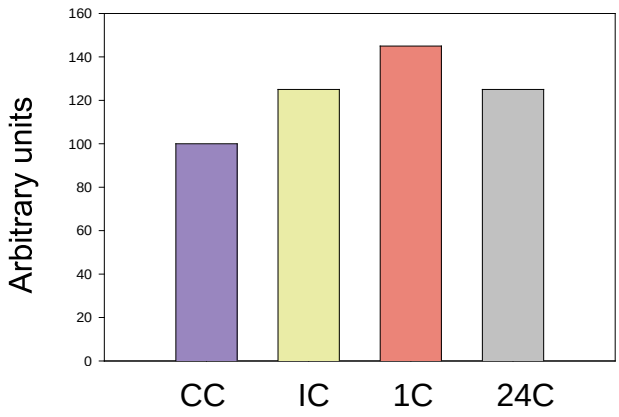
1H -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) Hippocampus.

24H -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) Hippocampus.

Fig 3.3 Western blot analysis of Control and Ischemic Cerebral cortex probed with Cytochrome *C* antibody



a) Western Blot



b) Densitometric scan

Cytochrome *C* level varied with varying periods of ischemia and reperfusion in cerebral cortex. Proteins from cytosolic fractions of cerebral cortex of ischemia and varying periods of reperfusion were separated on 12.5% SDS-PAGE and blotted on to nitrocellulose membrane. The blot was then probed with anti mouse cytochrome *C* antibody that recognizes a band at 14kDa. The band intensity was more in 0hr, 1hr and 24hr reperfusion samples when compared to control. The band intensities was further confirmed by densitometry.

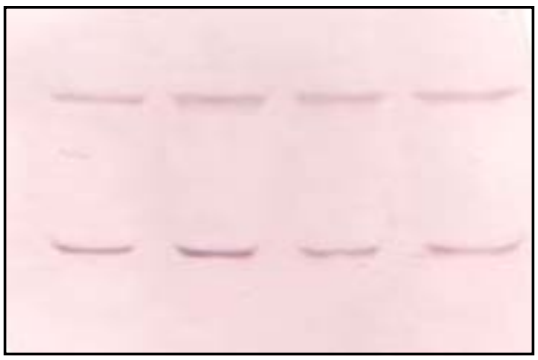
CC - Control (Sham operated) cerebral cortex.

IC - Ischemic (20 min occlusion no reperfusion) cerebral cortex.

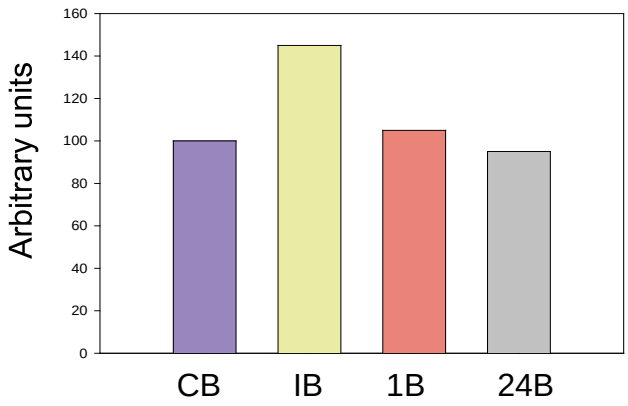
1C -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) cerebral cortex.

24C -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) cerebral cortex.

Fig 3.6 Western blot analysis of Control and Ischemic Cerebellum probed with Cytochrome *C* antibody



a) Western Blot



b) Densitometric scan

Cytochrome *C* level altered with varying periods of ischemia and reperfusion in cerebellum. Proteins from cytosolic fractions of cerebellum of ischemia and varying periods of reperfusion were separated on 12.5% SDS-PAGE and blotted on to nitrocellulose membrane. The blot was then probed with anti mouse cytochrome *C* antibody that recognizes a band at 14 kDa. The band intensity was more in 0hr reperfusion samples when compared to control. The band intensities was further confirmed by densitometry.

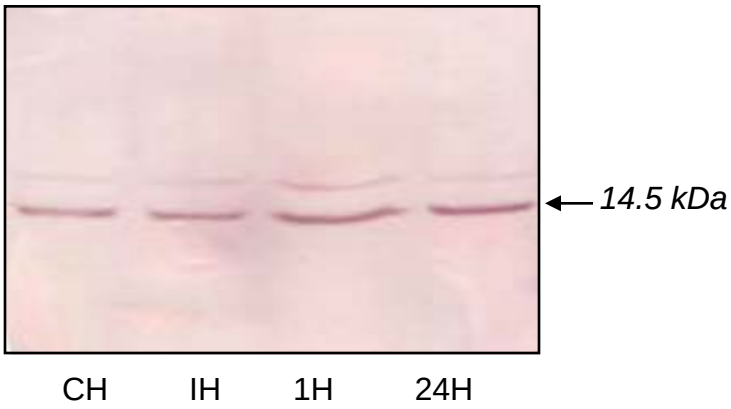
CB - Control (Sham operated) cerebellum.

IB - Ischemic (20 min occlusion no reperfusion) cerebellum.

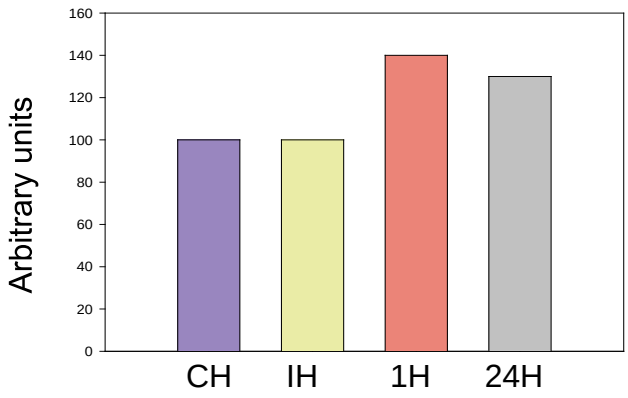
1B -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) cerebellum.

24B -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) cerebellum.

Fig 3.9 Western blot analysis of Control and Ischemic Hippocampus probed with Cytochrome *C* antibody



a) Western Blot



b) Densitometric scan

Cytochrome *C* level altered with varying periods of ischemia and reperfusion in hippocampus. Proteins from cytosolic fractions of hippocampus of ischemia and varying periods of reperfusion were separated on 12.5% SDS-PAGE and blotted on to nitrocellulose membrane. The blot was then probed with anti mouse cytochrome *C* antibody that recognizes a band at 14 kDa. The band intensity was more in 1hr reperfusion samples when compared to control. The band intensities was further confirmed by densitometry.

CH - Control (Sham operated) hippocampus.

IH - Ischemic (20 min occlusion no reperfusion) hippocampus.

1H -1 hour reperfusion (20 min occlusion and 1 hour reperfusion) hippocampus.

24H -24 hour reperfusion (20 min occlusion and 24 hour reperfusion) hippocampus.

Fig 3A Oxidative stress – cell death

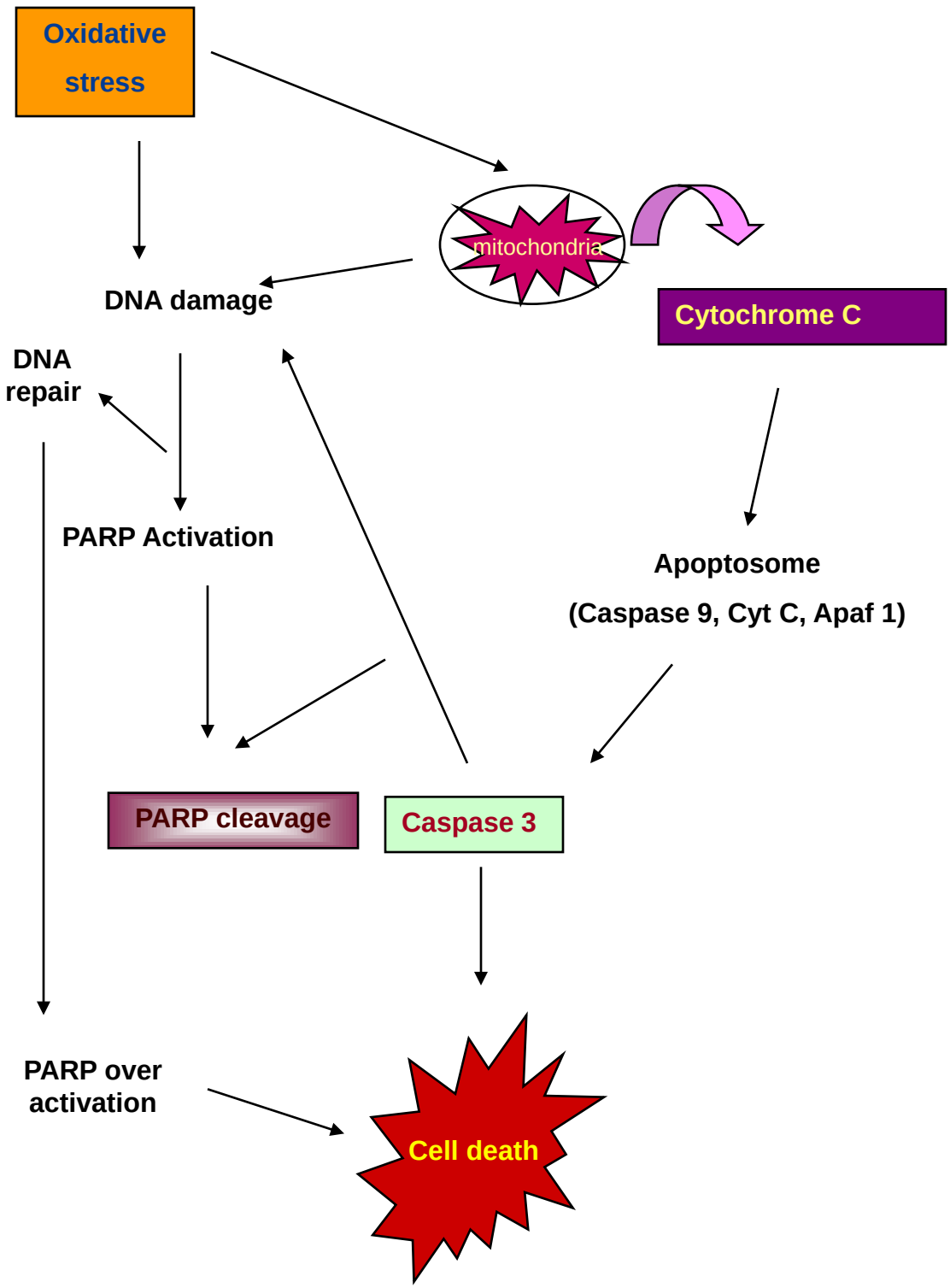
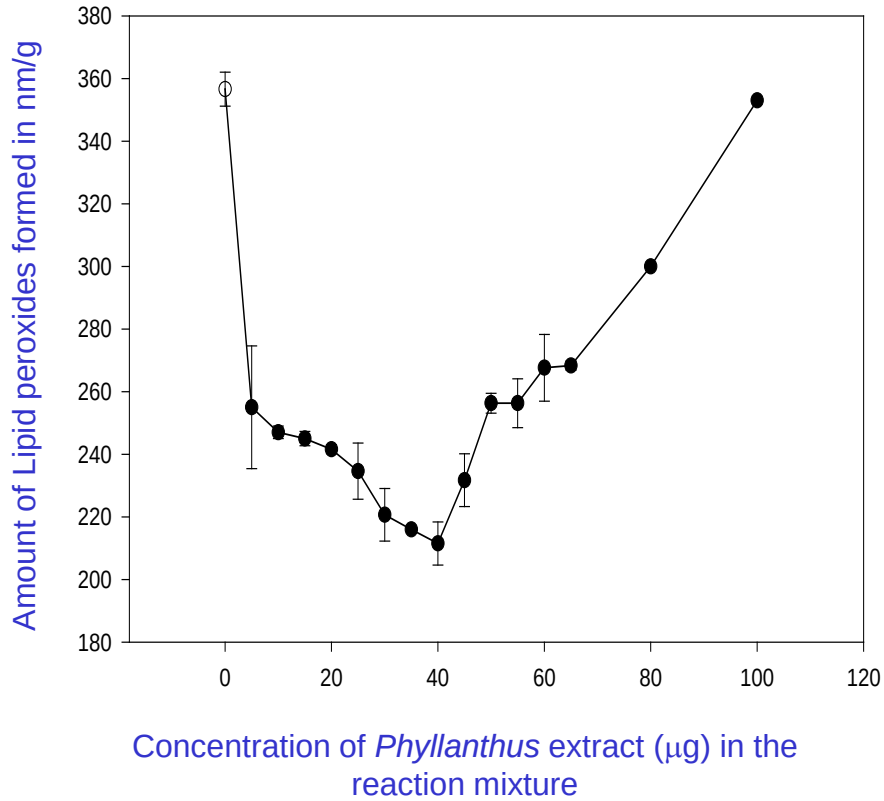


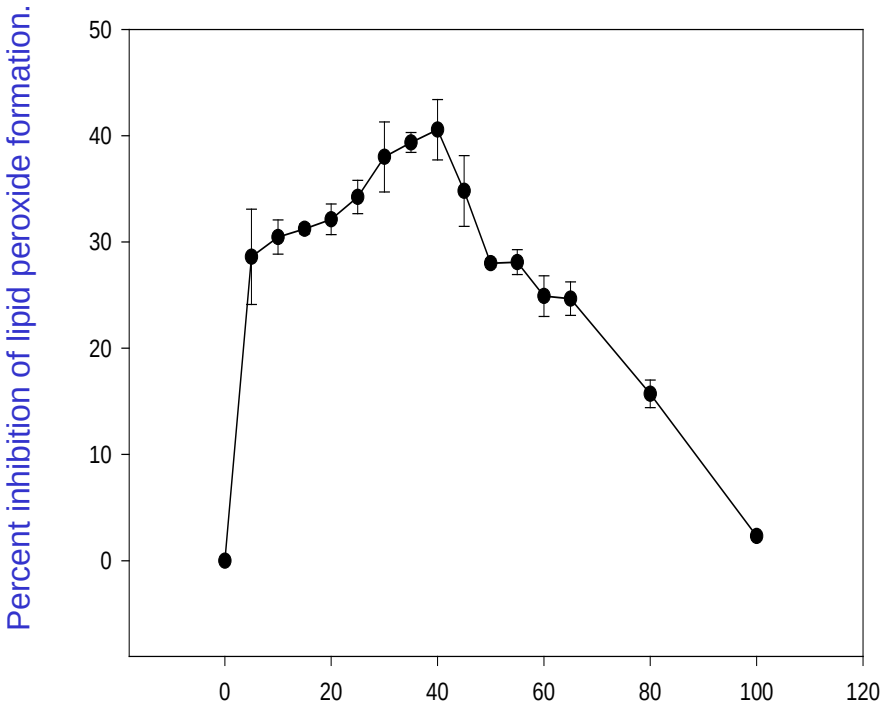
Fig 4.1a Inhibition of lipid peroxidation in rat liver homogenate by *Phyllanthus* extract



The figure represents the amount of lipid peroxide products formed with ferrous/ascorbate treatment and inhibitory effects of *Phyllanthus* extract in rat liver homogenate.

Lipid peroxidation (LPO) was induced in rat liver homogenate using ferrous/ascorbate treatment. Increasing concentrations of aqueous extract of *Phyllanthus* was added to the reaction mixture and its inhibitory effect on LPO was studied. The amount of lipid peroxides formed were estimated using the protocol of Okhawa *et al*. *Phyllanthus* showed a dose dependent inhibition of lipid peroxidation induced in rat liver homogenate. The inhibition was maximum at 40µg of the extract in the reaction mixture and further increase of the extract in the reaction mixture increased the induction of lipid peroxidation.

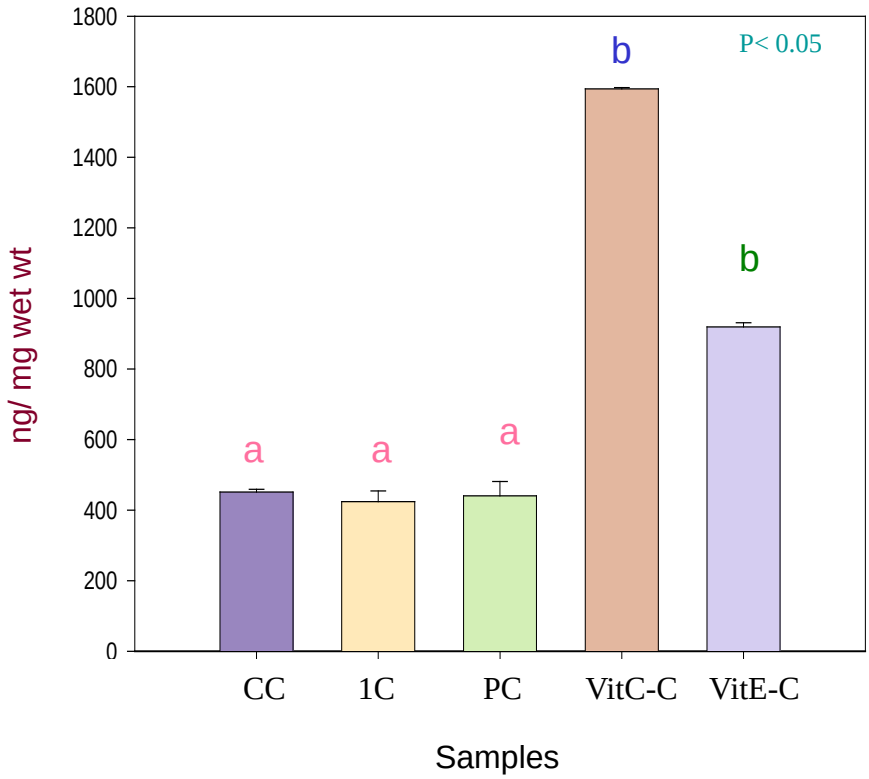
Fig 4.1b Percent Inhibition of lipid peroxidation in rat liver homogenate by *Phyllanthus* extract



The figure represents the amount of lipid peroxide products inhibited in percentage formed with ferrous/ascorbate treatment.

Lipid peroxidation (LPO) was induced in rat liver homogenate using ferrous/ascorbate treatment. Increasing concentrations of aqueous extract of *Phyllanthus* was added to the reaction mixture and its inhibitory effect on LPO was studied. The amount of lipid peroxides formed were estimated using the protocol of Okhawa *et al*. *Phyllanthus* showed a dose dependent inhibition on lipid peroxidation induced in rat liver homogenate. There was a 40% inhibition of lipid peroxidation at 40μg of the extract in the reaction mixture..

Fig 4.2a Levels of Reduced Glutathione (GSH) in ischemic and Antioxidant treated Cerebral cortex



GSH level did not show much variation in 1hr reperfusion cerebral cortex samples when compared to control but vitamin C treatment significantly increased GSH level. Vitamin E treatment also increased GSH level whereas treatment with *Phyllanthus* did not show much variation. Reduced Glutathione levels were estimated in cerebral cortex in 20 min ischemia and 1 hr reperfusion samples with antioxidant (vitamin C/vitamin E/*Phyllanthus*) treatment. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with level of significance set at P<0.05. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

CC - Control (Sham operated) cerebral cortex.

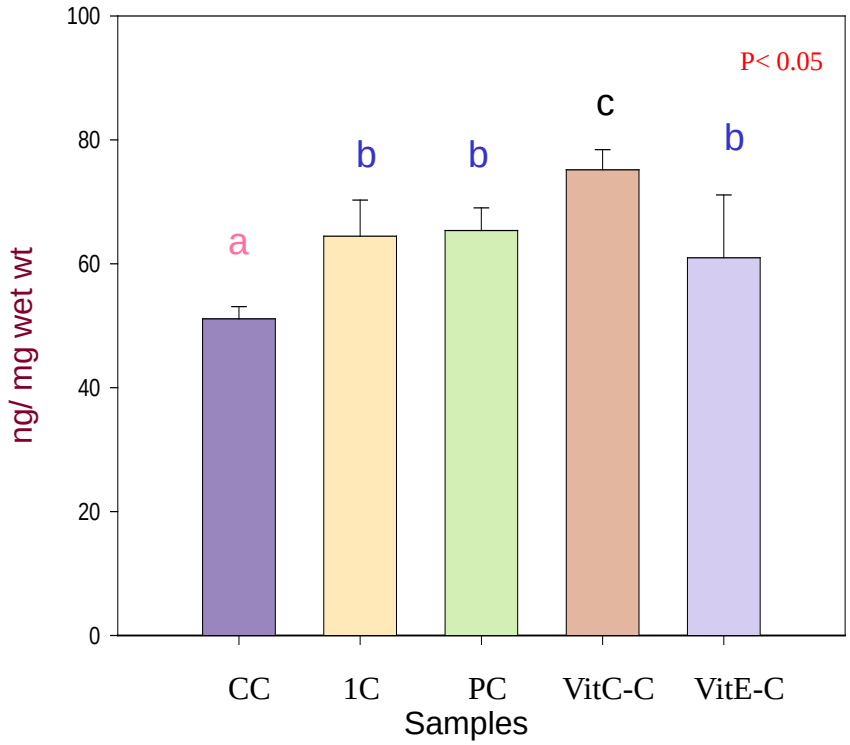
IC - 20 min Ischemia followed by 1 hr reperfusion cerebral cortex

PC - 20 min Ischemia followed by 1 hr reperfusion after 1 week *Phyllanthus* treatment cerebral cortex.

Vit C-C - 20 min Ischemia followed by 1 hr reperfusion after 1 week Vit C treatment cerebral cortex.

Vit E-C -20 min Ischemia followed by 1 hr reperfusion after 1 week Vit E treatment cerebral cortex.

Fig 4.2b Levels of Oxidized Glutathione (GSSG) in ischemic and Antioxidant treated Cerebral Cortex



GSSG level increased in 1hr reperfusion cerebral cortex samples when compared to control. There was a significant increase in GSSG level in ischemic samples which underwent vitamin C treatment. Vitamin E treatment decreased GSSG level but was not significant whereas *Phyllanthus* did not show much variation. Oxidized Glutathione levels were estimated in cerebral cortex in 20min ischemia and 1hr reperfusion samples with antioxidant (vitamin C/vitamin E/*Phyllanthus*) treatment. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with significance level set at P<0.05. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

CC - Control (Sham operated) cerebral cortex.

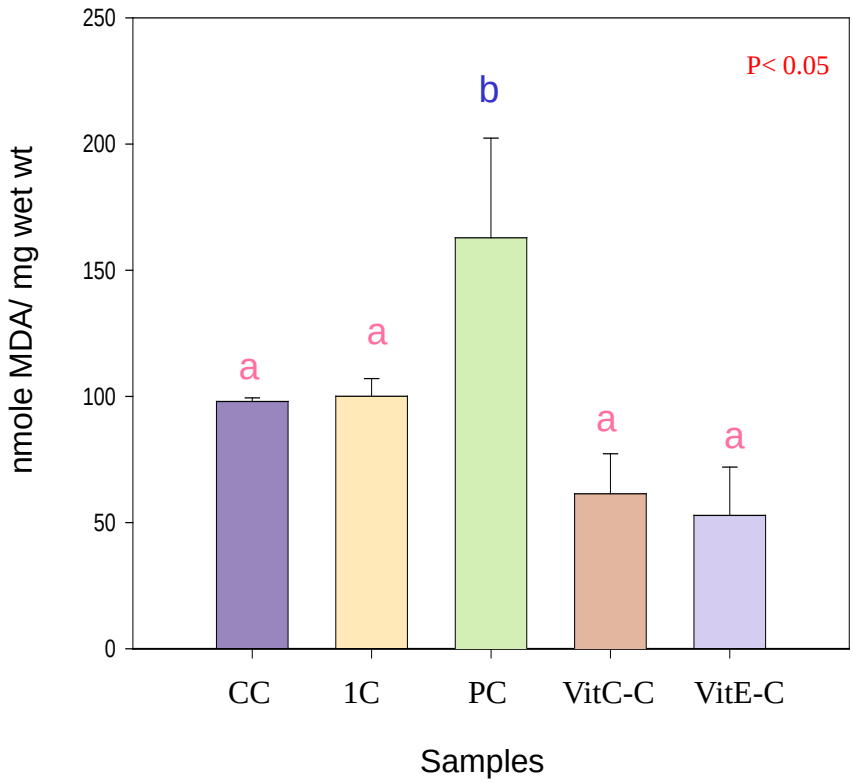
IC - 20 min Ischemia followed by 1 hr reperfusion cerebral cortex

PC - 20 min Ischemia followed by 1 hr reperfusion after 1 week *Phyllanthus* treatment cerebral cortex.

Vit C-C - 20 min Ischemia followed by 1 hr reperfusion after 1 week Vit C treatment cerebral cortex.

Vit E-C -20 min Ischemia followed by 1 hr reperfusion after 1 week Vit E treatment cerebral cortex.

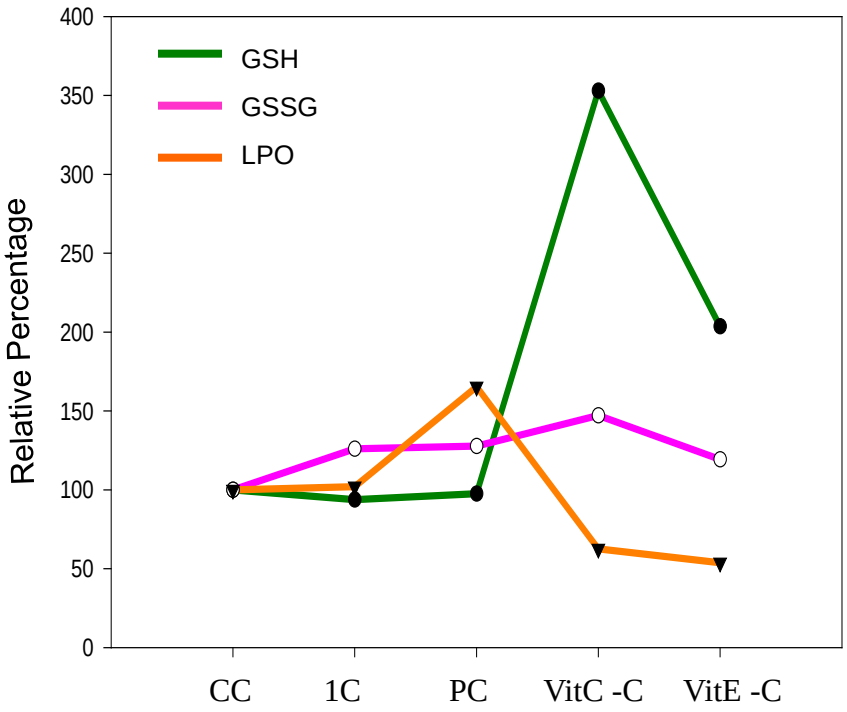
Fig 4.2c Levels of Lipid peroxides (LPO) in ischemic and Antioxidant treated Cerebral cortex



LPO levels increased after 1hr reperfusion whereas vitamin C and vitamin E treatments significantly decreased LPO levels. Lipid peroxide levels were estimated in cerebral cortex in 20min ischemia and 1hr reperfusion samples with antioxidant (vitamin C/vitamin E/*Phyllanthus*) treatment. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with significance level set at P<0.05. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

- CC - Control (Sham operated) cerebral cortex.
- IC - 20 min Ischemia followed by 1 hr reperfusion cerebral cortex
- PC - 20 min Ischemia followed by 1 hr reperfusion after 1 week *Phyllanthus* treatment cerebral cortex.
- Vit C-C - 20 min Ischemia followed by 1 hr reperfusion after 1 week Vit C treatment cerebral cortex.
- Vit E-C -20 min Ischemia followed by 1 hr reperfusion after 1 week Vit E treatment cerebral cortex.

Fig 4.2 GSH, GSSG and LPO (Percentage) levels in ischemic and Antioxidant treated Cerebral cortex



Vitamin C treatment increased the GSH level with a decrease in LPO level, thereby reducing the oxidative stress upon ischemia reperfusion in cerebral cortex.Treatment with vitamin E also increased GSH level and decreased LPO level. Whereas *Phyllanthus* treatment did not show much alteration. Vitamin C seems to be more effective in reducing oxidative stress in rat cerebral cortex when compared to vitamin E and *Phyllanthus*

CC - Control (Sham operated) cerebral cortex.

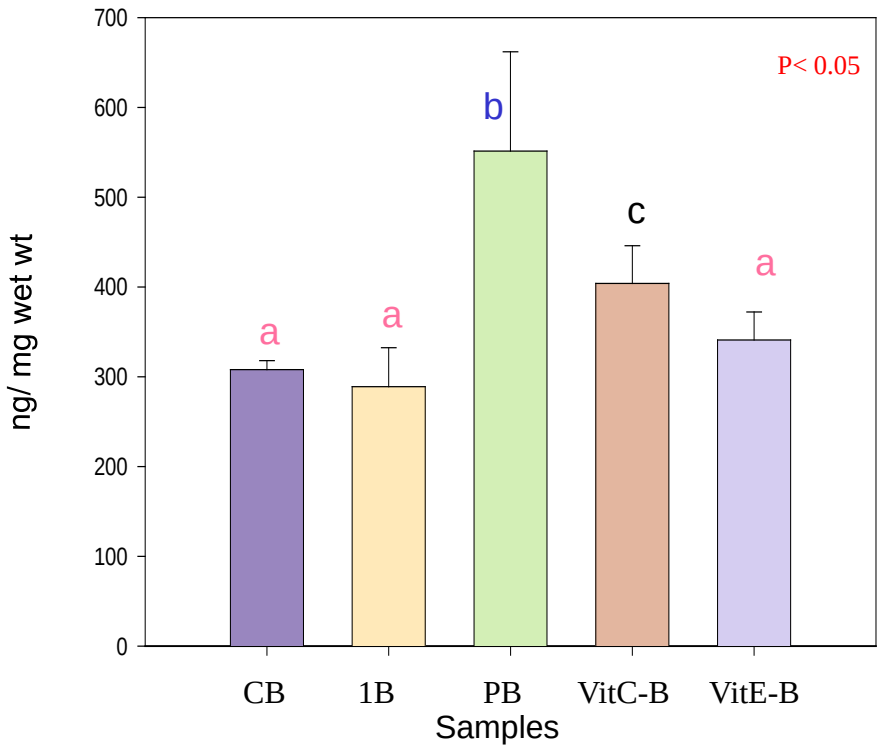
IC - 20 min Ischemia followed by 1 hr reperfusion cerebral cortex

PC - 20 min Ischemia followed by 1 hr reperfusion after 1 week *Phyllanthus* treatment cerebral cortex.

Vit C-C - 20 min Ischemia followed by 1 hr reperfusion after 1 week Vit C treatment cerebral cortex.

Vit E-C -20 min Ischemia followed by 1 hr reperfusion after 1 week Vit E treatment cerebral cortex.

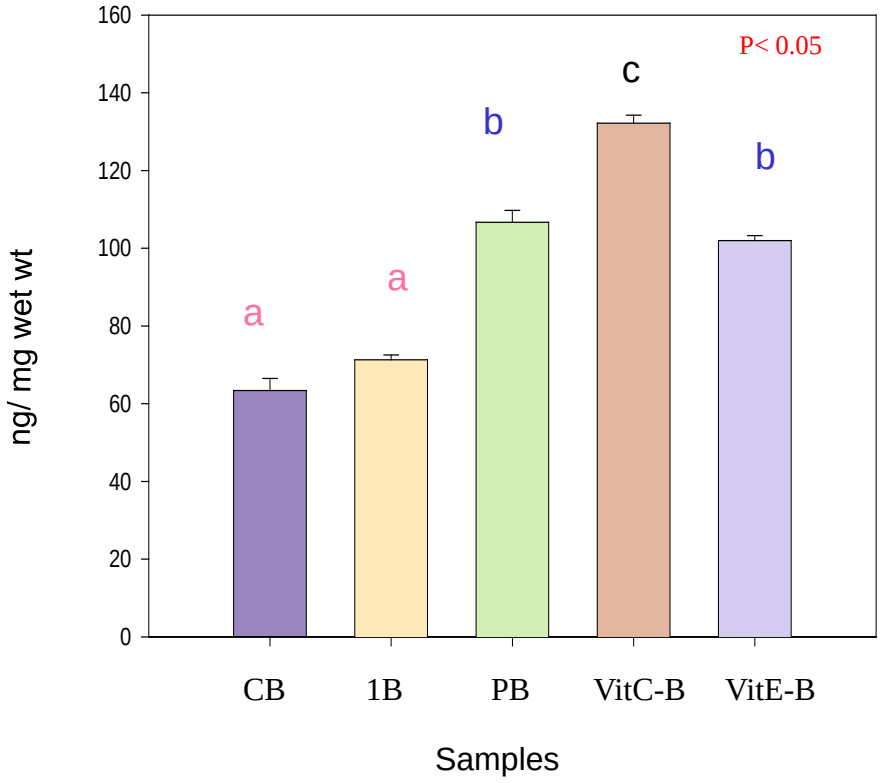
Fig 4.3a Levels of Reduced glutathione (GSH) in ischemic and Antioxidant treated Cerebellum



GSH level was decreased significantly in 1hr reperfusion when compared to control but *Phyllanthus* treatment significantly increased GSH level in cerebellum. Vitamin C and vitamin E treatments also increased GSH level but not as much as *Phyllanthus* treatment. Reduced Glutathione levels were estimated in cerebellum in 20min ischemia and 1hr reperfusion samples with antioxidant (vitamin C/ vitamin E/ *Phyllanthus*) treatment. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with significance level set at P<0.05. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

- CB - Control (Sham operated) cerebellum
- IB - 20 min Ischemia followed by 1 hr reperfusion cerebellum
- PB - 20 min Ischemia followed by 1 hr reperfusion after 1 week *Phyllanthus* treatment cerebellum
- VitC-B - 20 min Ischemia followed by 1 hr reperfusion after 1 week Vit C treatment cerebellum
- VitE-B -20 min Ischemia followed by 1 hr reperfusion after 1 week Vit E treatment cerebellum

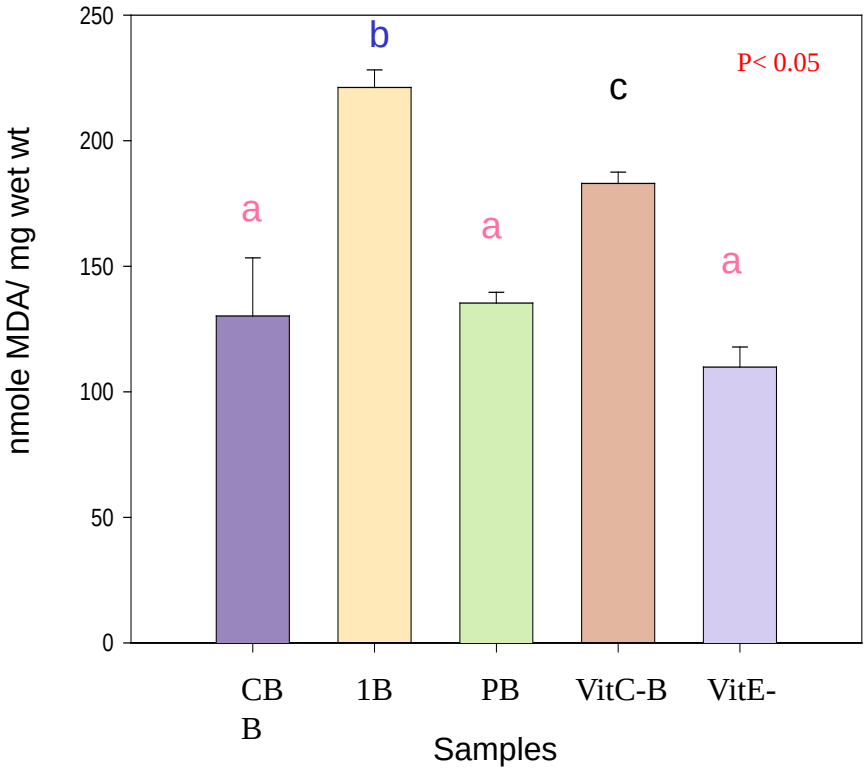
Fig 4.3b Levels of Oxidized Glutathione (GSSG) in ischemic and Antioxidant treated Cerebral Cortex



GSSG level increased significantly in 1he reperfusion as well in all the three antioxidant treated cerebellum samples when compared to control. Oxidized Glutathione levels were estimated in cerebellum in 20min ischemia and 1hr reperfusion samples with antioxidant (vitamin C/ vitamin E/ *Phyllanthus*) treatment. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and post hoc comparisons with level of significance set at P<0.05. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

- CB - Control (Sham operated) cerebellum
- IB - 20 min Ischemia followed by 1 hr reperfusion cerebellum
- PB - 20 min Ischemia followed by 1 hr reperfusion after 1 week *Phyllanthus* treatment cerebellum
- VitC-B - 20 min Ischemia followed by 1 hr reperfusion after 1 week Vit C treatment cerebellum
- VitE-B -20 min Ischemia followed by 1 hr reperfusion after 1 week Vit E treatment cerebellum

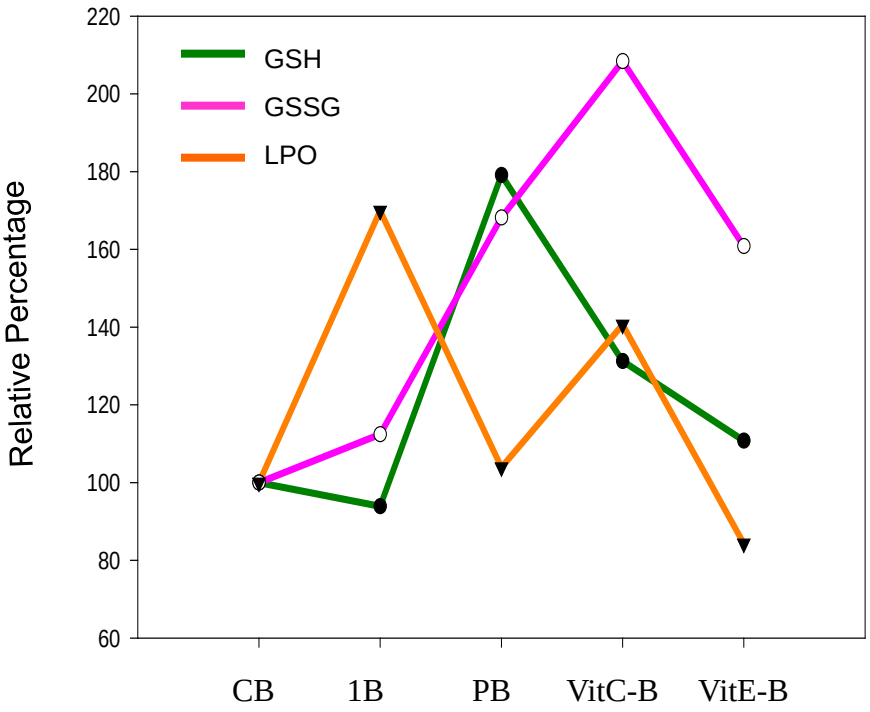
Fig 4.3c Levels of Lipid peroxides (LPO) in ischemic and Antioxidant treated Cerebellum



LPO levels increased significantly in 1hr reperfusion cerebellum when compared to control whereas Phyllanthus treatment significantly decreased LPO levels. Vitamin C and vitamin E treatments also reduced the level of LPO. Lipid peroxide levels were estimated in cerebral cortex in 20min ischemia and 1hr reperfusion samples with antioxidant (vitamin C/ vitamin E/ *Phyllanthus*) treatment. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with level of significance set at P<0.05. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

- CB - Control (Sham operated) cerebellum
- IB - 20 min Ischemia followed by 1 hr reperfusion cerebellum
- PB - 20 min Ischemia followed by 1 hr reperfusion after 1 week *Phyllanthus* treatment cerebellum
- VitC-B - 20 min Ischemia followed by 1 hr reperfusion after 1 week Vit C treatment cerebellum
- VitE-B -20 min Ischemia followed by 1 hr reperfusion after 1 week Vit E treatment cerebellum

Fig 4.3 GSH, GSSG and LPO (Percentage) levels in ischemic and Antioxidant treated Cerebellum



Phyllanthus treatment increased the GSH level with a decrease in LPO level, thereby reducing the oxidative stress upon ischemia reperfusion in cerebellum.Treatment with vitamin E also increased GSH level and decreased LPO level. Vitamin C treatment also reduced the oxidative stress but not as much as Phyllanthus or vitamin E treatments. *Phyllanthus* seems to be more effective in reducing oxidative stress in rat cerebellum when compared to vitamin C and vitamin E.

CB - Control (Sham operated) cerebellum

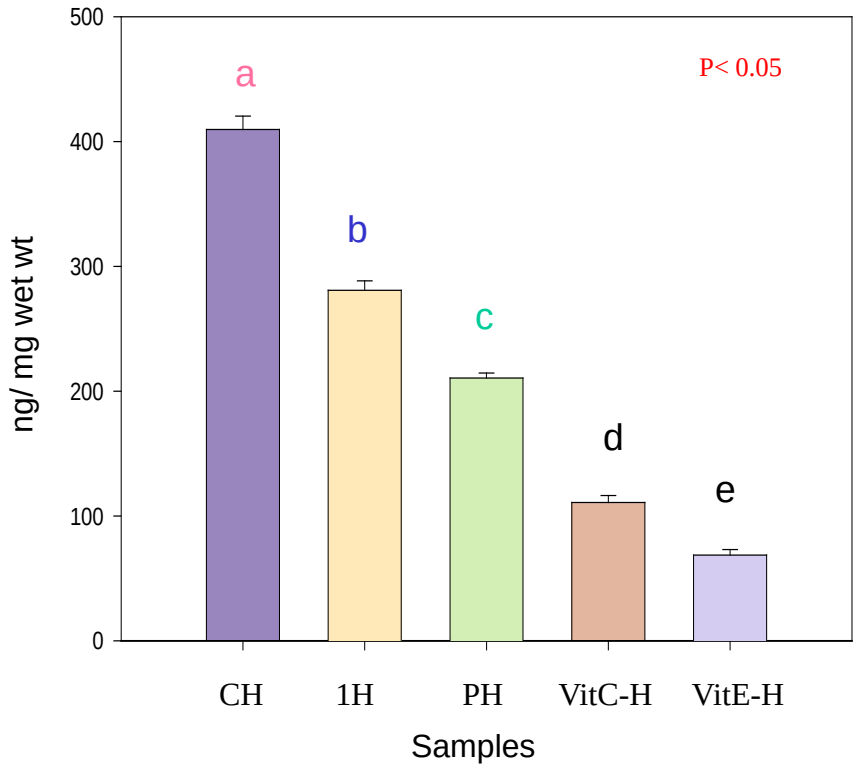
IB - 20 min Ischemia followed by 1 hr reperfusion cerebellum

PB - 20 min Ischemia followed by 1 hr reperfusion after 1 week *Phyllanthus* treatment cerebellum

VitC-B - 20 min Ischemia followed by 1 hr reperfusion after 1 week Vit C treatment cerebellum

VitE-B -20 min Ischemia followed by 1 hr reperfusion after 1 week Vit E treatment cerebellum

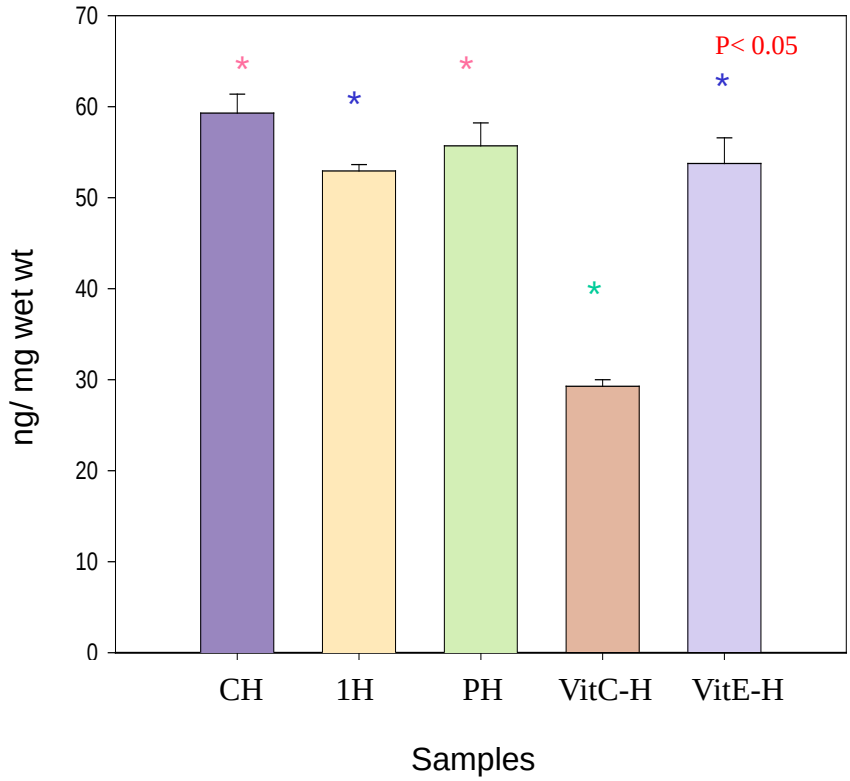
Fig 4.4a Levels of Reduced glutathione (GSH) in ischemic and Antioxidant treated Hippocampus



GSH level reduced significantly in 1hr reperfusion hippocampus as well as in all the three antioxidant treated samples when compared to control. Reduced Glutathione levels were estimated in hippocampus in 20min ischemia and 1hr reperfusion samples with antioxidant (vitamin C/vitamin E/*Phyllanthus*) treatment. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with level of significance set at P<0.05. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

- CH - Control (Sham operated) hippocampus
- IH - 20 min Ischemia followed by 1 hr reperfusion hippocampus
- PH - 20 min Ischemia followed by 1 hr reperfusion after 1 week *Phyllanthus* treatment hippocampus
- Vit C-H - 20 min Ischemia followed by 1 hr reperfusion after 1 week Vit C treatment hippocampus
- hit E-H -20 min Ischemia followed by 1 hr reperfusion after 1 week Vit E treatment hippocampus

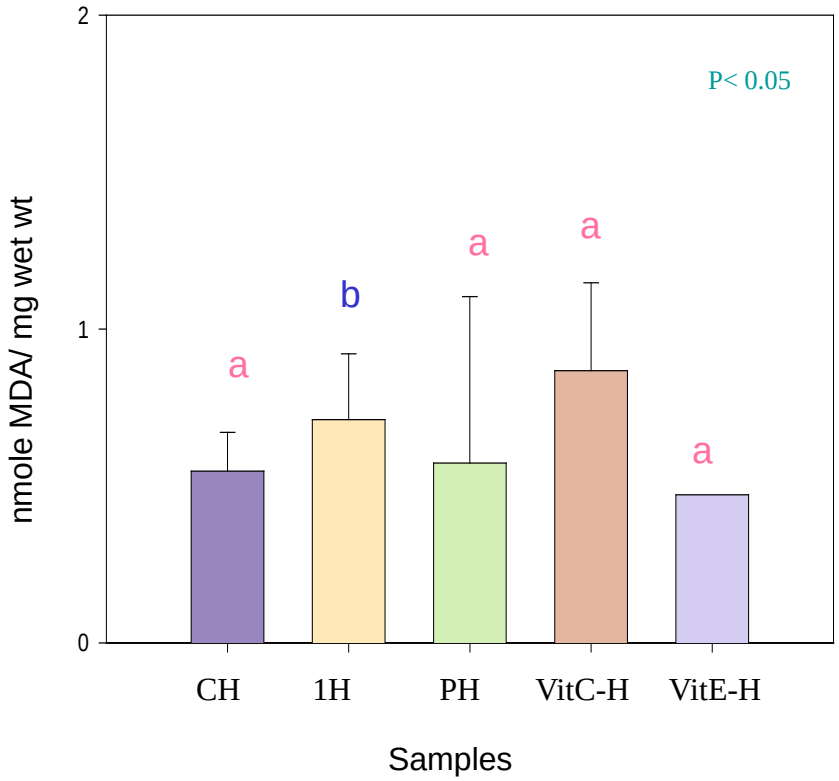
Fig 4.4b Levels of Oxidized Glutathione (GSSG) in ischemic and Antioxidant treated Hippocampus



There was a significant decrease in GSSG level in ischemic samples which underwent Vitamin C treatment. Oxidized Glutathione levels were estimated in hippocampus in 20min ischemia and 1hr reperfusion samples with antioxidant (vitamin C/vitamin E/*Phyllanthus*) treatment. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with level of significance set at P<0.05. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

- CH - Control (Sham operated) hippocampus
- IH - 20 min Ischemia followed by 1 hr reperfusion hippocampus
- PH - 20 min Ischemia followed by 1 hr reperfusion after 1 week *Phyllanthus* treatment hippocampus
- Vit C-H - 20 min Ischemia followed by 1 hr reperfusion after 1 week Vit C treatment hippocampus
- hit E-H -20 min Ischemia followed by 1 hr reperfusion after 1 week Vit E treatment hippocampus

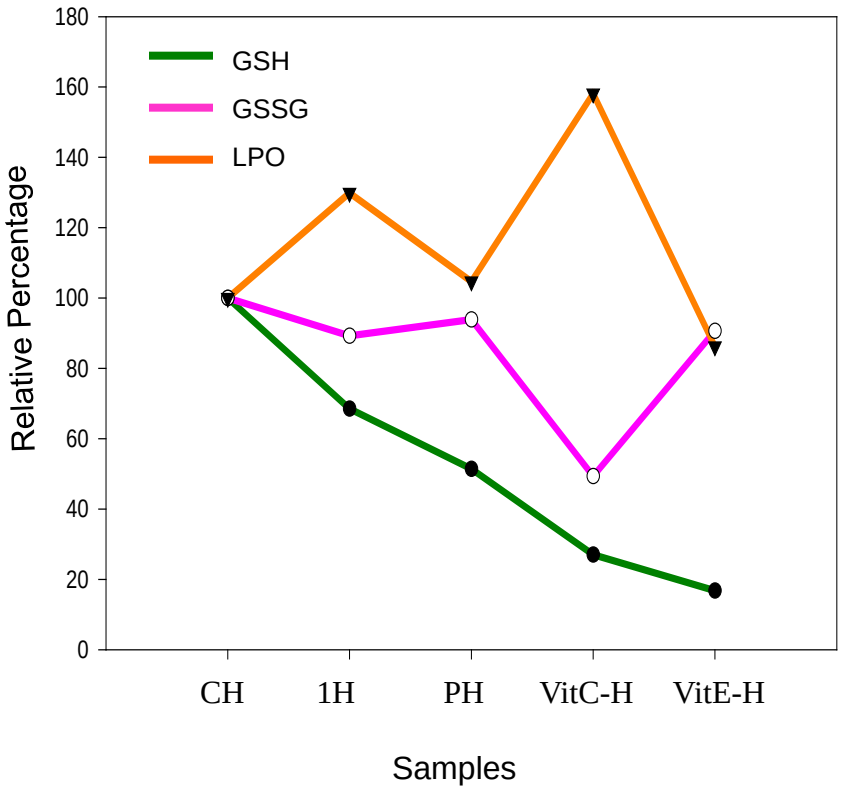
Fig 4.4c Levels of Lipid peroxides (LPO) in ischemic and Antioxidant treated Hippocampus



There was no significant alteration in the level of LPO after antioxidant treatments. Lipid peroxide levels were estimated in hippocampus in 20min ischemia and 1hr reperfusion samples with antioxidant (vitamin C/vitamin E/*Phyllanthus*) treatment. Data is expressed as Mean ± SEM (n=5). Significance was evaluated by one way ANOVA and multiple post hoc comparisons with level of significance set at P<0.05. Mean values labeled with different alphabets like a, b, c and d above each bar are significantly different from each other while those with the same alphabet are not significant.

- CH - Control (Sham operated) hippocampus
- IH - 20 min Ischemia followed by 1 hr reperfusion hippocampus
- PH - 20 min Ischemia followed by 1 hr reperfusion after 1 week *Phyllanthus* treatment hippocampus
- Vit C-H - 20 min Ischemia followed by 1 hr reperfusion after 1 week Vit C treatment hippocampus
- hit E-H -20 min Ischemia followed by 1 hr reperfusion after 1 week Vit E treatment hippocampus

Fig 4.4 GSH, GSSG and LPO (Percentage) levels in ischemic and Antioxidant treated Hippocampus



There was not significant alterations in oxidative stress parameters with antioxidant treatments in hippocampus. Antioxidant treatments seems to play no role in protecting rat hippocampus against ischemia induced oxidative stress.

CH - Control (Sham operated) hippocampus

IH - 20 min Ischemia followed by 1 hr reperfusion hippocampus

PH - 20 min Ischemia followed by 1 hr reperfusion after 1 week *Phyllanthus* treatment hippocampus

Vit C-H - 20 min Ischemia followed by 1 hr reperfusion after 1 week Vit C treatment hippocampus

hit E-H -20 min Ischemia followed by 1 hr reperfusion after 1 week Vit E treatment hippocampus