COMPARISON OF THE NEUROPROTECTIVE POTENTIAL OF THEANINE AND MINOCYCLINE

By

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DEDICATION

I would like to dedicate this thesis to my loving mother and grandmother whose prayers and faith in God were what brought me through to completing this project. Their sacrifice and love have made it possible for me to get this far with my education.

ABSTRACT

Stroke is one of the most common causes of disability and death worldwide. The most commonly experienced stroke in the clinical setting is focal ischaemia in which the middle cerebral artery (MCA) is occluded and leads to a complex series of various pathophysiological pathways that ultimately lead to neuronal cell death. Several studies have been conducted on various therapeutic agents in the search for a neuroprotective drug and various animal models have been used to carry out this research. While theanine, a component of green tea and minocycline, a tetracycline antibiotic, have been shown to possess some neuroprotective properties, the mechanisms by which these two agents carry out these effects still remains unclear. The objectives of this study were to investigate the mechanisms by which these drugs carry out these neuroprotective effects and their neuroprotective ability in a MCA occlusion model of focal ischaemia.

Ischaemia leads to oxidative stress due to the imbalance of free radicals and the endogenous antioxidant defence system. An antioxidant assay using the stable 2, 2-diphenyl-1-picryl-hydrazyl ($DPPH^{\bullet}$) radical was used to assess the antiradical properties of each drug. It was found that minocycline showed superior antioxidant activity *in vitro* when compared to theanine. Further studies on the drugs' ability to attenuate the Fenton reaction (in which iron catalyses the formation of reactive species) were elucidated using electrochemical analysis, UV/VIS studies, ferrozine and ferritin assays. It was found that minocycline, in contrast to theanine, was able to bind to iron ions and thus potentially prevent the participation of iron in metal catalysed radical reaction. The antioxidant activity of both drugs was further investigated by assessing their effect on cyanide-induced superoxide generation and quinolinic acid (QA)-induced lipid peroxidation (LP). Experimental evidence shows that both

drugs had no significant effect on the generation of superoxide *in vitro* and that there was a significant decrease in LP for minocycline *in vitro* and theanine *in vivo*. The metal binding and antioxidant properties were postulated to be a possible mechanism through which these agents reduced lipid peroxidation.

A study was conducted to determine the effects of the drugs on the biosynthesis of the neurotoxin, QA and it was found that minocycline increases the levels of holoenzyme activity of tryptophan-2, 3-dioxygenase (TDO) *in vitro* and that theanine reduces the levels of the same enzyme *in vivo* after treatment for 10 days. TDO is the enzyme that converts tryptophan to other products that enable enzymatic activity to change it to QA. Minocycline was thought to bring about this effect as it has been shown from preceding experimental studies that it is an effective reducing agent. Theanine on the other hand is hypothesised to bring about a reduction in holoenzyme activity by changing the binding of tryptophan to the enzyme or affecting the radicals that participate in the enzymatic degradation of tryptophan.

A focal ischaemic model of stroke was induced by occluding the MCA. Histological examination of the hippocampus post -ischaemia shows a reduction in the size of the infarct after pre-treatment with minocycline only. A further study into the effects of the drugs on the generation of superoxide and on the levels of the endogenous glutathione after a stroke was carried out. Pre-treatment of the animals with either theanine or minocycline showed no significant effects on the generation of the radical species or of the endogenous antioxidant which ruled out these as a mechanism of neuroprotection of both drugs, post-ischaemia.

The findings of this study provide novel information on the possible mechanisms by which both theanine and minocycline act to bring about neuroprotection. In particular in this study, pre-treatment with minocycline has shown promise in the focal ischaemic model of stroke.

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  Hosted by: University of Yaoundé 1, Yaoundé, Cameroon
  Entitled: Sleep, Epilepsy and Brain Dysfunctions
  Dates: 24-30th August 2009
  (Appendix I)

- Conference: 43rd Annual Congress 2009 of the South African Society for Basic and Clinical Pharmacology (SASBCP) held as the 5th International Conference on Pharmacy and Pharmaceutical Sciences (ICPPS), joined by the Academy of Pharmaceutical Sciences
  Hosted by: School of Pharmacy, Potchefstroom Campus of the North-West University, South Africa
  Dates: 23-26th September 2009
  (Appendix II)

LIST OF ABBREVIATIONS AND SYMBOLS

%	Percent
µg	Microgram
µl	Microlitre
ºC	Degrees Celsius
3-HAA	3-hydroxyanthranilic acid
3-HAO	3-Hydroxyanthranillic acid oxygenase
3-OH-KYN	3-hydroxy-kynurenine
5-HIAA	5-Hydroxyindole-3-acetic acid
5-HT	Serotonin
8-OH-G	8-Hydroxyguanine
A	Amperes
AD	Alzheimer's disease
AdSV	Adsorptive stripping voltammetry
Ag	Silver
Ag/AgCl	Silver/silver chloride
AMPA	α-Amino-3-hydroxy-5 methyl-4-isoxazole propionate
ANOVA	Analysis of variance
ATP	Adenosine-5'-triphosphate
BBB	Blood brain barrier
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
Ca^{2+}	Calcium (II) ion
CAT	Catalase
CCA	Common carotid artery
CCl_4	Carbon tetrachloride
Cl^-	Chloride anion
CNS	Central nervous system
Co	Cobalt
COX	Cyclo-oxygenase
CREB	Cyclic AMP response element-binding protein
Cu^+	Copper (I) ion
CuZnSOD	Copper and zinc superoxide dismutase
CYP450	Cytochrome P450
DNA	Deoxyribonucleic acid
DPPH	2, 2, Diphenyl-1-picryl-hydrazyl
DPPH•	2, 2, Diphenyl-1-picryl-hydrazyl radical
DTNB	5' 5' di-thiobis-(2-nitrobenzoic acid)
EDTA	Ethylene diamine tetra-acetic acid
ER	Endoplasmic reticulum
ET	Endothelium

FAD	Flavin adenine dinucleotide
Fe	Iron
Fe^{2+}	Iron (II) or ferrous ion
$Fe^{2+}SO_4$	Ferrous sulphate
$Fe^{2+}SO_4.7H_2O$	Hydrated ferrous sulphate
Fe^{3+}	Iron (III) or ferric ion
$Fe^{3+}Cl_3$	Ferric chloride
g	Grams
GAA	Glacial acetic acid
GABA	γ-Aminobutyric acid
GCE	Glassy carbon electrode
GSH	Glutathione
GSSG	Oxidised glutathione
H_2O_2	Hydrogen peroxide
HD	Huntington's disease
HOO•	Hydroperoxyl radical
i	Current
i.p.	Intraperitoneal
IDO	Indoleamine-2, 3-dioxygenase
K^+	Potassium (I) ion
KAT	Kynurenine amino transferase
KCl	Potassium chloride
KCN	Potassium cyanide
kg	Kilogram
KP	Kynurenine pathway
KP	Kynurenine pathway
KYN	Kynurenine
KYNA	Kynurenic acid
L	Litre
L-KYN	L-kynurenine
L-TRP	L-tryptophan
LOOH	Lipid peroxide
LP	Lipid peroxidation
L-TRP	L-tryptophan
M	Molar
MAO	Monoamine oxidase
MAPK	Mitogen activated protein kinase
MCA	Middle cerebral artery
MDA	Malondialdehyde
mg	Milligram
ml	Millilitre
mM	Millimolar
MnSOD	Manganese superoxide dismutase
MRSA	Methicillin resistant *Staphylococcus aureus*

mtPTP	Mitochondrial permeability transport pores
N	Nitrogen
n	Sample size
N_2	Nitrogen gas
Na^+	Sodium ion
Na+/K+ pumps	Sodium/potassium pumps
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NBD	Nitro-blue diformazan
NBT	Nitro-blue tetrazolium
nm	nanometres
NMDA	N-methyl-D-aspartate
nmol	nanomoles
NO	Nitric oxide
NO•	Nitric oxide radical
NOS	Nitrous oxide system
O	Oxygen
O_2	Oxygen gas
$O_2^{\bullet-}$	Superoxide anion
$^1O_2$	Singlet oxygen
•OH	Hydroxyl radical
$ONOO^-$	Peroxynitrite anion
PBS	Phosphate buffered saline
PD	Parkinson's disease
pmol	picomoles
PUFA	Polyunsaturated fatty acid
QA	Quinolinic acid
R•	Radical species
RNS	Reactive nitrogen species
ROO•	Peroxyl radical
ROS	Reactive oxygen species
RS•	Thiyl radical
SD	Standard deviation
SH	Thiol
-SH	Thiol group
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloracetic acid
TDO	Tryptophan 2, 3-dioxygenase
T_{max}	Time-to-peak
Tris-HCl	Tris hydroxymethyl amino methane

TRP	Tryptophan
UV/VIS	Ultraviolet-visible
V	Volts
v/v	Volume by volume
Vs^{-1}	Volts per second
w/v	Weight by volume
λmax	Lambda max
π	Pi bonding orbital
π*	Pi anti-bonding orbital

CHAPTER 1
LITERATURE REVIEW

1.1 STROKE

1.1.1 INTRODUCTION

Stroke is the most common cause of adult disability and death worldwide (Rantanen and Tatlisumak, 2004; Emre *et al.,* 2007; Yasuhara *et al.,* 2008). In the United States of America, it is responsible for 9.5 % of all deaths and with over 700 000 new cases of strokes occurring annually (Broderick, 1998; Tuhin, 2002). Approximately 80 % of all strokes are ischaemic (due to a brain infarction), 10 % are caused by intracerebral haemorrhage and the rest are caused by subarachnoidal haemorrhages (Feigen *et al.,* 2003; Hankey and Nelson, 2009). The disability after stroke, for example, paralysis, memory loss and pain and the cost of treatment including medication, hospitalisation and rehabilitation, causes a major socio-economic burden on society.

1.1.2 STROKE AETIOLOGY

An ischaemic stroke results from a temporary or permanent reduction or interruption in cerebral blood flow (Gupta and Briyal, 2004; Khatak, 2010). Obstruction to blood flow occurs when a major brain artery is blocked or has burst, thus depriving the brain of oxygen and essential nutrients such as glucose. The reduction of blood flow in most cases is caused by an embolism or by a thrombus obstructing the flow of blood in the artery (Dirnagl *et al.,* 1999; Zazulia, 2003).

With a reduction in blood flow, 2 areas of neuronal damage are formed around the site of the thrombus or embolism, namely the ischaemic core and the penumbral zone (Hilkle and Bowman, 2003). The ischaemic core is where the blood flow is completely interrupted resulting in irreversible cell damage due to lack of glucose and oxygen (Gupta and Briyal, 2004). The penumbra is the area surrounding the core in which ischaemia is incomplete due to perfusion from collateral vessels (Gupta and Briyal, 2004). If cerebral blood flow is

restored even at a sub-optimal level, it will provide an opportunity for the cells within the penumbra to recover and regain functional activity.

1.1.3 PATHOPHYSIOLOGY

1.1.3.1 The Cascade of Cerebral Ischaemia

Cerebral ischaemia leads to pathophysiological changes which contribute to brain injury due to the reduced glucose and oxygen (O_2) which occur collectively or individually. The pathophysiological changes include the increased production of free radicals, excitotoxicity, interruption of sodium (Na^+) and calcium (Ca^{2+}) influx, stimulation of the inflammatory process, endothelium (ET) release, delayed coagulation and endothelial dysfunction (Gupta and Briyal, 2004). The mechanisms that give rise to cell death following ischaemia are yet to be defined but it is clear that the 3 major factors which contribute to cell death are: acidosis; increased intracellular cytosolic Ca^{2+} and the production of free radicals (Gariballa, 2000; Pollock and Pollock, 2005).

The brain consumes a large amount of O_2 and glucose in order to produce energy by oxidative phosphorylation (Dirnagl *et al.,* 1999). Reduced O_2 and glucose to the tissues within the brain leads to an energy crisis and with the energy depletion comes a loss in membrane potential due to the interruption of ion pump activity (Dirnagl *et al.,* 1999; Gupta and Briyal, 2004). As the energy levels are depleted, energy dependent processes such as the presynaptic uptake of excitatory neurotransmitters are blocked, leading to an increase in glutamic acid in the extracellular space (Dirnagl *et al.,* 1999). Excess glutamic acid leads to the activation of N-methyl-D-aspartate (NMDA) receptors and metabotropic glutamate receptors leading to an increase in cytosolic Ca^{2+}. The accumulation of Ca^{2+} in the cells plays a role in the propagation of the irreversible neuronal damage through various processes such as lipid peroxidation. The cascade of events leading to ischaemic-induced neuronal death are summarised in Figure 1.1.

Extracellular oedema that develops in ischaemia is largely due to the activation of the Na^+ transport across the blood brain barrier (BBB) which reflects local brain damage caused by the damaged neurons, glia and endothelial cells (Lipton, 1999).

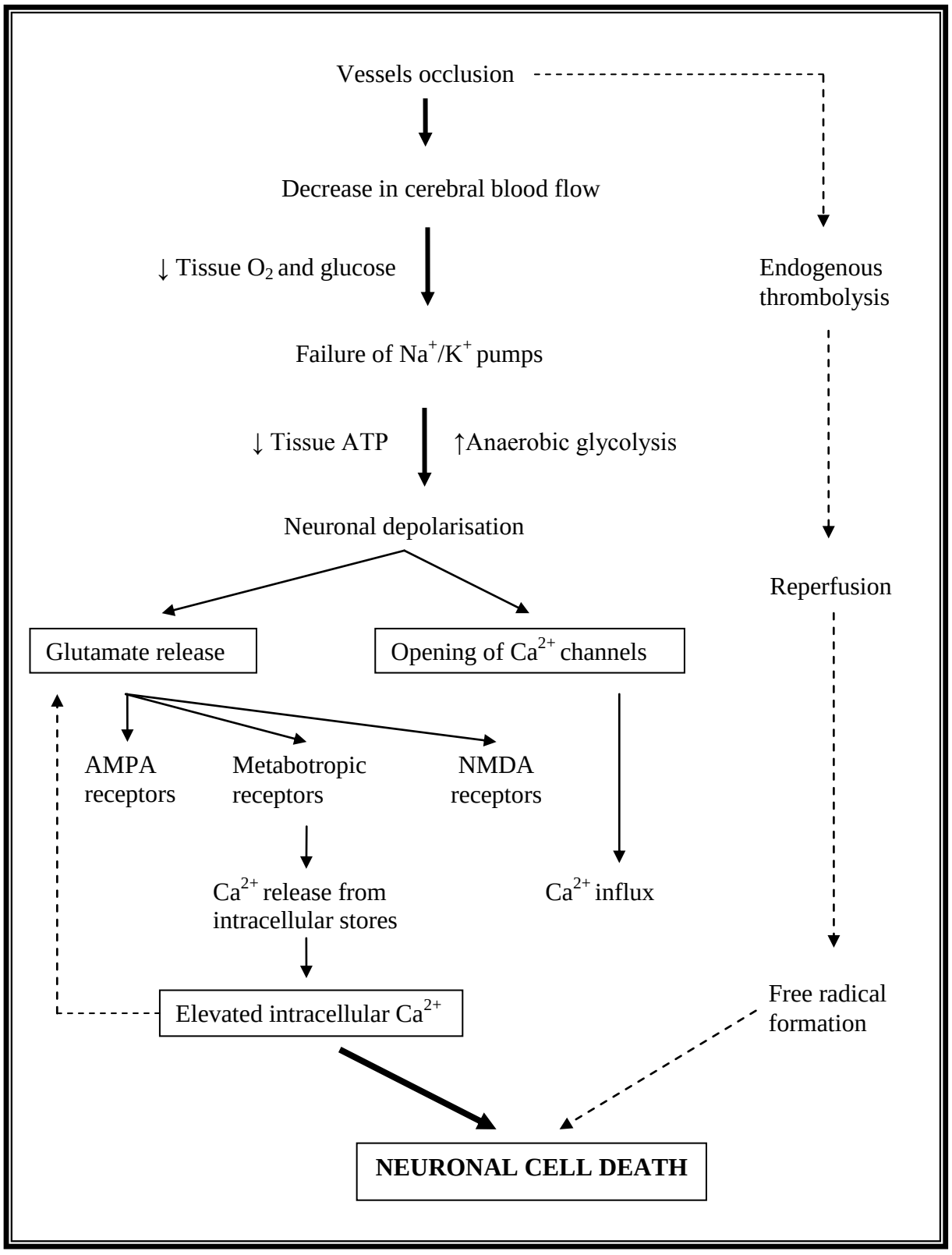

Figure 1.1: Summary of events that may lead to cell death after the onset of cerebral ischaemia redrawn from Gupta and Briyal, 2004.

1.1.3.2 Free radicals and oxidative stress

The central nervous system (CNS) and the brain are especially vulnerable to attack by free radicals because their membrane lipids are rich in polyunsaturated fatty acids (PUFAs). Additional reasons include the limited amount of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidise and catalase (Halliwell and Gutteridge, 1989). Certain areas in the brain such as the globus pallidus and substantia nigra, are rich in iron which catalyses radical reactions (Halliwell and Gutteridge, 1989). Free radical production has been implicated as an important mechanism of brain injury post-ischaemia and after reperfusion (Gariballa, 2000).

The potential mechanism for the production of free radicals during ischaemia, involves the limited supply of oxygen which leads to the electron transport chain of the inner mitochondrial membrane becoming highly reduced, resulting in oxygen radical production. The proposed mechanism for radical production during reperfusion may be due to the large quantities of superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide being produced as a result of the reperfusion and thus the peroxidation of membrane lipids in the brain (Gariballa, 2000).

The production of a greater number of free radicals than antioxidants within the brain leads to a state of oxidative stress (Bell *et al.*, 2002; Onténiente *et al.*, 2003). Oxidative stress may affect the free radical nitric oxide (NO•), which has an important role in biological processes in the brain such as vascular integrity (Draije *et al.*, 1995), the control of cerebral blood flow (White *et al.,* 1998), auto-regulation (White *et al.,* 2000) and modulation of neuronal activity and thromobogenesis (Şeneş *et al.,* 2007). Lipid peroxidation is a consequence of an increase in oxidative stress and studies have shown that there is an increase in lipid peroxidation in patients that have suffered an ischaemic stroke (Şeneş *et al.,* 2007).

1.1.3.3 Glutamate receptors and excitotoxicity in ischaemia

During ischaemia there is an increase in the levels of intracellular Ca^{2+} and extracellular excitatory amino acids such as glutamic acid and aspartate. The increase in excitatory neurotransmitters is responsible for the death of neurons within the penumbra zone (Takagi *et al.*, 1993; Lipton, 1999, Yepes *et al.*, 2000). An inverse relationship exists between the size of

the infarct and the magnitude of glutamic acid release (Takagi *et al.*, 1993). The excitotoxicity that arises as a result of the increase in excitatory neurotransmitters is induced by the overstimulation of glutamate receptors, in particular the NMDA receptors. The NMDA receptors are the major route of Ca^{2+} entry into the neuronal cell. Ca^{2+} is a secondary messenger in neurons where it plays a role in several physiological processes such as neurotransmitter release. However excess intracellular Ca^{2+} can lead to cell death. The calcium influx into cells during ischaemia is sufficient to lead to the activation of Ca^{2+} dependent processes and enzymatic routes involved in neuronal death.

There are two main energy-dependent pathways that are responsible for the elevation of extracellular excitatory neurotransmitter levels during ischaemia: a Ca^{2+} dependent pathway and the other pathway is independent of Ca^{2+} (Katayama *et al.*, 1991). The Ca^{2+} pathway occurs at the onset of ischaemia when there is sufficient energy to drive the pathway whilst the Ca^{2+} independent pathway is a delayed pathway due to the decline in energy levels as the stroke progresses (Katayama *et al.*, 1991). The increase in glutamic acid released during ischaemia results in a change in the glutamic acid transport system. The alteration of glutamic acid transportation is a consequence of 3 mechanisms namely: decreased uptake, reversed activation and decreased expression of protein transporters (Camacho and Massieu, 2006). The generation of free radicals inhibits glutamic acid uptake in the glia (Rao *et al.*, 2003). Swanson *et al.*, (1995) showed that anaerobic reactions that take place during ischaemia result in lactic acidosis and this may contribute to reduced glutamic acid uptake due to decreased functionality of glutamic acid transporters in astrocytes. Figure 1.2 summarises the failure of glutamic acid transportation that occurs during ischaemia.

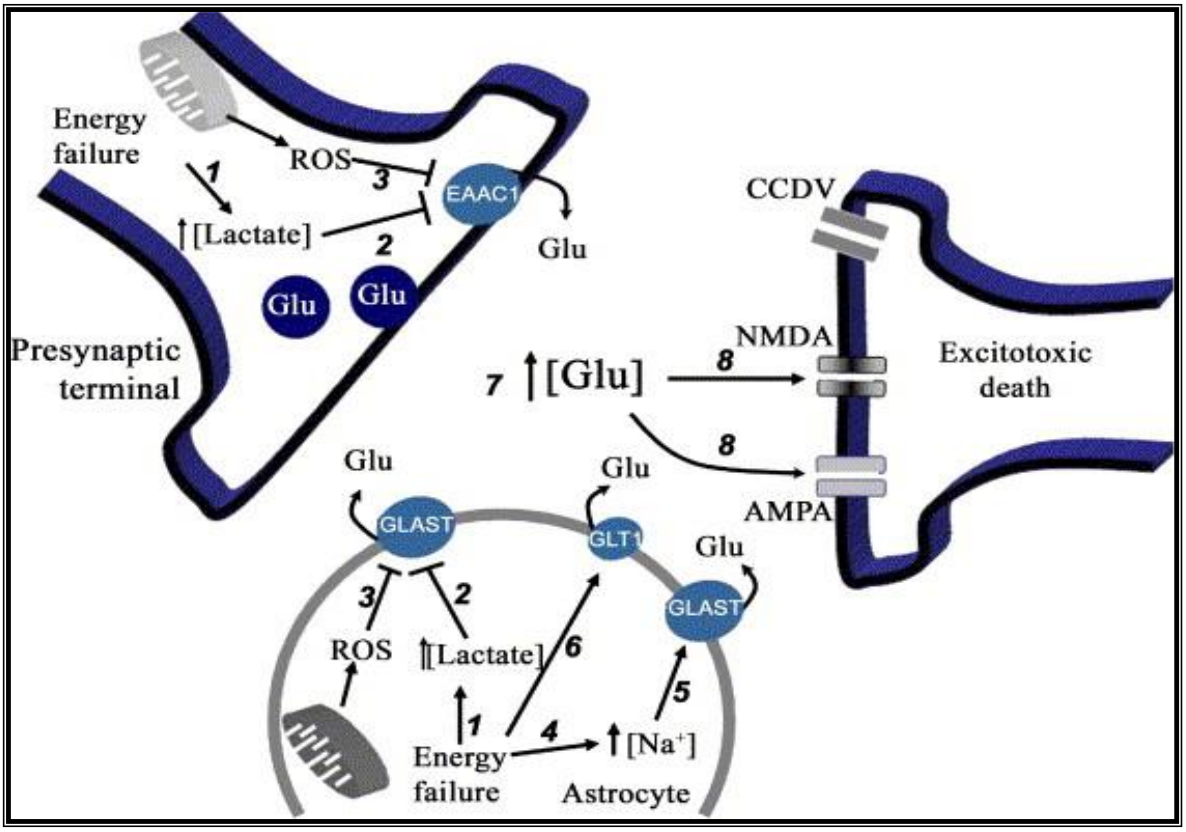

Figure 1.2: Possible alterations in the activity and the expression of glutamic acid transporters during cerebral ischemia (Camacho and Massieu, 2006).

The explanation of Figure 1.2: lack of glucose and oxygen during ischemia leads to energy failure and increased intracellular lactate (1), which inhibits glutamic acid uptake both in neurons and glial cells (2). Free radical production induced after glutamate receptor activation may oxidise cysteine residues on the glutamic acid transporter, thus inhibiting glutamic acid uptake (3). Low energy levels promote the increase in intracellular Na^+ (4) as well as the inverse functioning of glutamic acid transporters (5). Through a still unknown mechanism, down regulation of glutamic acid transporters at the plasma membrane occurs after the ischemic insult (6). The alterations shown above might be involved in the elevation of extracellular glutamic acid (7) and the development of neuronal death associated with ischemia (8) (Camacho and Massieu, 2006).

1.1.4 THE CURRENT THERAPEUTIC APPROACHES FOR THE MANAGEMENT OF STROKE

A clear understanding of the pathophysiological pathways involved in ischaemia will aid the development of effective drugs that will be useful in the prevention and management of stroke and the disability that results from it. Important objectives in the treatment of stroke entail limiting primary and secondary neuronal death by the inhibition of apoptotic mechanisms, enhancing the endogenous ability of the neuronal system to restore lost function and replacing lost cells (Onténiente *et al.,* 2003). An ideal drug for the management of ischaemia would improve blood flow, have a beneficial effect on biochemical pathophysiological pathways, and relieve the spasm of nutrient arteries and have antioxidant properties (Gariballa, 2000).

Currently, the only treatment for stroke is a thrombolytic agent. However, aside from its use being limited to administration within 3 hours of the stroke, only 5 - 8 % of patients qualify for this treatment within this period (Gupta and Briyal, 2004). Thrombolytic therapy has been shown to reduce neuronal damage and improve recovery after a stroke (Gupta and Briyal, 2004). However, large doses of the thrombolytic are associated with reperfusion-associated complications whilst low doses, although associated with lower risk have the potential of being less effective (Gupta and Briyal, 2004).

Neuroprotective agents have been investigated for their potential as drugs that may be used in ischaemic injury. Amongst these are free radical scavenging drugs such as the dietary supplement α-tocopherol (vitamin E) (Chaudhary *et al.,* 2003), the synthetic drug tamoxifen (Zhang *et al.,* 2005) and a combination of endogenous melatonin and synthetic meloxicam (Gupta *et al.,* 2002). These agents have been studied and been shown to be protective in animal models of ischaemia. Many drug models such as antagonists of voltage gated Ca^{2+} channels and antagonists of the excitatory amino acid receptors have also been studied and although these showed promise in animal models, these were found to be ineffective in human subjects (Gupta and Briyal, 2004). Therefore, there is still a need to discover a drug that possesses the ideal characteristics stated above that would be beneficial to human subjects.

1.1.5 EXPERIMENTAL MODELS OF ISCHAEMIA

1.1.5.1 Introduction

Several models exist as ischaemic experimental models for the *in vitro* and *in vivo* study of stroke. The *in vitro* models include cultured neurons, glia and cultured brain slices (Gupta and Briyal, 2004). However these models are limited as they are only able to demonstrate the level of cytotoxicity of the therapy being investigated.

In vivo experimental models are preferred as experimental models of ischaemia as these possess glia, neurons and vasculature that closely resemble the human system (Gupta and Briyal, 2004). A good *in vivo* experimental model of ischaemia will have the following characteristics (Gupta and Briyal, 2004; Woitzek *et al.*, 2006):

- The ischaemic process and the pathophysiological responses thereof must be consistent with human stroke, reproducible and reliable,
- A standard method of vessel occlusion,
- No barbiturates which have been shown to be neuroprotective, to be used during the course of the experiment,
- The infarct produced must not be extremely big and must be of a predictable average size,
- It must be a simple technique that is minimally invasive,
- Any physiological variations are to be controllable without difficulty and
- Economical and easy availability of animals of the same species.

3 main types of animal models exist to model ischaemia and these are focal global and forebrain ischaemia that are discussed below.

1.1.5.2 Forebrain ischaemia

Forebrain ischaemia as the name implies, is an experimental model of ischaemia that is limited to the forebrain. This model therefore is not an accurate representation of ischaemia that occurs in a clinical sense. There are 4 models of this type of ischaemia that have been

performed on certain species of animal models: bilateral common carotid artery (CCA) occlusion (in Mongolian gerbils), four vessel occlusion (in rats), bilateral common carotid occlusion (in spontaneously hypertensive rats) and two vessel occlusion (in rats with hypotension) (Gupta and Briyal, 2004).

1.1.5.3 Global ischaemia

In this model there is complete interruption of blood supply to the brain, leading to cerebral necrosis in isolated regions (Lipton, 1999). There are two ways in which it can be induced: total body ischaemia and global cerebral ischaemia (Gupta and Briyal, 2004). Total body ischaemia includes decapitation and cardiac arrest without resuscitation, cardiac arrest and resuscitation and systemic hypotension (Gupta and Briyal, 2004). Global cerebral models of global ischaemia can be induced by increased intracranial pressure, cervical compression and a combination of occlusion of the major arteries that supply blood to the brain (Gupta and Briyal, 2004).

1.1.5.4 Focal ischaemia

Focal ischaemia is the most common type of stroke encountered by humans. This is therefore the most useful model for the clinical setting. Furthermore, this model may be performed to bring about either permanent or temporary occlusion of the middle cerebral artery (MCA). Inducing focal ischaemia leads to a moderate to extreme reduction in blood flow in the artery (Sironi *et al.,* 2003). Temporary occlusion allows for the reperfusion of blood into the vasculature and in permanent occlusion there is no restoration of blood flow. The most commonly used models of focal ischaemia are thromboembolic, suture MCA occlusion, surgical MCA occlusion, photochemically-induced thrombosis and endothin-1-induced MCA occlusion (Gupta and Briyal, 2004; Durukan *et al.,* 2008).

This model has the advantage over global ischaemia of producing heterogeneous pathology which includes the core and penumbra (Section 1.1.2) (Gupta and Briyal, 2004). Overall this is the most suitable model of ischaemia as it fulfils the following criteria discussed in Section 1.1.1.1: it is a simple technique, it is applicable to more than one animal species, the

ischaemic lesion size is reproducible and most importantly, it is relevant to human stroke (Durukan *et al.*, 2008).

1.2 NEURODEGENERATION

1.2.1 NEURONAL CELL DEATH

1.2.1.1 Introduction

Neuronal death can occur by two distinct pathways namely: necrosis and apoptosis (Vermes and Haanan, 1994, Festjens *et al.*,2006). The mitochondria of the cell play a role in necrosis and apoptotic neuronal cell death (Sas *et al.,* 2007) which implies that both processes are energy dependent (Love, 2003). The role of the mitochondrion in both types of neuronal death is dependent on the initial insult. Necrotic cell death occurs in severe and toxic insults whereas apoptosis occurs in mild insults (Sas *et al.,* 2007). Differences that occur within a cell in both processes are shown in Figure 1.3.

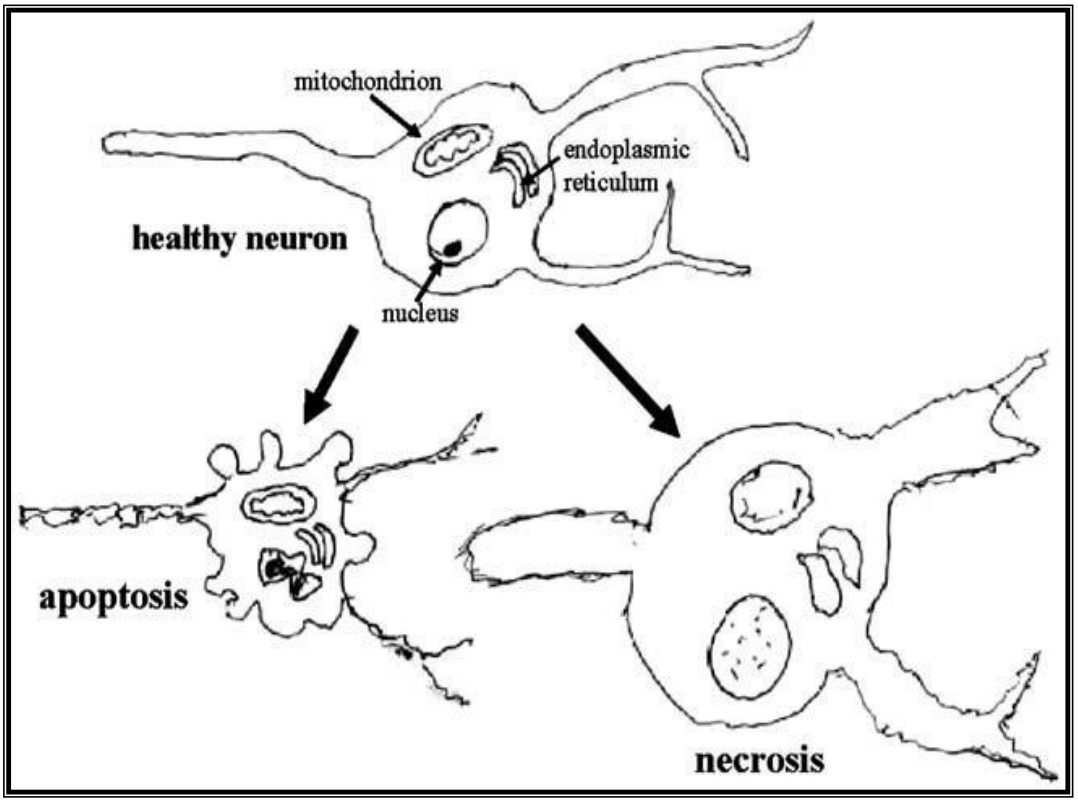

Figure 1.3: The distinguishing features of necrosis and apoptosis (Mattson, 2006)**.**

1.2.1.2 Necrosis

Necrosis, as previously mentioned, is a result of severe insult and occurs when cells are exposed to extreme conditions such as hypothermia, hypoxia and ischaemia (Wyllie *et al.,* 1998). It affects a number of cells and leads to swelling of the cytoplasm, mitochondria and other organelles within the cell and this leads to cell lysis (Sas *et al.,* 2007). Necrosis triggers an inflammatory reaction where the affected cells are phagocytised by macrophages (Wyllie *et al.,* 1998; Sas *et al.,* 2007).

1.2.1.3 Apoptosis

Apoptosis is a form of cell death that involves scattered discrete cells rather than groups of contiguous cells (Kerr *et al.,* 1972). It is a well regulated process of morphological and biochemical events. The following morphological changes occur in apoptosis: cell shrinkage as the cytoplasm shrinks and the nucleus is condensed, formation of membrane bound vesicles and the fragmentation of the cell into multiple small membrane-bound bodies that contain intact organelles (Wyllie *et al.,* 1981; Wyllie *et al.,* 1998). The apoptotic process is responsible for cell growth and development, differentiation of immune cells and the elimination of abnormal and unwanted cells without adversely affecting surrounding cells (Mattson, 2006; Sas *et al.,* 2007). No inflammatory response accompanies this type of neuronal death (Fadeel and Orrenium, 2005). Apoptosis has a role in the pathogenesis of various diseases as its exaggeration or failure in the human body can lead to diseases such as cancer and neurodegenerative disorders respectively (Sas *et al.,* 2007).

Oxidative stress can lead to an excessive influx of Ca^{2+} into the cell. Ca^{2+} regulates several important steps in the apoptotic pathway such as in early signalling events and in chromatic cleavage (McConkey and Orrenius, 1997). The process of apoptosis is mediated by caspases (cysteine-requiring aspartate-directed proteases) that are responsible for programmed cell death in mammals (Love, 2003). In models of experimental stroke, the up-regulation of caspases (specifically of caspase-3) precedes the death of neurons (Broughton *et al.,* 2009).

1.2.2 MECHANISMS OF NEURODEGENERATION

1.2.2.1 Introduction

Oxygen is essential to life for many living organisms and plays an important role in several biological processes such as the efficient production of energy. At higher levels than that found in air, oxygen can be poisonous. Aerobic organisms survive only because they have antioxidant defence systems to protect them from the damaging effects of oxygen. Cell death is attributed to the formation of free radicals especially superoxide anion which is a very toxic oxygen containing free radical (Halliwell and Gutteridge, 1984a).

Oxidative stress is a state of imbalance between radical species produced in the tissues and the antioxidant defences (Halliwell, 2007). It is manifested by excess radical production, oxidation to proteins and nucleic acids, loss of reductive potential of cells and lipid peroxidation. The brain is particularly vulnerable to oxidative damage as it consumes about 20 % of the oxygen inspired, it is rich in polyunsaturated fatty acids and metal ions and it has relatively low levels of antioxidant defences (Papa, 1996; Faraci, 2004; Butterfield, 2006).

1.2.2.2 Free radicals

Halliwell and Gutteridge (1989) define a free radical as 'any species that is capable of independent existence that contains one or more unpaired electrons'. The presence of an unpaired electron makes the free radical highly reactive. The reaction of a free radical with a non radical species leads to the generation of different radicals which may be more or less reactive than the original reacting radical (Halliwell and Gutteridge, 1986). When two free radicals react, the species generated are 'neutral' and unreactive.

The respiratory chain in the mitochondria is one of the major sources of free radicals in the human body (Sas *et al.,* 2007). Other pathways responsible for the production include xanthine oxidase, monoamine oxidase (MAO), cytochrome P450 (CYP450), nitrous oxide systems (NOS) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Sas *et al.,* 2007). Free radicals generated within the body damage lipids, proteins, deoxyribonucleic acid (DNA) and other biomolecules (Halliwell et al., 1995). There are different types of free

radical species but these can be generally divided into oxygen and nitrogen containing species termed reactive oxygen species (ROS) and reactive nitrogen species (RNS) respectively. ROS and RNS are important in neuronal signalling and physiology but at elevated levels these can lead to dysfunction of neurons and death (Butterfield, 2006; Faraci, 2006). ROS are produced inside cells and have a short life span, thus their toxic effects are often limited to the cell in which they were produced or cells in close proximity to the place of their production (Kontos, 2001). ROS are important mediators in cell death as these are involved in the two major causes of cell death which are apoptosis and excitotoxicity (Sas *et al.,* 2007) or by acting synergisistically with pro-inflammatory mediators (Slater *et al.,* 1987).

1.2.2.3 Superoxide anion radical

The superoxide anion radical ($O_2^{\bullet-}$) is the primary product when oxygen is reduced by accepting an electron from a reducing agent. Of the oxygen consumed by the body, 17 % of it is converted into $O_2^{\bullet-}$ (Halliwell and Gutteridge, 1986). The levels of $O_2^{\bullet-}$ within a cell are determined by the rate of its production, the activity of antioxidant enzymes and the availability of oxygen (Kontos, 2001). The $O_2^{\bullet-}$ is highly reactive and is able to reduce and oxidise other compounds whereas other reactive species such as hydrogen peroxide and the hydroxyl radical are only able to act as oxidising agents (Kontos, 2001). The $O_2^{\bullet-}$ is unable to cross biological membranes and very few compounds and enzymes are able to react with it (Şenes *et al.,* 2007).

The $O_2^{\bullet-}$ causes damage to biomolecules by inducing the oxidation of the lipids within cell membranes and their interaction with hydrogen peroxide or organic peroxides can generate highly reactive entities that damage DNA, lipids and other essential cell components (Halliwell and Gutteridge, 1986). Iron (III) release from ferritin where it is stored, is facilitated by $O_2^{\bullet-}$, which reduces it into iron (II). The reduced iron is then available to catalyse iron-dependent radical reactions and lipid peroxidation. Overgeneration of $O_2^{\bullet-}$ during an ischaemic stroke (Şenes *et al.,* 2007) coupled with the production of $O_2^{\bullet-}$ that occurs due to the accumulation of monocytes and macrophages after ischaemia (Saito *et al.,* 1993) results in neuronal damage.

Not all the effects of $O_2^{\bullet-}$ are deleterious. During an inflammatory reaction, $O_2^{\bullet-}$ contributes to the death of bacteria that has been engulfed by granulocytes (Halliwell and Gutteridge, 1986). The effects of $O_2^{\bullet-}$ are more damaging in the presence of hydrogen peroxide.

1.2.2.4 Hydrogen peroxide

Any biological system that generates $O_2^{\bullet-}$ will produce hydrogen peroxide (H_2O_2) by the enzymatic conversion of $O_2^{\bullet-}$ by superoxide dismutase and other enzymatic reactions (Halliwell and Gutteridge, 1989). Unlike $O_2^{\bullet-}$, H_2O_2 is able to cross biological membranes but its reactivity is limited (Halliwell *et al.*, 1995; Kontos, 2001). H_2O_2 is not highly toxic after it diffuses across cellular membranes, but in the presence of $O_2^{\bullet-}$ it can react with metal ions such as Fe^{2+} and Cu^{+} to produce the hydroxyl radical which is a highly reactive free radical (as explained in Section 1.2.2.5).

1.2.2.5 Hydroxyl radical

The hydroxyl radical (•OH) is an extremely reactive radical that is the product of the Haber-Weiss reaction (Equation 1.3) which is the reaction between $O_2^{\bullet-}$ and H_2O_2 in the presence of transition metal ions such as Fe^{3+}, Fe^{2+} and Cu^{+} (Halliwell and Gutteridge, 1989). The Fenton reactions involve the reduction of iron (III) to iron (II) in the presence of $O_2^{\bullet-}$ (Equation 1.1) and then oxidation of iron (II) back into iron (III) facilitated by the homolytic fission of H_2O_2 (Equation 1.2). The net reaction of the two Fenton reactions (Equations 1.1 and 1.2) is the Haber-Weiss reaction.

Beckman and colleagues (1990) reported that the protonation and decomposition of peroxynitrite forms a free radical that is of similar reactivity to •OH. This finding was later confirmed by van der Vliet and co-workers (1994a) who showed that the radical was indeed •OH.

$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$	**Equation 1.1**	**Fenton reaction**
$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$	**Equation 1.2**	
$O_2^{\bullet-} + H_2O_2 \xrightarrow{Metal\ catalyst} OH^- + \bullet OH + O_2$	**Equation 1.3**	**Haber-Weiss reaction**

The •OH is a very short lived radical that is limited to the site of its catalytic production (Valko *et al.,* 2005) but it can initiate chain reactions that yield secondary radicals that may expand the range of its toxic action. The •OH reacts immediately with all molecules within its vicinity by hydrogen abstraction, electron transfer or by addition reactions (Slater, 1978) and because of its high rate constant it will react with all types of molecules within a living organism such as DNA, amino acids and phospholipids (Halliwell *et al.,* 1995).

1.2.2.6 Peroxyl radical

The peroxyl radical (ROO•) is mainly formed as a product of the chain propagation step in lipid peroxidation (Halliwell and Gutteridge, 1989) and non lipid systems (Dean *et al.,* 1993). The simplest ROO• that can be formed is the hydroperoxyl radical (HOO•) which can react with thiols to produce thiyl radicals (RS•) (Halliwell *et al.,* 1995) and in the chain reactions in the oxidation of lipids.

1.2.2.7 Singlet oxygen

This species is mainly formed by photosensitisation reactions. The formation of singlet oxygen ($^1O_2$) can take place in the retina of the eye and certain diseases such as porphyrias lead to excess $^1O_2$ formation (Halliwell and Gutteridge, 1989). Due to the absence of unpaired electrons within its structure, $^1O_2$ is not regarded as a free radical but is classified as a powerful non-radical oxidising agent (Halliwell *et al.,* 1995). Examples of compounds that readily react with $^1O_2$ are DNA, tryptophan (TRP) and NADPH. The generation of $^1O_2$ by photosensitisation reactions has been used medically (called photodynamic therapy). For example, photodynamic therapy is used in the treatment of genital sores caused by *Herpes Simplex* (Halliwell and Gutteridge, 1989).

1.2.2.8 Nitric oxide radical

Under normal physiological conditions, the nitric oxide free radical (NO•) serves an important role as a mediator in various processes such as cerebral blood flow (White *et al.*, 1998, Toda *et al.*, 2009), autoregulation and cerebral vasodilation (White *et al.*, 2000). However, when its concentrations are elevated during ischaemia, peroxynitrite is formed by the reaction of NO• and $O_2^{\bullet-}$ (Zalba *et al.*, 2000) and it is the peroxynitrite anion that leads to further oxidative damage such as oxidation of lipids.

1.2.2.9 Peroxynitrite anion

The reaction of NO• and $O_2^{\bullet-}$ produces the peroxynitrite anion ($ONOO^-$) (Beckman *et al.*, 1990) which is a powerful oxidising agent that has powerful bactericidal and parasiticidal properties (Beckman *et al.*, 1990). Van der Vliet and colleagues (1994b) showed that $ONOO^-$ depleted antioxidants in the blood as it rapidly oxidised ascorbic acid, uric acid and plasma thiol groups. These authors also discovered that $ONOO^-$ contributed to oxidative damage by oxidising lipids and proteins. The $ONOO^-$ can further cause oxidative damage when it is decomposed to •OH (Beckman *et al.*, 1990).

1.2.2.10 Mitochondrial dysfunction

The mitochondria are responsible for supplying cells with energy and thus it consumes most of the oxygen inspired. Other than supplying energy to the cell, it also plays an important role in other cell processes such as Ca^{2+} homeostasis, regulation of cell cycles, free radical generation, thermogenesis and signalling processes which are important for cell death (apoptotic and necrotic), aging and development processes (Sas *et al.*, 2007). Mitochondrial dysfunction is thus marked by disturbances of Ca^{2+}, adenosine-5'-triphosphate (ATP) or the metabolism of ROS (Brookes *et al.*, 2004). Excitotoxicity (Section 1.2.3.11) and respiratory toxins such as cyanide (Section 1.2.6) lead to mitochondrial dysfunction. Parkinson's disease (PD), Alzheimer's disease (AD) and ischaemia-reperfusion injury are diseases marked by oxidative stress and mitochondrial dysfunction (Simanian and Coyle, 1996).

When the function of one or more respiratory chain complexes within the mitochondria is damaged, there is enhanced free radical production. This results in oxidative damage of the mitochondria and opening of the mitochondrial permeability transport pores (mtPTPs) ultimately leading to the disruption of mitochondrial function (Sas *et al.,* 2007). The opening of the mtPTPs leads to the induction of apoptosis. During normal mitochondrial function, a small proportion of electrons leak out during oxidative phosphorylation. When there is a problem with mitochondrial functioning, excess electrons leak out and thus combine with oxygen to generate ROS which ultimately leads to a state of oxidative stress within the cell (Beal, 2000). Oxidative stress then leads to the opening of the mtPTPs which results in a cycle of events leading to further damage of the mitochondria and ultimately to pathways that result in cell death (Hong *et al.,* 2004).

1.2.2.11 Excitotoxicity

Glutamic acid is a major excitatory neurotransmitter that plays an important role in memory and learning (Kakuda, 2002). It is released from neuronal cells via an impulse and stimulates glutamate receptors and opens ion channels leading to permeability of sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}). There are three types of ionotropic glutamate receptors namely: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and kainite receptors. When the ATP levels are depleted due to an injury, the mitochondria become dysfunctional and depolarisation of the neuronal membrane occurs (Section 1.2.3.10). This results in excess glutamic acid being released into the extracellular space, leading to glutamic acid binding to NMDA and non-NMDA receptors, increasing the cell permeability to Ca^{2+} and Na^+ resulting in neuronal cell death (Figure 1.4) (Kakuda, 2000; Kakuda *et al.,* 2000; Arundine and Tymianski, 2004). Excitotoxicity is therefore neuronal death mediated by the overstimulation of the glutamate receptors.

Two components make up excitotoxicity: a Na^+ and chloride ion (Cl^-) dependent component which is characterised by immediate cell swelling and the other is Ca^{2+}-dependent cell degeneration component (Rothman and Olney, 1987; Arundine and Tymianski, 2004). An increase in the influx of Na^+ and Cl^- creates an osmotic imbalance between the neuron and the extracellular environment (Rothman and Olney, 1987). The osmotic imbalance causes water

to flow into the cell, causing cell swelling, damage and potential cell lysis, which are characteristics of cell necrosis (Epstein and Gendelman, 1993).

Excessive Ca^{2+} influx is a result of the overstimulation of glutamate receptors mediated by prolonged activation of the glutamate receptors or by neuronal injury inducing changes in receptor functioning (Arundine and Tymianski, 2004). Ca^{2+} overload ultimately leads to an increase in the production of ROS and RNS, activation of genetic signals, leading to apoptosis and mitochondrial dysfunction.

Stimulation of the NMDA receptor resulting in a Ca^{2+} influx in the neuronal cell decreases the membrane potential of the mitochondria leading to the opening of mtPTP (Schinder *et al.*, 1996). This then results in the uncoupling of the electron transfer from ATP synthesis and this hampers energy metabolism and increases free radical production (Beatrice *et al.*, 1980).

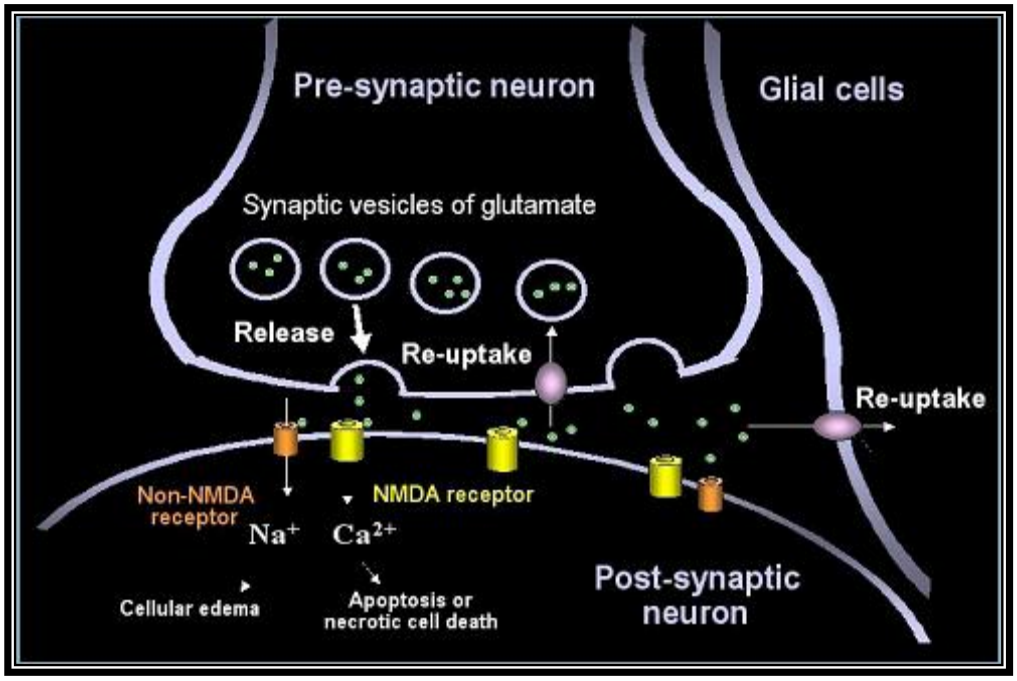

Figure 1.4: A schematic diagram showing mechanisms of excitotoxicity. *Stimulation of NMDA and non-NMDA receptors and cellular pathways ultimately leading to excitotoxicity* (Epstein and Gendelman, 1993).

Neuronal cell death is also mediated by AMPA receptor-mediated Ca^{2+} influx by the activation of phosphorylation of cyclic AMP response element-binding protein (CREB) via the stimulation of the mitogen activated protein kinase (MAPK). Thus the overstimulation of the glutamate receptors by glutamic acid accumulation has proven to be neurotoxic and the

metabolic disturbances that result from it have been implicated in many diseases such as AD (Hynd *et al.*, 2004) and depression (Hayley *et al.*, 2005; Yao and Reddy, 2005).

1.2.3 OXIDATION OF MACROMOLECULES

1.2.3.1 Introduction

Oxidative damage is the damage that radical species cause on biomolecules such as lipids, DNA and proteins within living organisms (Hallliwell, 2007). The alteration of physiologically critical molecules such as these is associated with various pathologies (Laguerre *et al.*, 2007).

1.2.3.2 Oxidation of nucleic acids

Free radicals can cause the damage of DNA in several ways and the damage that occurs is preferentially localised in certain genes (Lu *et al.*, 2004b). Damage to DNA can also occur by direct damage by H_2O_2, decreased levels of ATP and NAD^+; a decrease in the ratio of GSH to GSSH; an increase in intracellular Ca^{2+}(Halliwell and Gutteridge, 1986), ROS directly reacting with DNA (Ames *et al.*, 1993) or when ROS-derived lipid-hydroperoxides breakdown into genotoxins (Marnett, 2000). ROS such as $ONOO^-$ can lead to covalent modifications of DNA (Lee and Blair, 2001; Sas *et al.*, 2007). Lipid hydroperoxides are derived from ROS and produced enzymatically by the action of cyclo-oxygenases (COX) on PUFAs (Lee and Blair, 2001).

The presence of oxidised DNA bases is often used as a marker for free oxidative DNA damage (Helbock *et al.*, 1999). The guanine base is usually used as a biological marker for oxidative damage to DNA as it is particularly sensitive to oxidation and is converted to 8-hydroxyguanine (8-OH-G) in the presence of the •OH (Figure 1.5).

Under normal conditions, lesions that result from genotoxins derived from ROS and lipid hydroperoxides are repaired in order to maintain the integrity of DNA. If the lesions are not repaired, this leads to DNA replication and subsequently mutation (Marnett, 2000; Lee and Blair, 2001). Irreparable damage to DNA triggers apoptosis and necrosis (Sas *et al.*, 2007). In

extreme oxidative stress conditions, extensive damage to DNA leads to the activation of apoptotic machinery and switches the mode of cell death from apoptosis to necrosis (Virág, 2005). With age, mitochondrial DNA becomes more susceptible to oxidative stress due to a decrease in repair capacity and its proximity to the respiratory chain (Sas *et al.*, 2007).

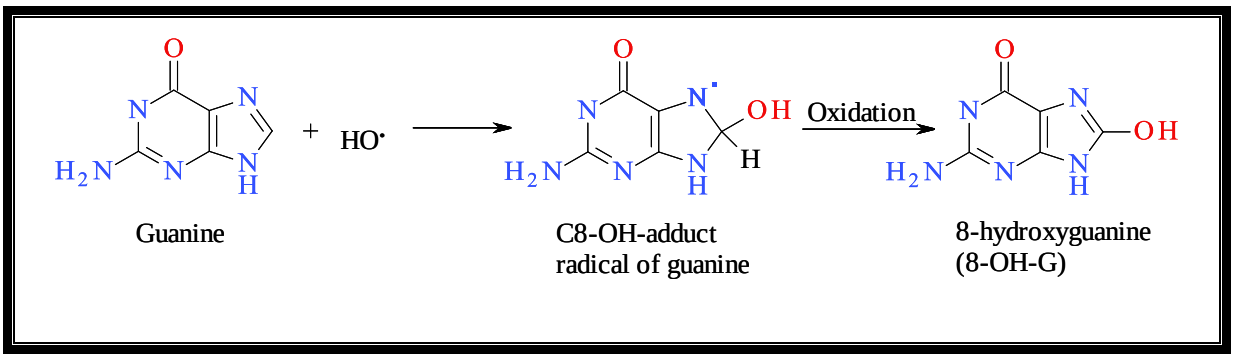

Figure 1.5: Showing the radical attack to the guanine base (Shigenaga *et al.*, 1989).

1.2.3.3 Oxidation of proteins

Oxidative stress that leads to the damage of proteins depends on many factors which include: the nature and location of the free radical, the proximity of the radical to the protein and the concentration of antioxidants (Grune *et al.*, 1997). The most common products of protein oxidation are aggregates of covalently and non-covalently cross-linked proteins (Grune *et al.*, 1997).

Oxidation of proteins is initiated in several ways: electron leakage, metal-ion dependent reactions, auto-oxidation of lipids (Dean *et al.*, 1997), elevation of levels of intracellular Ca^{2+} in response to oxidative stress (McConkey and Orrenius, 1997) and when lipid hydroperoxides lead to the production of bifunctional electrophiles (Lee and Blair, 2001). Figure 1.6 shows the postulated mechanisms of protein oxidation. Fortunately, only a small number of proteins are damaged in cells subjected to oxidative stress (Halliwell, 2007) but the damage results in the modification of protein structure and function (Lee and Blair, 2001; Butterfield, 2006). Oxidised proteins are not physiologically inactive and their unfolding makes them susceptible to proteinases (Dean *et al.*, 1997). Protein nitration leads to the modification of protein function which is thought to be important in the pathomechanism of several diseases such as Huntington's disease (HD), AD and PD.

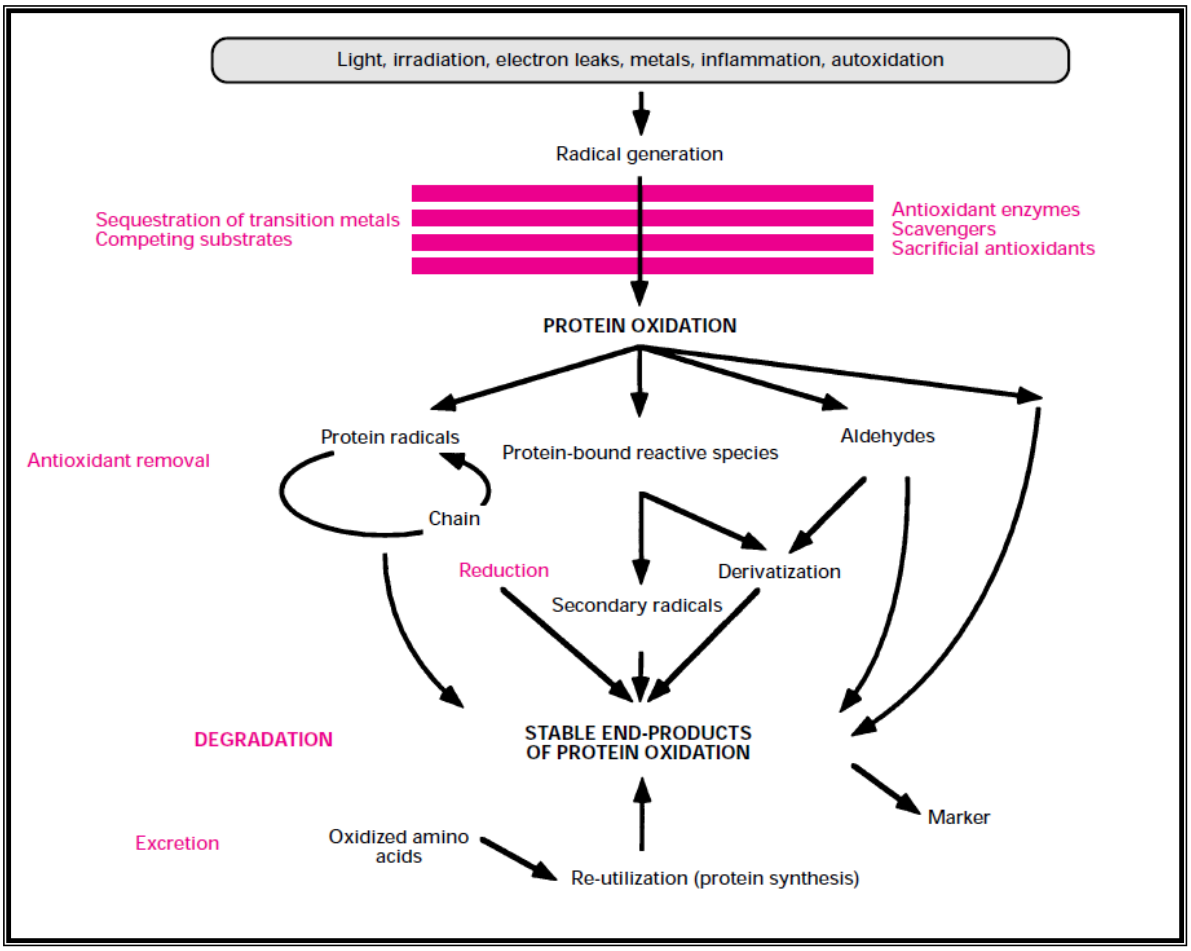

Figure 1.6: Mechanisms of protein oxidation *in vivo* (Dean *et al.*, 1997).

1.2.3.4 Lipid peroxidation

Lipid peroxidation (LP) refers to the oxidation of lipids within the body as a result of oxidative stress. It occurs by three reaction pathways:

- Non-enzymatic chain auto-oxidation mediated by free radicals,
- Non-enzymatic and non-radical photo-oxidation and
- Enzymatic oxidation.

Biological lipids are susceptible to LP due to the presence of double bonds within their structure. LP occurs in 3 stages namely chain initiation, chain propagation and chain termination (McBrien and Slater, 1982; Laguerre *et al.*, 2007). Chain initiation occurs when the initiator such as a chemical agent or free radical, causes the homolytic fission of a hydrogen atom on the lipid leading to the formation of lipid and hydrogen radicals.

Propagation is the next step in LP that involves the conversion of lipid radicals into lipid peroxide radicals and the other radical species into ROS. The duration of a propagation reaction is dependent on the fatty acid composition, concentration of oxygen and presence of antioxidants within the membranes (Gutteridge and Halliwell, 1990). Termination is the final stage in which two radicals react or the radical species react with another species to form a non-reactive product.

During cell injury, enzymes such as COX and lipo-oxygenases are activated and the release of transition metal ions from injured cells takes place. Enzymatic-induced LP takes place when the cell injury-induced enzymes and the released transition metal stimulate the production of fatty acid peroxides (Gutteridge and Halliwell, 1990). LP is often a late event of cell injury that accompanies rather than causes final cell death (Smith *et al.*, 1983; Gutteridge and Halliwell, 1990).

LP causes physical and chemical changes within the membrane which alter the integrity of the membrane (Ottino and Duncan, 1997), impair membrane function, decrease fluidity, increase non-specificity in permeability to ions such as Ca^{2+} and the inactivation of membrane-bound receptors and enzymes (Gutteridge and Halliwell, 1990). An increase in intracellular Ca^{2+} due to oxidative stress increases the availability of transition metals which initiate LP and thus increases LP especially in injured tissue (Halliwell and Gutteridge, 1986). The degree of oxidative stress in lipids is assessed by determining the concentration of lipid oxidation products in the body fluids or by the susceptibility of lipids to peroxidation induced *ex vivo* (Dotan *et al.*, 2004)

Many studies have shown that damage to cells caused by ROS and LP play a crucial role in ageing and in the pathogenesis of several chronic and acute diseases such as PD, cancer and lupus (Halliwell and Gutteridge, 1984a; Esterbauer, 1993). There are several health risks that are associated with consumption of the products of LP (Esterbauer, 1993).The body can be protected from the harmful effects of LP by antioxidants, low oxygen tension and metal chelators (Barber and Bernheim, 1967).

1.2.4 METAL IONS

Transition metal ions are able to participate in radical reactions that lead to the generation of ROS (Halliwell and Gutteridge, 1989). Injury induced increases in intracellular Ca^{2+} lead to the increase in availability of transition metals within the cell (Halliwell and Gutteridge, 1986). Iron is responsible for the Fenton reaction that generates the toxic •OH radical (Section 1.2.3.5). Cu^{+} also reacts with H_2O_2 to generate the •OH but with a much greater rate constant than Fe^{2+} does (Halliwell and Gutteridge, 1984a). Cu^{+} in the blood does not generate "free" •OH because any Cu^{+} is bound to proteins in the blood and is thus made unavailable to react with H_2O_2 (Rowley and Halliwell, 1983). Of all transitional metals ions, Fe^{2+} is the most powerful pro-oxidant (Shimada *et al.,* 1992).

Transition metals are found at the active site of many enzymes and because these are able to accept and donate single electrons by overcoming spin restriction (Hill, 1981). These are efficient in the catalysis of the oxidation of biomolecules as they simultaneously bind to biomolecules and dioxygen, thus effectively serve as a bridge between the oxygen and the biomolecule (Miller *et al.,* 1990).

Transition metal ions have the ability to stimulate the oxidation of lipids by decomposing lipid peroxides (LOOH) into radicals that continue the chain propagation step of LP (Equation 1.4) (Halliwell and Gutteridge, 1984b; Gutteridge and Halliwell, 1990). The alkoxyl and peroxyl radicals lead to further LP by abstracting hydrogen atoms from neighbouring lipids. In general, metals such as cobalt (Co), copper (Cu) and iron (Fe) with 2 valency states and suitable redox potentials increase the rate of LP (Barber and Berheim, 1967).

$$LOOH \xrightarrow{Metal\ ion} \underset{\text{(alkoxyl radical)}}{LO\bullet} + \underset{\text{(peroxyl radical)}}{LOO\bullet} \qquad \textbf{Equation 1.4}$$

Excessive amounts of transition metals, as well as deficiencies in them, have been implicated in several diseases. For example, excess copper in certain parts of the brain have been shown

to contribute to the initiation and/or the progression of several neurodegenerative diseases such as AD, whilst copper deficiency leads to an alteration of glucose metabolism (Uriu-Adams and Keen, 2005).

1.2.5 CYNANIDE

Cyanide is a very potent respiratory toxin (Yen *et al.,* 1995). The main mechanism in which cyanide acts involves the inhibition of cytochrome oxidase aa_3 which is a terminal oxidative enzyme of the electron transport chain (Johnson *et al.,* 1987). The cyanide-induced histotoxic hypoxia that occurs, results in elevated levels of brain Ca^{2+} (Johnson *et al.,* 1986) which is an important mediator in neuronal death and changes in intracellular ionic regulation (Johnson *et al.,* 1986). Johnson and colleagues (1987) showed that a single dose of cyanide causes significant LP in the brain and this leads to both functional and morphological manifestations of neurotoxicity. Cyanide also causes DNA fragmentation leading to cell death that resembles apoptosis (Mills *et al.,* 1996; Mills *et al.,* 1999). An agent that will attenuate the toxic effects of cyanide would have to prevent such reactions from occurring. Chlorpromazine has been shown by Moduh and colleagues (1988) to be an effective antidote for cyanide poisoning by its ability to prevent Ca^{2+} influx and LP.

1.2.6 QUINOLINIC ACID

1.2.6.1 Introduction

Quinolinic acid (2, 3-pyridine dicarboxylic acid; QA) is an endogenous amino acid that is a metabolite of the tryptophan-kynurenine pathway that is usually present in nanomolar concentrations in the human brain (Moroni, 1999). QA unlike its bioprecursors TRP and kynurenine (KYN) is unable to cross the BBB and it may be thus assumed that it is synthesised within the brain (Foster *et al.,* 1984). QA production is regulated by the bioavailability of the iron (II) ion, the compartmentation of metabolic enzymes and the presence of 3-hydroxyanthranilic acid oxygenase (3-HAO) (Foster *et al.,* 1986). Substantial increases in QA level have been found in infections and inflammatory neurological disorders (Heyes *et al.,* 1996) as it is produced by immune activated macrophages (Heyes *et al.,* 1998).

1.2.6.2 Biosynthesis of quinolinic acid

1.2.6.2.1 Introduction

TRP is one of the twenty standard amino acids in the human body. The largest proportion of TRP within the central nervous system (CNS) is converted to indoleamines (indoles or indole derivatives containing amine groups) or used in protein synthesis (Saito *et al.,* 1993). The majority of TRP is metabolised by the kynurenine pathway (KP) (Figure 1.7) which represents the major catabolic route for TRP in the brain and in the periphery (Sas *et al.,* 2007). The conversion of TRP to L-kynurenine (L-KYN) is initiated by the oxidation of the indole ring of TRP under aerobic conditions (Knox and Mehler, 1950).

The major metabolites of KP are kynurenic acid (KYNA), QA and 3-hydroxy-kynurenine (3-OH-KYN) (Sas *et al.,* 2007), all of which are neuroactive. KYNA is a non-competitive α-7-nicotinic acetylcholine receptor blocker (Hilmas *et al.,* 2001) and has affinity for the NMDA receptor and α-amino-3-hydroxy-5 methyl-4-isoxazole propionate (AMPA) receptor (Sas *et al.,* 2007). QA, a potent neurotoxin, is an N-methyl-D-aspartate (NMDA) excitotoxic receptor agonist (Saito *et al.,* 1993; Šťastný *et al.,* 2004). Whereas QA is neurotoxic, KYNA is neuroprotective. Rios and Santamaria (1991) reported that KYNA blocks QA-induced LP.

Some TRP metabolites of KP are powerful antioxidants. Christen *et al.,* (1990) reported that TRP metabolites such as 3-hydroxyanthranilic acid (3-HAA) and 3-OH-KYN have been shown to possess free radical scavenging properties by the scavenging of the peroxyl radical. Imbalances in KP metabolites (particularly KYNA, QA and 3-OH-KYN) may lead to disturbances in normal brain functioning and this may play a role in the pathology of some brain disorders (Sas *et al.,* 2007).

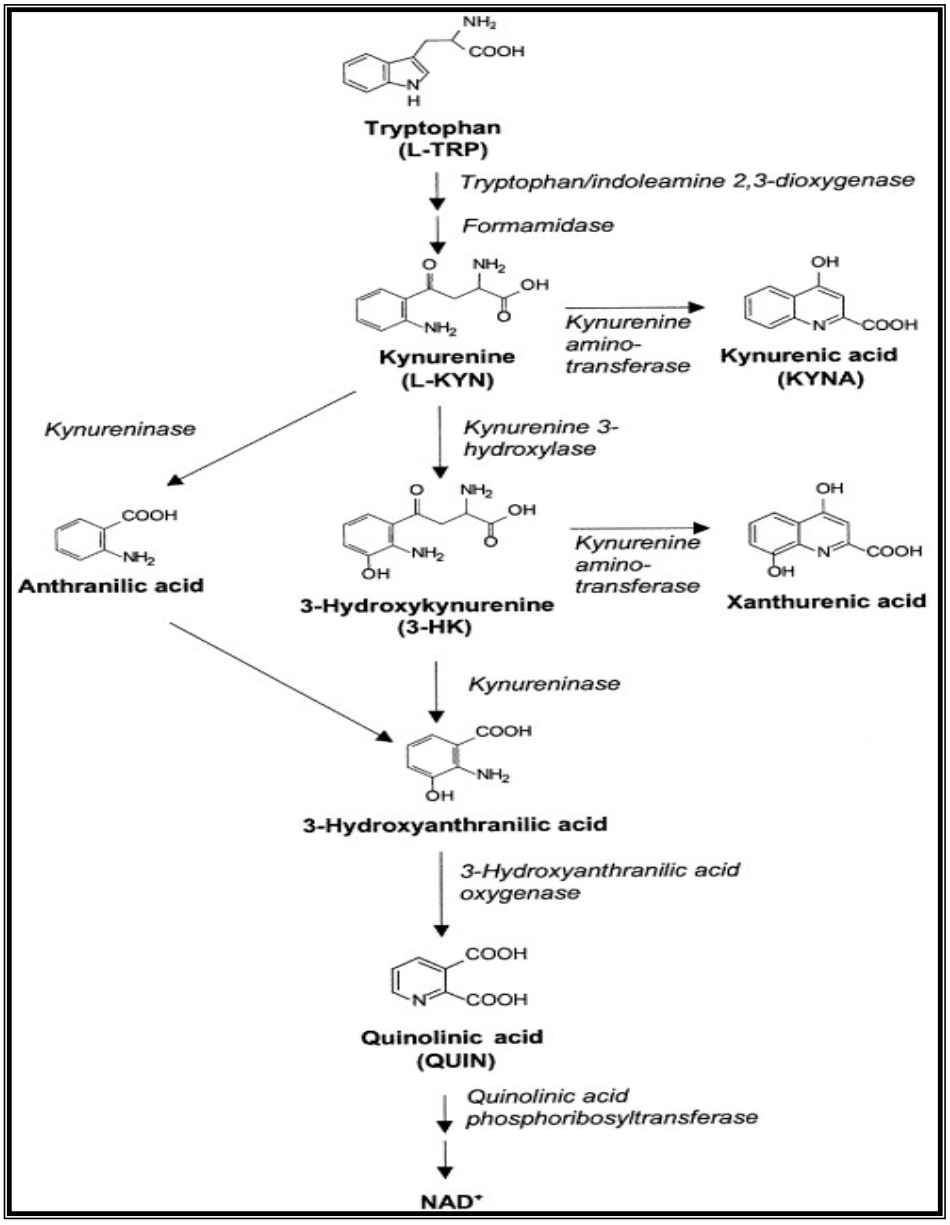

Figure 1.7: Graphical representation of the KP. Tryptophan 2, 3-dioxygenase (TDO) or indoleamine 2, 3-dioxygenase (IDO) catalyse the formation of kynurenine (Sas *et al.*, 2007)

Some neurological diseases and pathological states occur when there is a change to KP metabolites (Sas *et al.*, 2007). Increases in the toxic metabolite 3-OH-KYN imply low levels of KYN which contributes to the susceptibility of neurons to damage. Elevated levels of KYNA exert an inhibitory effect on NMDA receptors and this may explain the cognitive deficits in patients with Down's syndrome (Baran *et al.*, 1996) and the cognitive deterioration that occurs in AD.

1.2.6.2.2 Enzymes regulating the Kynurenine Pathway

1.2.6.2.2.1 Tryptophan-2, 3-dioxygenase

This enzyme is also known as tryptophan pyrrolase or L-tryptophan-oxygen 2, 3-oxidoreductase. In humans it is expressed in the liver and not in other tissues (Takikawa, 2005). Tryptophan-2, 3-dioxygenase (TDO) is specific for L-TRP and is responsible for the conversion of L-TRP to L-KYN. TDO exists in two forms namely the already active holoenzyme and the apoenzyme form which requires haeme and haematin for its activation *in vivo* and *in vitro* respectively (Badawy and Evans, 1975). Haeme is a co-factor of TDO and regulates the activity of TDO (Badawy and Evans, 1975).

Haeme is required for activity of TDO and reversibly binds the inactive apoenzyme to form the oxidised and inactive holoenzyme containing ferrihaeme (Equation 1.5) (Knox and Piras, 1966). TRP then binds to the inactive holoenzyme reducing it to the fully active form which contains haeme in the ferrous form which results in TRP becoming a free radical (Equation 1.6). Oxygen that is present then binds to the TRP radical complex and $O_2^{\bullet-}$ is generated. The $O_2^{\bullet-}$ produced results in the oxidation of the ferric ion in the haeme to the ferrous form (Equation 1.7).

$$Apoenzyme + Haeme - Fe^{3+} \rightarrow Holoenzyme - Fe^{3+}$$

(inactive) (inactive)

Equation 1.5

$$Holoenzyme - Fe^{3+} + TRP \rightarrow Holoenzyme - Fe^{3+} - TRP$$

(inactive) (inactive)

$$\downarrow$$

$$Holoenzyme - Fe^{2+} - TRP \bullet$$

(active)

Equation 1.6

$$Holoenzyme - Fe^{2+} - TRP \bullet + O_2 \rightarrow Holoenzyme - Fe^{2+} - TRP \bullet$$

$$\downarrow \qquad\qquad | \; O_2$$

$$Holoenzyme - Fe^{3+} - TRP \bullet$$

$$\downarrow \qquad\qquad | \; O_2^{\bullet -}$$

$$Holoenzyme - Fe^{3+} + TRP\ peroxide$$

Equation 1.7

1.2.6.2.2.2 Indoleamine-2, 3-dioxygenase

Indoleamine-2, 3-dioxygenase (IDO) is a haeme containing dioxygenase similar to TDO. Human IDO has a number of substrates it oxidises which include D- and L-TRP (Yamamoto and Hayaishi, 1967), D- and L-5-hydroxy KYN and TRP (Shimizu *et al.,* 1978) unlike TDO which has only one substrate L-TRP. IDO acts on its indoleamine substrates by cleaving the pyrrole ring by utilising oxygen and $O_2^{\bullet -}$ as a cofactor (Hayaishi *et al.,* 1957). The KYN produced by IDO can be further metabolised by KP into various phenolic compounds (Christen *et al.,* 1990).

IDO is expressed in a number of peripheral organs such as the brain, colon, kidney, large and small intestines, spleen and stomach whereas TDO synthesis is limited to the liver. IDO activity is induced by the activation of dendritic cells such as macrophages and astrocytes and

this results in the decline of TRP levels systemically and locally (Takikawa, 2005). Interferons, which are soluble proteins that are released by cells in the immune system, appear to use the mechanism of TDO induction which ultimately leads to lower TRP to inhibit growth of bacteria (Takikawa, 2005). Interferon-γ, which has been used to treat renal tumours (Dermott and Atkins, 2005), causes IDO-induced TRP depletion which in turn causes a decrease in serotonin (5HT) synthesis and thus accounts for interferon-γ side effects such as depression, diarrhoea and anxiety (Ebbinghaus *et al.*, 2005). Other than the catabolism of TRP, other physiological functions of IDO include the suppression of various intracellular pathogens and viral infections (Takikawa, 2005).

1.2.6.2.2.3 Formamidase

TRP is converted by either IDO or TDO to N-formyl-KYN which is degraded by formamidase to L-KYN. The KYN produced is further metabolised in three ways, by kynurenine amino transferase (KAT), KYN 3-hydroxylase and kynureninase.

1.2.6.2.2.4 Kynurenine 3-hydroxylase

KYN 3-hydroxylase is a flavin adenine dinucleotide (FAD)-dependent monooxygenase, which is responsible for the hydroxylation of L-KYN to 3-OH-KYN (Schwarcz and Pellicciari, 2002). Astrocytes generally lack KYN 3-hydroxylase and thus favour the synthesis of KYNA (Guillemin *et al.*, 2001). Thus astrocytes are neuroprotective in terms of maximising the production of KYNA and minimising QA synthesis (Stone, 2001b).

1.2.6.2.2.5 Kynureninase

Kynureninase is a pyridoxal phosphate-dependent enzyme (Schwarcz and Pellicciari, 2002). It catalyses the hydrolysis L-KYN and 3-OH-KYN to 3HAA.

1.2.6.2.2.6 Kynurenine amino transferase

Kynurenine amino transferase (KAT) is responsible for the conversion of KYN to KYNA and the conversion of 3-OH-KYN to xanthurenic acid. There are two types of KATs in the

brain and distributed mainly in astrocytes but also in the hippocampus and striatum (Okuno *et al.,* 1991). Microglial cells contain little KAT and thus produce intermediates that favour the QA branch of KP (Guillemin *et al.,* 2001).

1.2.6.2.2.7 3-Hydroxyanthranilic acid oxygenase

3-Hydroxyanthranilic acid oxygenase (3-HAO) is produced in peripheral organs although higher concentrations of it are found in the liver and kidney. 3-HAO catalyses the conversion of 3-HAA to an unstable intermediate α-amino-β-carboxymuconoic acid semialdehyde, which rearranges itself non-enzymatically to QA by cyclisation and dehydration or is enzymatically converted to picolinic acid (Decker *et al.,* 1961). Neuroprotection shown by 3-HAO inhibitors has been attributed to the prevention of QA formation and thus decrease in brain levels of QA (Sas *et al.*, 2007). 3-HAO is sometimes responsible for regulating the rate of substrate influx through KP (Saito *et al.*, 1993).

1.2.6.3 The neurotoxicity of QA

QA is a weak but specific competitive agonist of the NMDA receptor. When it binds to NMDA receptors, it leads to an increase in intracellular calcium and this leads to the initiation of many reactions that contribute to cell death and the promotion of LP (Štipek *et al.*, 1997; Iwahashi *et al.,* 1999; Šťastny *et al.*, 2004). LP is involved in the neurotoxic effects of QA (Santamaria and Rios, 1993). By the activation of the NMDA receptor and induction of LP, QA leads to changes in the permeability of the BBB (Šťastný *et al.,* 2004).

QA has the ability to generate free radicals such as reactive oxygen species (ROS). The increase in intracellular Ca^{2+} caused by QA stimulating NMDA receptors leads to a cascade of events that lead to the generation of free radicals. Beal *et al.,* (1986) discovered that when QA is injected intracerebrally, it causes a decrease in γ-aminobutyric acid (GABA) which leads to the generation of •OH. There is no effective removal mechanism for QA in the extracellular space, the outcome of which contributes to QA has long lasting effects and high *in vivo* potency (Foster *et al.,* 1984).

QA has been implicated in the aetiology or pathology of various neurological diseases especially those that involve inflammatory reactions (Sas *et al.,* 2007) such as ischaemia brought on by strokes (Stone, 2001a).

1.3 NEUROPROTECTION

1.3.1 INTRODUCTION

Neuroprotection occurs when neuroprotective agents are able to prevent the effects of toxins to neuronal cells. Free radicals have a role in the pathology of several human conditions such as cerebral ischaemia and AD and thus agents that effectively inhibit radical formation or reactions will be found to be protective (Gutteridge and Smith, 1988).

1.3.2 ANTIOXIDANT THERAPY

1.3.2.1 Introduction

An antioxidant is defined by Halliwell *et al.,* (1995) as a substance that when present at lower concentrations compared to those of an oxidisable substrate significantly delays or prevents the oxidation of that substrate. Antioxidants are either synthesised in the body e.g. enzymes, or are taken in as part of the diet (Halliwell, 2007). It is possible that an antioxidant may effectively protect one system from oxidative stress but fail to protect or even lead to the damage of another system (Halliwell *et al.,* 1995). The antioxidants are categorised according to their mechanism: preventing the formation of active oxidants; scavenging (donation of electrons to reactive species); transforming reactive species into inert compounds; stabilising biological membranes; removal of substances that catalyse free radical-induced damage; quenching and removal of active oxidants and repair of damage caused by oxidation products and adaptive responses (Gutteridge *et al.,* 1983; Etsuo *et al.,* 2005; Ligumsky *et al.,* 2005). Antioxidants work in controlling the levels of oxidative species rather than completely eliminating them because it is energetically cheaper to repair or replace damaged biomolecules rather than maintaining excess antioxidant defence levels (Halliwell, 2007).

1.3.2.2 Enzymatic antioxidant systems

There are many enzymes that exist in the mammalian body that protect the body from the deleterious effects of free radicals and these are shown in Tables 1.1 and 1.2 according to the body compartment in which they are found. These enzymatic antioxidants are classified as preventative antioxidants as they prevent the reactive species from initiating toxic reactions.

Table 1.1: Intracellular antioxidant defence systems in mammals (Halliwell and Gutteridge, 1986).

Intracellular Antioxidants	**Function**
Superoxide dismutase	Removal of $O_2^{\bullet-}$
Catalase	Removal of H_2O_2
Glutathione peroxidise Glutathione-S-transferase Related GSH-requiring enzymes	Removal of H_2O_2 and lipid peroxides (without reactive aldehyde formation)

Table 1.2: Extracellular antioxidant defence systems in mammals (Halliwell and Gutteridge, 1986).

Extracellular Antioxidants	**Function**
Extracellular superoxide dismutase	Removal of $O_2^{\bullet-}$ (but present in very low concentrations)
Uric acid	Scavenging of •OH and $^1O_2$. Binds to iron and copper ions in forms that are poorly reactive in radical reactions
Caeruloplasmin	Inhibition of LP; quickly oxidises Fe^{2+} and Fe^{3+} that can bind to transferrins
Albumin	Binds metals (especially to copper but, also, weakly, iron). Inhibits copper-dependent radical reactions
Haptoglobin/ Haemopexin	Bind free haemoglobin/haem

H_2O_2 is a non-radical oxidising species that can give rise to the formation of the highly reactive radical •OH (Section 1.2.3.4). There are 2 types of enzymes that are responsible for the removal of H_2O_2 from cells and these are catalases and peroxidases. Catalase (CAT) enzymes contain either iron or manganese (Mn) within their structure and convert H_2O_2 into water and O_2 (Halliwell and Gutteridge, 1989). Glutathione (GSH) peroxidase catalytically converts 2 GSH which contains a thiol (-SH) group and a H_2O_2 into oxidised glutathione (GSSG) and water respectively (Equation 1.8). GSSH is the oxidised form of GSH and consists of 2 GSH molecules joined together. GSH peroxidise is specific for GSH and it donates a hydrogen to GSH.

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$ **Equation 1.8**

GSH peroxidase has other substrates other than H_2O_2 and these include hydroperoxides and peroxynitrite. Other enzymes exist that protect the body from the harmful effects of H_2O_2, these include cytochrome c peroxidise and NADH peroxidise among others (Halliwell ad Gutteridge, 1989).

Superoxide dismutase (SOD) is the enzyme that catalyses the dismutation of $O_2^{\bullet-}$ to form H_2O_2 (Halliwell *et al.,* 1995; Didion *et al.,* 2002). SOD is able to react with $O_2^{\bullet-}$ at a very rapid rate. Its levels in the plasma and synovial fluid are very low (Halliwell and Gutteridge, 1986). Uric acid is the end product of purine metabolism; it is present in the serum and scavenges $O_2^{\bullet}$, ROO• and •OH radicals. Besides being an effective radical scavenger, it is a good chelator of metal ions (Davies *et al.,* 1986).

1.3.2.3 Metal chelators

Metal chelators form complexes with metal ions and are classified as preventative antioxidants as these prevent metal ion-catalysed generation of reactive species (Benzie and Szeto, 1999; Laguerre *et al.,* 2007). Chelators of metal ions include ethylene diamine tetra-acetic acid (EDTA), albumin which forms complexes with copper ions, ferritin which complexes with iron ions and haemopexin and haptoglobin which form complexes with haem and haem proteins respectively (Gutteridge and Halliwell, 1990).

Iron storage and transport proteins such as ferritin and transferrin are regarded as part of the antioxidant defence systems in the body as they store iron within their structure and prevent it from otherwise participating in the Fenton reaction (Section 1.2.2.5) if it was in the "free form" (Halliwell and Gutteridge, 1989). Metallothioneins are proteins that are present in the liver and kidney which are able to enter the nucleus of the cell and bind to zinc, copper and mercury ions, thus preventing them from participating in harmful reactions that transition metals would otherwise participate in (Section 1.2.5) (Halliwell and Gutteridge, 1989). Haeme is an iron containing molecule that is able to participate in radical reactions that can

lead to oxidation of lipids, proteins and DNA. Haemopoxin is able to remove it from the circulatory system of the body and it is in this manner that it can be termed a preventative antioxidant (Gutteridge and Smith, 1988).

1.3.2.4 Free radical scavengers

1.3.2.4.1 Introduction

Free radical scavengers are antioxidants that are preferentially oxidised in place of biomolecules (Halliwell and Gutteridge, 1989). Free radical scavengers act by donating an electron to reactive radical species. In so doing, these prevent the progression of radical-mediated oxidative damage (Halliwell and Gutteridge, 1989).

1.3.2.4.2 Endogenous scavengers of free radicals

Endogenous antioxidants are localised in either the lipophilic or hydrophilic compartments of the body. Lipophilic compartments such as membranes and lipoproteins have lipophilic antioxidants such as vitamin E distributed within their structure (Etsuo *et al.*, 2005) and the antioxidant is able to scavenge radicals that arise from within them. For example, during LP, radicals such as ROO• and chain initiating species are scavenged by lipophilic radicals. The hydrophilic antioxidants such as vitamin C are distributed in the aqueous compartments of the body and are able to scavenge radicals that arise there (Etsuo *et al.*, 2005). Vitamin C is able to directly scavenge •OH even at millimolar concentrations (Halliwell and Gutteridge, 1989). LP of low density lipids is effectively inhibited by the combination of vitamins C and E (Etsuo *et al.*, 2005).

1.3.2.4.2.1 Melatonin

Melatonin (5-methoxy-N-acetyl-tryptamine) a product of tryptophan metabolism, is an endogenous neurotransmitter that is produced in the pineal gland (Reiter, 1991) and is responsible for the regulation of circadian rhythms (Cagnacci, 1996). It has several neuroprotective properties (summarised in Figure 1.8), including the ability to significantly reduce LP induced by several methods (Sewerynek *et al.*, 1995; Daniels *et al.*, 1998;

Southgate and Daya, 1999). A study by Limson and colleagues (1998) showed that melatonin is able to bind to aluminium, cadmium, lead and zinc. This could mean that melatonin could potentially inhibit metal ion-induced oxidative reactions. In addition to this, exogenously administered melatonin is able to reduce the degree of tissue and neurovascular damage and behavioural deficits associated with strokes (Reiter *et al.,* 2003; Macleod *et al.,* 2005; Reiter *et al.,* 2005; Chen *et al.,* 2006). Melatonin has been shown to be neuroprotective in several neurodegenerative disorders such as AD and HD (Tunez and Montilla, 2007).

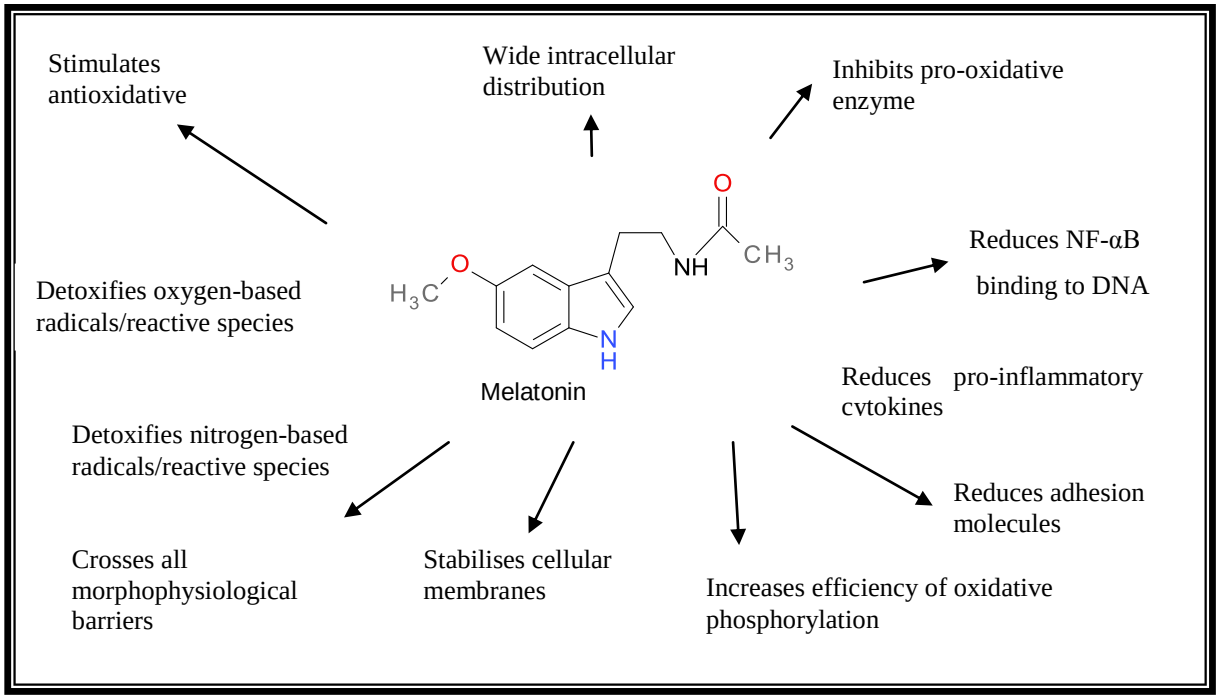

Figure 1.8: Mechanisms of the neuroprotective properties of melatonin (Reiter *et al.,* 2002).

Melatonin is a potent antioxidant, because it is able to donate electrons to toxic radicals (Reiter, 1998) and is able to scavenge up to 4 or more reactive species per molecule (Tan *et al.,* 2002). It has been shown to scavenge •OH (Tan *et al.,* 1993) and stimulate the CAT enzyme that is responsible for the removal of H_2O_2 (Montula *et al.,* 1997). Its anti-inflammatory property is attributed to melatonin's ability to scavenge NOO^- and to reduce the formation of NO• (Cuzzocrea *et al.,* 1998) as these 2 species are believed to have a role in mediating the inflammatory process (Beckman and Koppenol, 1996). The metabolites of melatonin also possess antioxidant properties (Maharaj *et al.,* 2007).

1.3.2.5 Green tea

1.3.2.5.1 Introduction

Tea is a common beverage and of the total tea consumed worldwide, 20 % of it is green tea (Jankun *et al.,* 1997). Green tea (*Camilla sinesis*) has many components that have physiological and pharmacological actions. These include catechins, flavonoids, caffeine and GABA. Catechins possess antioxidant, anti-obesity and anticancer properties; caffeine processes neuroactive properties as it affects the autonomic and dopaminergic nervous systems and GABA is an inhibitory neurotransmitter (Kakuda, 2001; Kakuda, 2002; Yamada *et al.,* 2005). Theanine is a non-proteinous amino acid that is also found in green tea that produces the taste in it.

1.3.2.5.2 Theanine

1.3.2.5.2.1 Introduction

Theanine (L-theanine; γ-glutamylethylamide or γ-ethylamino- L-glutamic acid) (Figure1.9) reportedly accounts for 50 % of all the amino acids in green tea leaves and comprises between 1-2 % of the total dry weight of green tea leaves (Goto *et al.,* 1996). It is synthesised from ethylamine and glutamic acid (also known as glutamate) in the roots of tea plants and from there it is transported to the leaves of the plant (Buckowski *et al.,* 1999) where it may be converted by the plant to catechins (Eschenauer and Sweet, 2006). It is an analogue of the excitotoxic neurotransmitter glutamic acid (Figure 1.8) and it is because of this that theanine's potential neuroprotective properties have been widely studied.

As a glutamic acid analogue, theanine is able to bind to glutamate receptors such as NMDA, kainate and AMPA receptors as an antagonist. Theanine reduces neuronal death after ischaemia by binding to the above mentioned glutamate receptors (Kakuda, 2002; Egashira *et al.,* 2007). Kakuda *et al.* (2008) showed that theanine inhibits glutamine transport in neurons in the brain. At a concentration of 500 μM, theanine suppresses glutamic acid-induced neuronal toxicity (Kakuda, 2002). The effect of theanine on neurotransmitters has also been studied. It has been shown to reduce the levels of brain 5-hydroxyindoles (Yokogoshi *et al.,*

1995), decrease overall 5HT levels in the brain (Yokogoshi *et al.*, 1997) and increase the release of dopamine (Yokogoshi *et al.*, 1998). Theanine possesses anxiolytic and cognitive enhancing properties (Nathan *et al.*, 2006) and has a hypotensive effect in spontaneously hypertensive rats (Yokogoshi and Kobayashi, 1997).

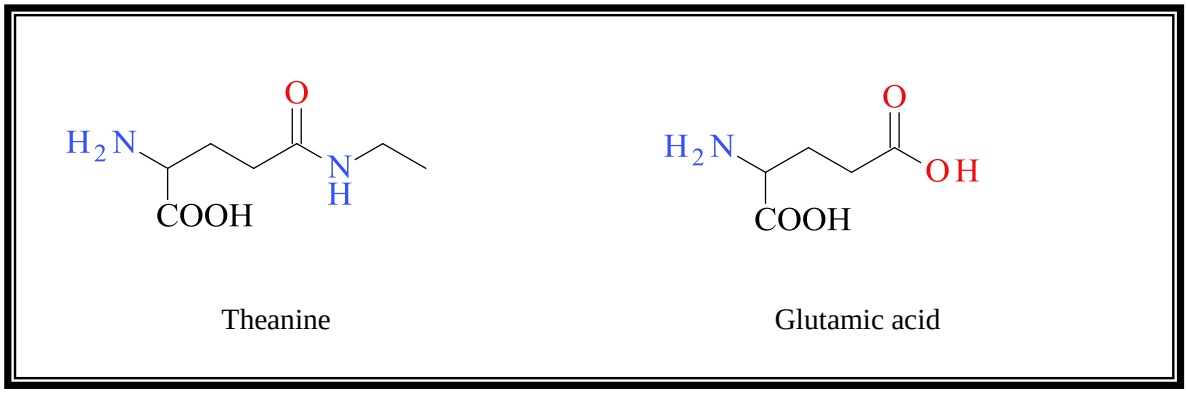

Figure 1.9: Chemical structure of theanine and glutamic acid (Yamada *et al.*, 2005).

1.3.2.5.2.2 Pharmacology

1.3.2.5.2.2.1 Dosing

Approximately 4 cups of green tea is equivalent to between 50-200 mg of theanine (Eschenauer and Sweet, 2006). Doses of up to 4000 mg/kg of theanine were tested in rats and were found to have no adverse effects (Borzelleca *et al.*, 2006).

1.3.2.5.2.2.2 Adverse effects

A 13 week study on theanine to assess the toxicokinetic and toxicity was carried out by Borzelleca and colleagues (2006) on rats using doses of theanine of 0, 1500, 3000 and 4000 mg/kg. The blood and urine samples were collected, body weights and food consumption and behavioural observations were made to assess the effects of theanine. No adverse effects and treatment-related deaths were observed. This study and the fact that theanine has been widely consumed for several decades implies that theanine is a safe drug.

1.3.2.5.2.2.3 Drug interactions

Theanine has been shown to have a positive interaction when administered with the chemotherapeutic agent doxorubicin. Sadzuka *et al.,* (1996) reported that theanine enhances the efficacy of doxorubicin. It does this by inhibiting the efflux mechanism from tumour cells thereby increasing the inhibitory effect of the anticancer drug on tumour growth (Eschenauer and Sweet, 2006). Theanine also reduced the adverse reactions of doxorubicin such as the induction of lipid peroxide level and the reduction of glutathione peroxidase activity (Sugiyama and Sadzuka, 2004). Other potential drug interactions with theanine are with antihypertensives and with lipid lowering agents which result in an additive decline in blood pressure and lipid levels respectively (Eschenauer and Sweet, 2006).

1.3.2.5.2.3 Pharmacokinetics

1.3.2.5.2.3.1 Absorption

The absorption of theanine occurs via a common Na^+ coupled co-transporter in the brush border membrane in the intestinal tract (Kitaoka *et al.,* 1996). Its levels in the blood and liver rapidly increased an hour after administration and thereafter were sharply decreased. Theanine is taken up by the brain by a leucine-preferred transport system (Yokogoshi *et al.,* 1995). After intraperitoneal (i.p.) admission, it takes 30 minutes for theanine to be taken up by the brain (Kimura and Murata, 1971) and it reaches peak concentrations within 5 hours in the brain (Terashima *et al.,* 1999). Serum concentrations of theanine were reached 0.5-2 hours after the administration of a 200 mg dose in rats (Unno *et al.,* 1999).

1.3.2.5.2.3.2 Distribution

Theanine levels in tissues such as blood, liver and the brain were detected as early as an hour after administration (Terashima *et al.,* 1999).

1.3.2.5.2.3.3 Metabolism

Orally administered theanine is metabolised to glutamic acid and ethylamine in the rat kidney (Unno *et al.,* 1999; Terashima *et al.,* 2003). The enzyme responsible for its degradation is a phosphate-independent glutaminase (Tsuge *et al.,* 2003).

1.3.2.5.2.3.4 Excretion

As theanine is metabolised mainly by the kidney, a large percentage of theanine is immediately excreted through urine (Tsuge *et al.,* 2003). Theanine was detected in the urine 5 hours after administration and its concentration in the urine continued to increase during the next 24 hours (Terashima *et al.,* 2003).

1.3.2.6 Tetracyclines

1.3.2.6.1 Introduction

Tetracyclines are a class of broad spectrum antibacterials. The first tetracycline to be isolated and used in the clinical setting in 1948 was chlortetracycline (Duggar, 1948). These agents are divided into two classes according to their mechanism of action namely typical and atypical tetracyclines. Typical tetracyclines act by binding to the 30S ribosomal subunit of bacteria and inhibit protein synthesis (Dormercq and Matute, 2004; Bryskier, 2005) and the atypical tetracyclines act by interfering with the electrochemical gradient of the bacterial cell membrane which subsequently promotes cell lysis and thus cell death (Bryskier, 2005).

The use of tetracyclines became popular but their use has recently been limited due to the emergence of bacterial resistance to these agents (Byskier, 2005). The manner in which bacteria develop resistance to antibacterial agents is by developing an efflux mechanism, protecting their ribosomal units, mutations in the 30S ribosomal unit and by enzymatically inactivating the antibiotic (Byskier, 2005). Semisynthetic tetracycline analogs doxycycline and minocycline have been used more frequently clinically due to their efficacy and the susceptibility of many bacterial infections to them. The tetracyclines remain the treatment of choice for Lyme disease, several sexually transmitted diseases and cholera. The combination

of tetracyclines with other drugs has also formed effective therapeutic agents in the treatment of diseases such as amyloidosis (when combined with chloramphenicol) (Bryskier, 2005).

1.3.2.6.2 Minocycline

1.3.2.6.2.1 Introduction

Minocycline (7-dimethyl-amino-6-demethyl-deoxytetracycline hydrochloride) (Figure 1.10) is a second generation semi-synthetic tetracycline that belongs in the "typical" class of tetracyclines. Its superiority to most tetracyclines is due to the fact that it is a long acting antibiotic that readily crosses the BBB as it has enhanced lipophilicity at physiological pH (Barringer *et al.,* 1974). Both gram negative and gram positive bacteria are susceptible to minocycline because it resistant to the efflux mechanisms in both these types of bacteria (Bryskier, 2005).

Minocycline is effective in treating upper and lower respiratory infections with several strains of bacteria such as *Staphylococcus aureus* (*S. aureus*) and *Streptococcus pneumoniae* being susceptible to it (Steigbigel *et al.,* 1968). Being lipophilic in nature, minocycline readily accumulates in sebaceous follicles in the skin and can thus be used to treat skin and soft tissue infections especially acne vulgaris (Eady *et al.,* 1990; Freeman *et al.,* 1994). Organisms such as *Neisseria gonorrhoea* and *Chlamydia trachomatis* are susceptible to minocycline *in vitro.* Minocycline may be used as a prophylaxis against gonorrhoea. However, the tetracycline doxycycline and other antibiotics are clinically superior to minocycline (Herfindal *et al.,* 1992). Minocycline is useful in the treatment of non-gonoccocal urethritis (Romanowski *et al.,* 1993). *S. aureus* infections particularly methicillin resistant *S. aureus* (MRSA) have been shown to be sensitive to minocycline (*in vitro*). No clinical studies have been carried out to evaluate the clinical use of minocycline in the treatment of MRSA but minocycline's inhibition of beta-lactamase production and its penetrability into tissues has made it effective in *S. aureus* infections of the bone, lungs and heart valves (Freeman *et al.,* 1994). Although rifampicin is the first choice in the prophylaxis of meningococcal meningitis, minocycline may be used as an alternative to it (Bryskier, 2005).

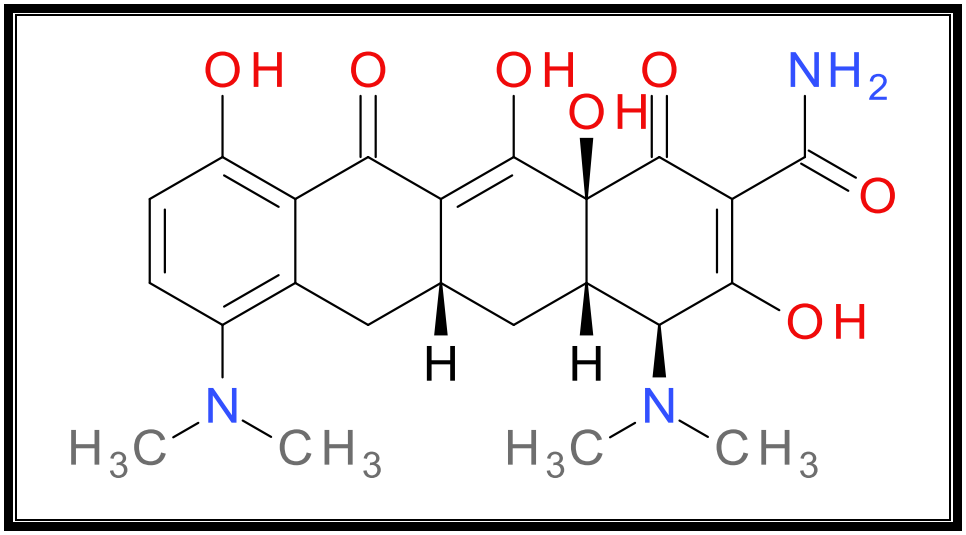

Figure1.10: Chemical structure of minocycline
(http://www.medicinescomplete.com/mc/martindale/2009/images/CLK1103C001.gif)

1.3.2.6.2.2 Pharmacology

1.3.2.6.2.2.1 Dosing

Due to its long half life, minocycline is usually orally administered at a dose of 200 mg once on the first day then 100 mg twice daily (Bryskier, 2005) and it is better adsorbed with food (Herfindal *et al.*, 1992). Minocycline can also be administered by intravenous infusion with a 200 mg dose being administered over a period of 30-60 minutes (Bryskier, 2005).

1.3.2.6.2.2.2 Adverse effects

Most adverse effects of minocycline (and other tetracyclines) are caused by their toxic degradation products (Bryskier, 2005). The adverse effects associated with the use of minocycline are: photosensitivity reactions in which a rash appears on the regions on skin exposed to sunlight, hypersensitivity reactions which rarely occur but include oedema and rashes, hyperpigmentation associated with prolonged use of minocycline and permanent discolouration of bones and teeth. Gastrointestinal side effects such as oesophageal ulcers which develop mainly when minocycline is taken with little or no fluid, dizziness and ataxia have also been reported with the use of minocycline in therapeutic doses (Herfindal *et al.*, 1992; Bryskier, 2005). Minocycline is able to cross the placenta and distribute in the foetus

and thus interfere with bone and tooth development. It is also secreted in breast milk. It is because of these reasons that the use of minocycline in pregnant and lactating women is prohibited.

1.3.2.6.2.2.3 Drug interactions

In general tetracyclines are not to be administered with antacids, dairy products and other preparations containing calcium, magnesium, zinc and iron as chelation with the drug will occur and thus reduce the oral bioavailability of the drug (Herfindal *et al.*, 1992; Bryskier, 2005). Combination with diuretic agents may cause an increase in serum levels of minocycline and with oral contraceptives may reduce the efficacy of the contraceptives (Bryskier, 2005). Minocycline also interacts with warfarin (Gasse *et al.*, 2005).

1.3.2.6.2.3 Pharmacokinetics

1.3.2.6.2.3.1 Absorption

Minocycline is well absorbed orally with approximately 95 % of the concentration being reached in the plasma within less than an hour (MacDonald *et al.,* 1973) and bioavailability is improved when it is taken on an empty stomach or at least 1 hour before a meal (Bryskier, 2005). Time-to-peak (T_{max}) is reached approximately 2-3 hours after administration and its serum half life was found to be approximately 16 hours (MacDonald *et al.*, 1973). Minocycline is highly protein-bound (76 %).

1.3.2.6.2.3.2 Distribution

Findings by MacDonald *et al.* (1973) showed that due to minocycline's lipophilicity, it is widely distributed with its concentrations in tissues being higher than serum concentrations. This characteristic makes it therapeutically effective, as its penetration into and around tissues combined with its long half-life support the clinical recommendation to reduce the dose frequency of minocycline (Freeman *et al.,* 1994). Minocycline has the highest partition coefficient of the tetracyclines and this characteristic makes it a superior drug within its class.

1.3.2.6.2.3.3 Metabolism

Nelis and De Leenheer (1981) showed that the major pathways of minocycline metabolism occur mainly by hydroxylation and N-demethylation with its principle metabolite being 9-hydroxyminocycline by a microsomal biotransformation that is yet to be elucidated.

1.3.2.6.2.3.4 Excretion

Minocycline is minimally excreted in the urine (6-19 %) with most of it being eliminated in bile. It is for this reason that it is recommended that a daily dose of 200 mg not to be exceeded (Freeman *et al.,* 1994). 4-epiminocycline, which is considered the degradation product of minocycline, is formed by the epimerisation of minocycline in the acidic environment of the kidneys (Nelis and De Leenheer, 1981). Faecal and urinal examination show that minocycline is partially metabolised to inactive substances (MacDonald *et al.,* 1973).

1.4 RESEARCH OBJECTIVES

Stroke evokes several toxic pathways that lead to a state of oxidative stress. The toxic events that occur as a result of stroke include an increase in free radical production, LP, an increase in the activity of neutrophils and macrophages which eventually lead to neurodegeneration and cell death (Gariballa, 2000). These processes lead to the biosynthesis of toxic endogenous toxins such as QA, which further contribute to ischaemic-induced injury. Exogenous substances that are taken therapeutically or as part of the diet (Yasuhara *et al.,* 2008) have a role in potentiating or preventing the damage that may result after ischaemic insult. Recent studies have shown that theanine (Kakuda, 2002) and minocycline (Yrjänheikki *et al.,* 1999) have neuroprotective properties in a stroke.

This study was undertaken to elucidate the potential neuroprotective mechanism of theanine and minocycline using various inorganic studies and biological assays. These included:

- Measuring the potential free radical and antioxidant of the above-mentioned agents in the presence of neurotoxins in rat brain homogenate,

- An electrochemical and UV/Vis analysis and iron binding assays to investigate the metal binding properties of the drugs,
- Establish the effects of the agents on the liver enzyme TDO and thus its effect on the biosynthesis of the neurotoxin QA,
- Examination of the effect that the drugs have on the brain post-ischaemia and
- The influence on the generation of $O_2^{\bullet-}$ and the endogenous levels of GSH after ischaemic insult.

The results of this study will contribute to the understanding of the mechanisms by which these drugs act and to determine whether the drugs are useful in preventing neuronal damage that occurs after a stroke.

CHAPTER 2
ANTIRADICAL AND ANTIOXIDANT ACTIVITY

2.1 INTRODUCTION

Free radicals that are generated *in vivo* result in damage of DNA, small molecules and lipids (Halliwell et al, 1995). Lipid peroxidation results from free radicals attacking membrane lipids which lead to the destruction of the cell membrane, cell integrity and the proper functioning of membrane bound enzymes and receptors as illustrated in Figure 2.1. Free radicals have also been shown to play a role in cardiovascular diseases (Dorman *et al.*, 2003). Reactive oxygen species (ROS) are oxygen containing free radicals produced by the sequential univalent reduction of oxygen (Fridovich, 1978). The most commonly formed ROS are the superoxide anion radical ($O^{\bullet}_{2}{}^{-}$), hydroxyl radical ($HO^{\bullet}$) and hydrogen peroxide (H_2O_2) a non radical ROS.

An antioxidant has been defined by Halliwell *et al.*, (2007) as any substance that delays, prevents or removes oxidative damage to a target molecule. The importance of an antioxidant *in vivo* cannot be emphasised enough as these agents protect the human body against damage by ROS. An antioxidant may carry out its antioxidant activity in four ways: it may prevent the formation of active oxidants; scavenge the free radicals or quench and remove active oxidants and repair damage done by oxidants (Halliwell *et al.*, 1995).

The free radical scavenging activity of a potential antioxidant is evaluated *in vitro* by its ability to scavenge the stable 2, 2-diphenyl-1-picryl-hydrazyl ($DPPH^{\bullet}$) radical. This radical is stable due to the delocalisation of the spare electron over the whole molecule which gives rise to its characteristic deep violet colour in an alcoholic solution with an absorption band at 517nm (Molyneux, 2004).

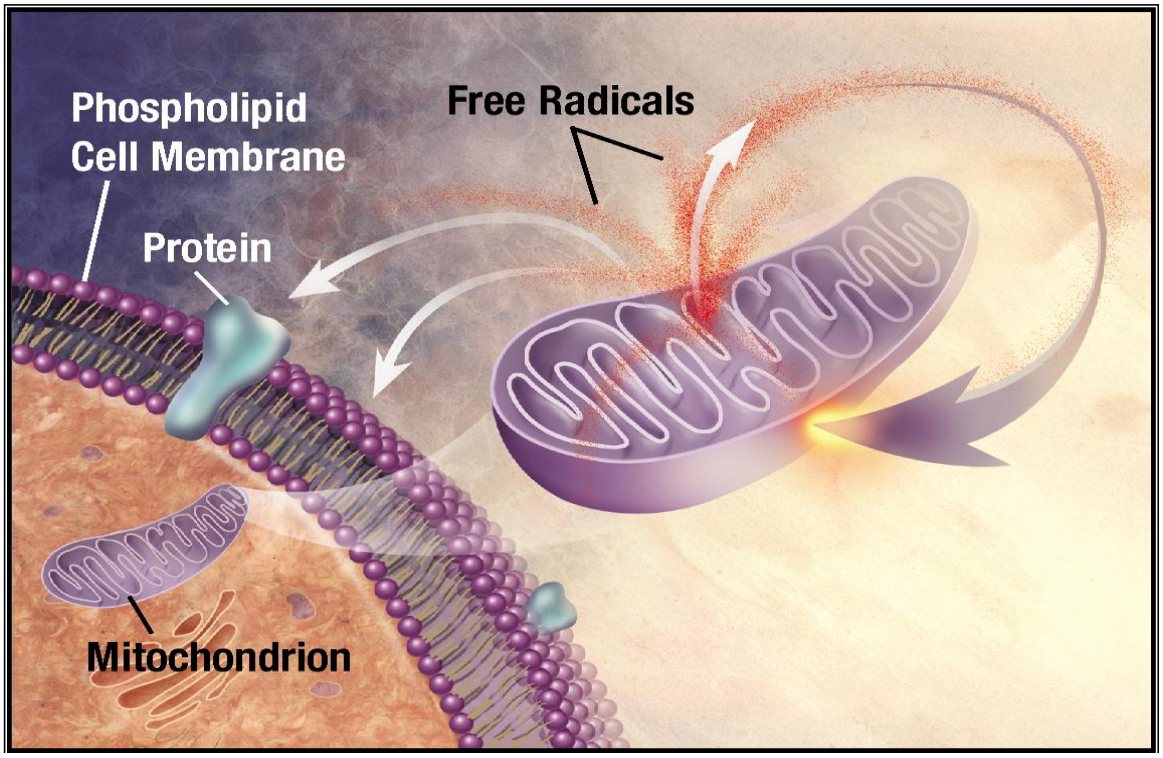

Figure 2.1: Illustration of free radicals attacking the cell membrane and proteins within the membrane (www.nia.hih.gove/Alzheimers/Resources/HighRes.htm).

The DPPH radical scavenging activity is influenced by the structure of the radical scavenger and its ability to donate hydrogen atoms as shown in equations 2.1 and 2.2 (Saito and Kawabata, 2005). In the presence of a hydrogen atom donor (RH) or a radical species ($R^{\bullet}$) (Figure 2.2), the violet colour of the $DPPH^{\bullet}$ solution changes to a pale yellow.

$$DPPH\bullet + RH \rightarrow DPPH - H + R\bullet \qquad \textbf{Equation 2.1}$$

$$DPPH\bullet + R\bullet \rightarrow DPPH - R \qquad \textbf{Equation 2.2}$$

The DPPH thus acts as both an oxidisable substrate and as a reaction indicator molecule (Dorman *et al*, 2003).

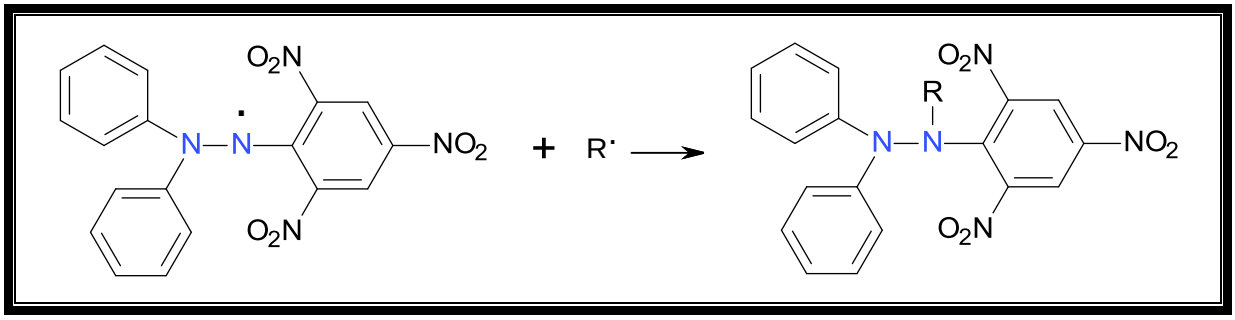

Figure 2.2: Showing the manner in which DPPH reacts with a radical substance**.** (http://upload.wikimedia.org/wikipedia/commons/4/4d/DPPHInhibition.png)

A substance that is able to scavenge DPPH radicals will be able to prevent abstraction of hydrogen from susceptible polyunsaturated fatty acids (PUFAs) (Dorman *et al.,* 2003).

Antioxidants derived from natural products such as plants have generated much interest globally. Green tea which is a popular herbal tea has been shown to have many naturally occurring antioxidants which include ascorbic acid, phenols and flavonoids (Sang *et al.,* 2002; Lin *et al.,* 2008). It has also been shown to have the highest hydrogen atom donating ability when compared to other teas (Gadow *et al.,* 1997; Benzie and Szeto, 1999; Atoui *et al.,* 2005) and that regular intake increases antioxidant status *in vivo* and the plasma antioxidant capacity (du Toit *et al.,* 2001) and thus reduces coronary heart disease (Benzie and Szeto 1999). Whether theanine exhibits free radical scavenging activity in a dose dependent manner needs to be established. Theanine is a glutamic acid analogue and glutamic acid causes significant cell death in rat neurons. However, it has been shown that simultaneous exposure of the rats neurons to theanine and glutamic acid resulted in the suppression of cell death due to the presence of theanine (Kakuda, 2002). Theanine has shown to prevent lipid peroxidation (Tsuge *et al.,* 2003). Investigation of the antibiotic minocycline, as a neuroprotective agent has shown promising results (Ravina *et al.,* 2003).

2.2 ANALYSIS OF THE FREE RADICAL AND ANTIOXIDANT ACTIVITY *IN VITRO*

2.2.1 INTRODUCTION

The brain is susceptible to damage by free radicals as it is rich in PUFAs and its high utilisation of oxygen (Şenes *et al.,* 2007). Free radical related damage to DNA, proteins and lipids (such as the PUFAs in the brain), has been proposed to play an important role in the development of several neurodegenerative disorders (Halliwell *et al.,* 1989). It is therefore important to search for agents that have antioxidant properties and thus potentially delay the rate at which neurodegeneration can occur. The antioxidant properties of theanine and minocycline were investigated using increasing concentrations of the drugs and these were compared to a known antioxidant vitamin C.

2.3 MATERIALS AND METHODS

2.3.1.1 Chemicals and reagents

Theanine, minocycline and 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) were purchased from Sigma Chemical Corporation, St Louis, Missouri, United States of America. The above chemicals were dissolved in methanol. Methanol and ascorbic acid (vitamin C) were purchased from Saarchem, Wadeville, Gauteng, South Africa.

2.3.1.2 Determination of antioxidant activity using the DPPH radical scavenging method

A modified method of free radical scavenging of Brand-Williams *et al.,* (1995) described below, was used to measure the free radical scavenging ability of theanine and minocycline.

DPPH (2 ml, 0.1 mM) was dissolved in methanol and incubated for 30 minutes with 2 ml theanine or minocycline at final concentrations of 50, 100, 200, 300, 400, 450 and 500 µg/ml. The maximum effective working volume for this experiment is recommended to be 4 ml with equivalent volumes of DPPH and reductant for optimum analytical accuracy (Molyneux,

2004). The test compounds were not dissolved in water as water gives rise to poor absorption readings (Molyneux, 2004) but rather in methanol as it gives rise to more sensitive absorption profiles of DPPH with no interference with the reaction (Molyneux, 2004; Sharma and Bhat, 2009). Vitamin C was used as a positive control to check if the procedures were working correctly (Brand-Williams *et al.,* 1995; du Toit *et al.,* 2001). Due to the photosensitivity of both DPPH and minocycline, all operations were done under dim light (Ozcelik *et al.,* 2003; Sharma and Bhat, 2009). After incubation, the absorbance was then read at 517 nm using a Shimadzu UV mini 1240 UV/VIS spectrophotometer. The absorbance of the samples using methanol as the blank was recorded.

As previously mentioned, in the presence of a hydrogen ion donor, there is a change in colour of the DPPH solution from violet to yellow as well as a decrease in absorbance. After the absorbance values were recorded, the DPPH radical scavenging activity was calculated using the following equation:

$$Scavenging\ activity\ (\%) = \left\{\frac{(A_0 - A_1)}{A_o}\right\} \times 100 \qquad \textbf{Equation 2.3}$$

Where,

A_0 = the absorbance of control reaction which was made up of DPPH in the absence of the test compound and

A_1 = the absorbance of DPPH in the presence of the test compound.

This equation shows that the higher the percentage inhibition, the higher the hydrogen atom donating ability and thus the more efficient the test compound is as an antioxidant.

2.3.1.3 Statistical Analysis

The results were analysed using a one-way Analysis of variance (ANOVA) followed by the Student Newman-Keuls Multiple Range Test using the Graph Pad 4 ® program. The level of significance used was $p < 0.05$ (Zar, 1974).

2.4 RESULTS

The free radical scavenging activity of the theanine, minocycline and vitamin C analysed over a concentration range is shown in Figure 2.3. The results illustrate that an increase in the percentage of DPPH radical scavenged correlates with an increase in the hydrogen donating ability and thus an increase in antioxidant activity.

Figure 2.3 shows that although there is a change in the percentage of DPPH radical scavenged at different concentrations, there is no statistically significant change in percentage of free radical scavenging when the concentration is increased from 50µg/ml for all drugs. There is a statistically significant difference ($p < 0.001$) between the free radical scavenging properties of theanine in comparison to minocycline and vitamin C and no statistically significant difference between the free radical scavenging properties of minocycline and vitamin C.

The colour changes of the solutions when the test compounds were added to DPPH were noted. The violet colour of the DPPH solution immediately changed to yellow on adding vitamin C and changed to yellow after 18 minutes on adding minocycline. With theanine however, the violet coloured solution only changed to a yellow solution after more than 6 hours.

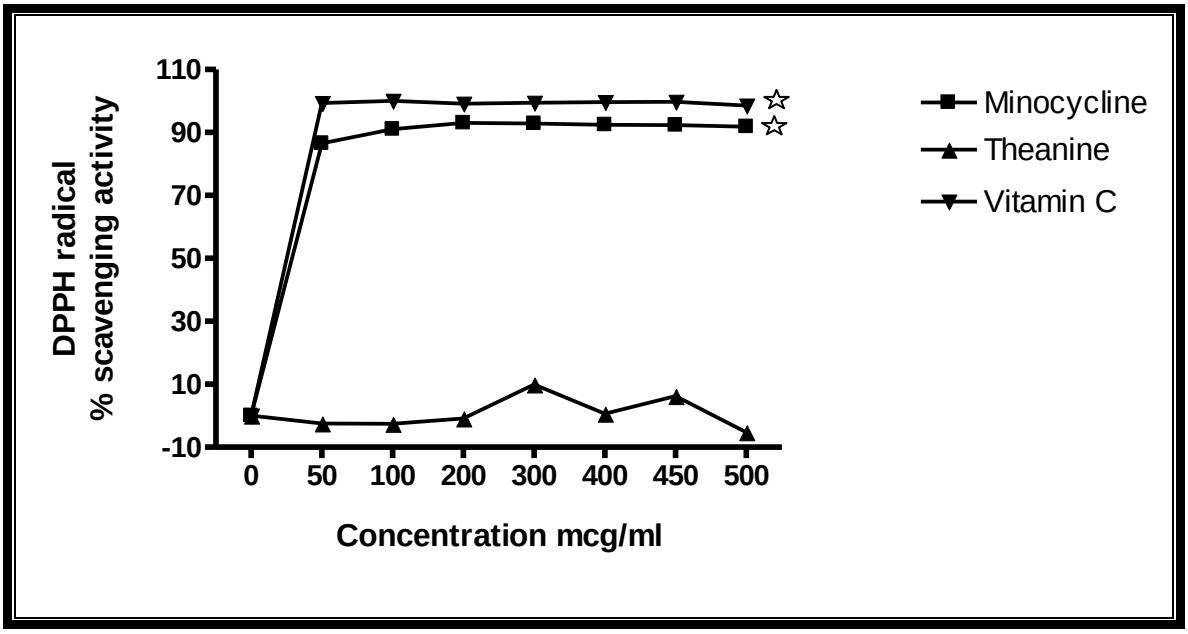

Figure 2.3: DPPH$^{\bullet}$ scavenging activity of theanine, minocycline and vitamin C at increasing concentrations. Each point represents the mean ± SD (n=3). ☆ ($p<0.001$) in comparison to theanine (ANOVA followed by Student Newman-Keuls Multiple Range Test).

2.5 DISCUSSION

The DPPH$^{\bullet}$ scavenging activity is influenced by the chemical structure of the radical scavenger (Sharma and Bhat, 2009). On a visual level, a slow colour change from the violet coloured DPPH radical solution to yellow implies that the reductant has poor hydrogen donating ability (Shimada *et al.*, 1992).

Vitamin C is a highly effective scavenger of the DPPH radical as evidenced by the violet coloured DPPH solution immediately changing colour to yellow on addition of vitamin C. Vitamin C has two sites for H abstraction thus two molecules of DPPH$^{\bullet}$ are scavenged per molecule of vitamin C (Figure 2.4) rendering it a very effective scavenger of DPPH and thus has been described as having rapid reaction kinetics with steady state being reached in less than 1 minute (Shimada *et al.*, 1992; Brand-Williams *et al*, 1995; Molyneux, 2004).

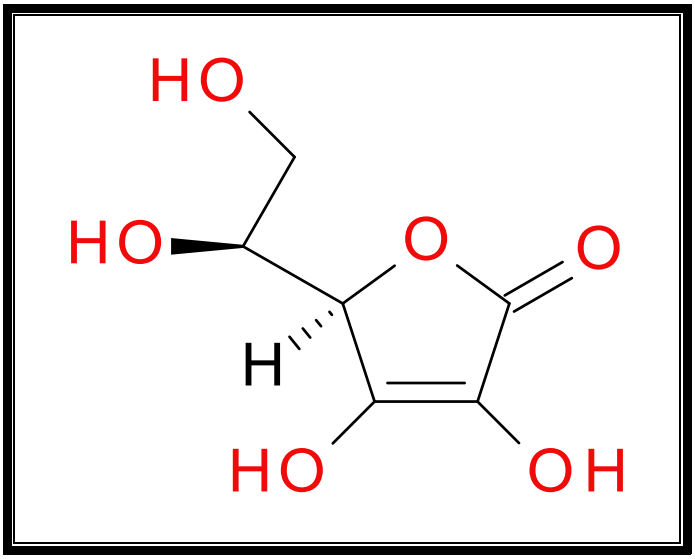

Figure 2.4: Structure of vitamin C (http://www/upload.wikimedia.org/wikipedia/commons/8/81/Ascorbic_acid_structure.png).

Brand-Williams *et al.*, (1995) have shown that certain compounds reactivity with DPPH corresponds to the number of available hydroxyl groups, where the number of available hydroxyl (OH) groups is related to the number of DPPH molecules scavenged. A proposed mechanism of $DPPH^{\bullet}$ scavenging activity by minocycline could be that the hydrogen is abstracted from a phenolic OH group and this leads to the formation of the diphenyl picryl hydrazine radical a phenol-derived radical (Lebeau *et al.*, 2000; Saito and Kawabata, 2005) (Figure 2.5). Within its structure, minocycline has a dimethylamino substituent which provides for increased resonance stabilisation of the phenol-derived radical and increases steric stabilisation of the phenol-derived radical produced by the reaction (Kraus *et al.*, 2005). The violet colour of the DPPH solution changes to yellow after 18 minutes incubation when minocycline is added, this indicates intermediate reaction kinetics when steady state is reached between 5-30 minutes (Brand-Williams *et al*, 1995).

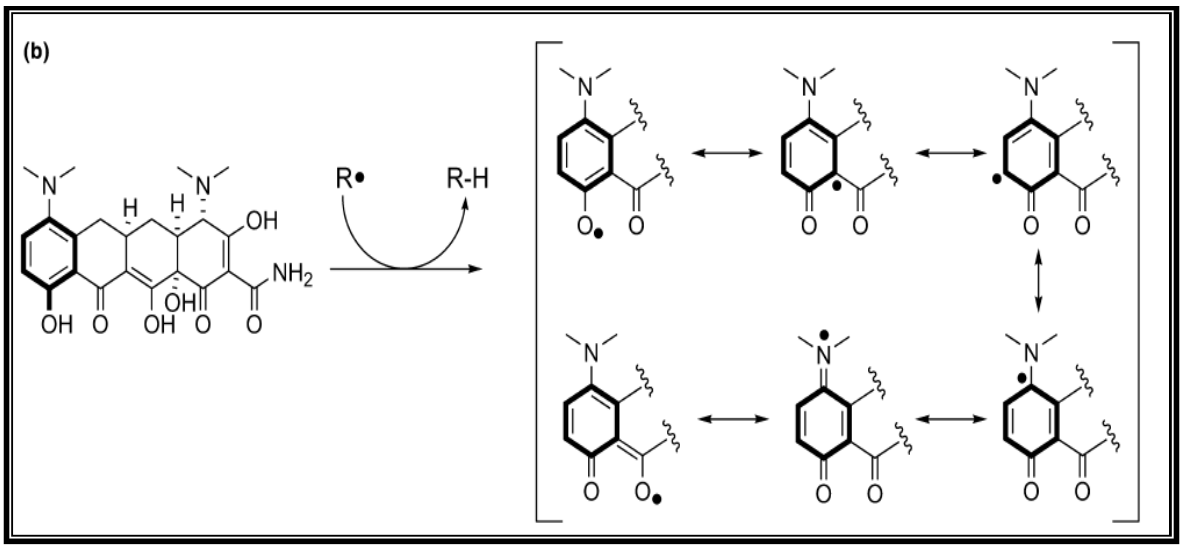

Figure 2.5: Mechanism of radical scavenging by minocycline (Kraus *et al.*, 2005).

It has been shown in a previous study that radical scavenging capacity of half a cup of green tea is equivalent to 200 mg of vitamin C and that green tea has the greatest antioxidant power when compared to oolong and black teas (Miller *et al.*, 2000; Gadow *et al.*, 1997). Flavonoids in tea account for most of the antioxidant activity in tea (du Toit *et al.*, 2001). Therefore, the presence of theanine in green tea also contributes to the antioxidant capacity of tea. Although theanine possesses free radial scavenging properties, it has very slow reaction kinetics with steady state being reached in more than 6 hours. The colour change of the violet coloured DPPH radical solution is hardly changed when it is added.

Free radicals are involved in lipid peroxidation and play a role in cardiovascular diseases (Dorman *et al.*, 2003). Effective free radical scavengers must also be investigated for potential lipid peroxidation inhibitors as these may prevent the abstraction of hydrogen from susceptible PUFAs (Dorman *et al.*, 2003) as well as cardiovascular protective properties. Minocycline has shown more promising results *in vitro* than theanine and its free radial scavenging properties are comparable to naturally occurring vitamin C.

CHAPTER 3
IRON CHELATION STUDIES

3.1 INTRODUCTION

Iron (Fe) is an essential element for all forms of life. Two thirds of body Iron is found in haemoglobin and transferrin (a transport protein) and the remaining iron is stored in ferritin and haemosiderin (Halliwell and Gutteridge, 1985). In the mammalian body, iron is used for the utilisation of oxygen and is a component of numerous oxidases and oxygenases (Puntarulo, 2005). Iron's bioavailability is hindered by its tendency to become oxidised, hydrolysed and polymerised into insoluble ferric hydroxide (Boyer *et al.,* 1988a).

Iron is a catalyst for the Haber-Weiss reaction (Section 1.2.2.5) by facilitating the conversion of superoxide anion radicals ($O_2\bullet^-$) and hydrogen peroxide (H_2O_2) to hydroxyl radicals (•OH) (Halliwell and Gutteridge, 1986). •OH is highly reactive and the damage it can do to the body is site specific depending on the location of the iron, which does not exist in a "free form" but is bound to proteinous membranes, nucleic acids and low molecular weight chelating agents (Halliwell and Gutteridge, 1986). For example, if the iron catalyst is attached to DNA, the Haber-Weiss reaction will lead to •OH dependent base modification and if the metal catalyst is attached to cell membranes, this will lead to lipid peroxidation (LP) (Halliwell and Gutteridge, 1986). Iron dependent LP requires chelated ferric ion (Fe^{3+}) and ferrous-dioxygen-ferric chelate complex as initiating species (Bucher *et al.,* 1983; Decker and Welch, 1990). There is no obvious physiological mechanism of disposing excess iron (other than menstrual periods), thus in a case of iron overload, the excess iron will lead to the generation of free radicals which will lead to harmful effects on cells (Halliwell and Gutteridge, 1986). Iron has been shown to play a role in neurodegenerative diseases such as Parkinson's disease (Babincová and Babinec, 2005) and an increase in iron in the substantia nigra is associated with oxidative stress due to the presence of free, redox active iron (Boyer *et al.,* 1988b). Damaged to tissue by injury, infection makes it particularly vulnerable to radical reactions.

Ferritin is the major storage protein for iron in bacteria, plants and mammals (Figure 3.1). It maintains iron in a soluble, non toxic form in which it may be easily mobilised (Boyer and McCleary, 1987; Babincová and Babinec, 2005). Ferritin is synthesised in response to iron

and acts as an iron "sink" (Halliwell and Gutteridge, 1985). Ferritin is composed of 24 tightly packed subunits and each molecule may store up to 4500 iron atoms as hydrous ferric oxide. It has narrow channels within it that lead to a central core (Funk *et al.,* 1985). It is through these pores that Fe^{2+} moves during its mobilisation and deposition into the core in its oxidised form as Fe^{3+} (Gutteridge *et al.,* 1983; Funk *et al.,* 1985). A strong external chelator and reducing agent is required for the removal of iron from ferritin (Jameson *et al.,* 2004). However, it is still unclear which agents are responsible for the removal of iron *in vivo*. Ferritin iron has been shown to participate in the initiation of LP by the reduction of Fe^{3+} within the core by reductants such as $O_2\bullet^-$ (Gutteridge *et al.,* 1983; Puntarulo, 2005). Puntarulo (2005) has observed that elevated levels of ferritin are associated with the severity of a stroke as well as elevated levels of $O_2\bullet^-$ and H_2O_2.

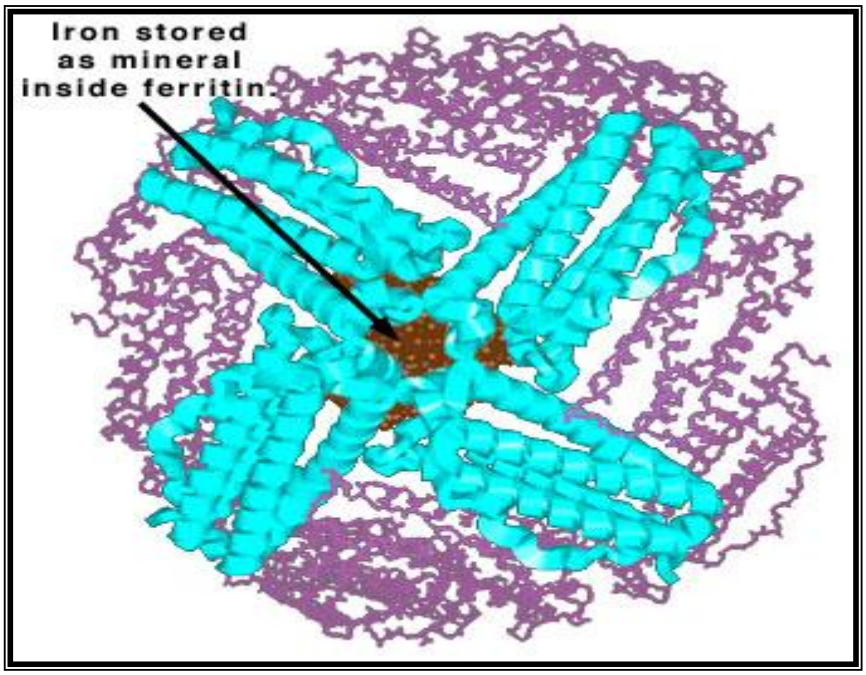

Figure 3.1: Ferritin molecule showing where iron is stored within its structure (http://www.javeriana.edu.co/Facultades/Ciencias/neurobioquimica/libros/perinatal/ferritin1.jpg).

Antioxidants are useful in scavenging free radicals and can thus prevent cell damage and there is a place for their use therapeutically in degenerative diseases. In plasma, antioxidants play a role in removing free radicals as well as preventing iron-mediated catalysis of free radical reactions (Halliwell and Gutteridge, 1986). In this way, antioxidants prevent or delay the primary and secondary effects of iron. The use of ferritin has been suggested as another therapeutic means of ridding the body of excess iron. Metal chelators have a therapeutic role

in cases of iron overload and these rid the body of potentially toxic iron by binding to iron compartmentalised metal ions (Halliwell and Gutteridge, 1986; Puntarulo, 2005). Nitric oxide is effective as an antioxidant as it chelates and scavenges free radicals (Puntarulo, 2005).

3.2 ULTRAVIOLET AND VISIBLE SPECTROSCOPIC ANALYSIS OF THEANINE AND MINOCYLCINE ALONE AND IN COMBINATION WITH IRON (II) AND IRON (III)

3.2.1 INTRODUCTION

The fundamental principle that underlies spectrophotometric techniques is that all chemicals to some extent absorb electromagnetic radiation of specific wavelengths (Newman, 1965). This allows for the qualitative and quantitative determination of chemicals according to the extent to which they absorb electromagnetic radiation. Ultraviolet and visible (UV/VIS) spectroscopy is a spectrophotometric technique that measures the absorbance of electromagnetic radiation in the UV/VIS range in the electromagnetic spectrum which extends from 200 nm to 800 nm.

When a chemical absorbs UV or VIS radiation, this causes the molecules within the solution to become "excited", that is to say, the molecules assume a higher energy state (Silverstein *et al.,* 1981). The electrons in the molecule are promoted from a bonding or non-bonding orbital into an empty anti-bonding orbital (Silverstein *et al.,* 1981). Within the UV/VIS spectra, the electron is promoted from either a non bonding or pi (π) orbital to a pi anti-bonding (π^*) orbital. This causes a release of energy in the form of heat or fluorescence (Newman, 1964).

The chemical groups that absorb light in the UV/VIS range are those that possess atoms with non-bonding orbitals (in other words a lone pair of electrons) or π bonds (Silverstein *et al.,* 1981). Molecules such as unsaturated organic compounds, compounds with atoms such as oxygen (O) and nitrogen (N) which posses a lone pair of electrons and conjugated double bonds are most likely to have π bonding orbitals (Silverstein *et al.,* 1981).

Different molecules absorb light in different wavelengths so a qualitative analysis of an unknown chemical is possible from the absorption spectrum it produces (Newman, 1964). The wavelength of the light absorbed depends on the energy required for the electrons within its structure to make a transition to a higher energy state. An absorption band is defined by its intensity and position (Silverstein *et al.,* 1981). The position of the absorption band corresponds to the wavelength of radiation and the intensity of the absorption band depends

on two factors namely, the probability of interaction between radiant energy and the electronic system and the difference between the ground and excited state of the electron (Silverstein *et al.,* 1981).

Any change in the structure or composition of a known system will result in a change in the spectrum in wavelength or intensity (Newman, 1965). It is on this principle that the interaction of theanine and minocycline with iron (II) and iron (III) will be investigated.

3.2.2 MATERIALS AND METHODS

3.2.2.1 Chemicals and reagents

Theanine and minocycline were purchased from Sigma Chemical Corporation, St Louis, Missouri, United States of America. Hydrated ferrous sulphate ($Fe^{2+}SO_4.7H_20$) and ferric chloride ($Fe^{3+}Cl_3$) were purchased from Saarchem, Wadeville, Gauteng, South Africa. All solutions were prepared using deaerated milli Q water to prevent auto oxidation of the iron.

3.2.2.2 UV/VIS Spectroscopy

The interaction between theanine and minocycline with iron (II) (Fe^{2+}) and iron (III) (Fe^{3+}) was studied by comparing the absorption spectra of the test compound alone and in combination with the iron solutions in equimolar concentrations of 0.1 mM to 1 mM. The absorption spectra were determined using a GBC UV/VIS 916 spectrophotometer using quartz cuvettes with a path length of 1 cm.

3.2.3 RESULTS

No peaks corresponding to an absorption band were observed for theanine in the ultraviolet and visible range of the spectra.

Minocycline produces a yellow coloured aqueous solution. Figures 3.2 and 3.3 show that the absorption bands of 1mM minocycline occur between 308 nm and 343 nm and between 343 nm and 395 nm with λ_{max} values of 349 nm and 359 nm. The figures also show that a

decrease in absorptive intensity occurs when Fe^{2+} and Fe^{3+} respectively were added to minocycline. A concentration of 0.1mM of minocycline produced the same results shown below.

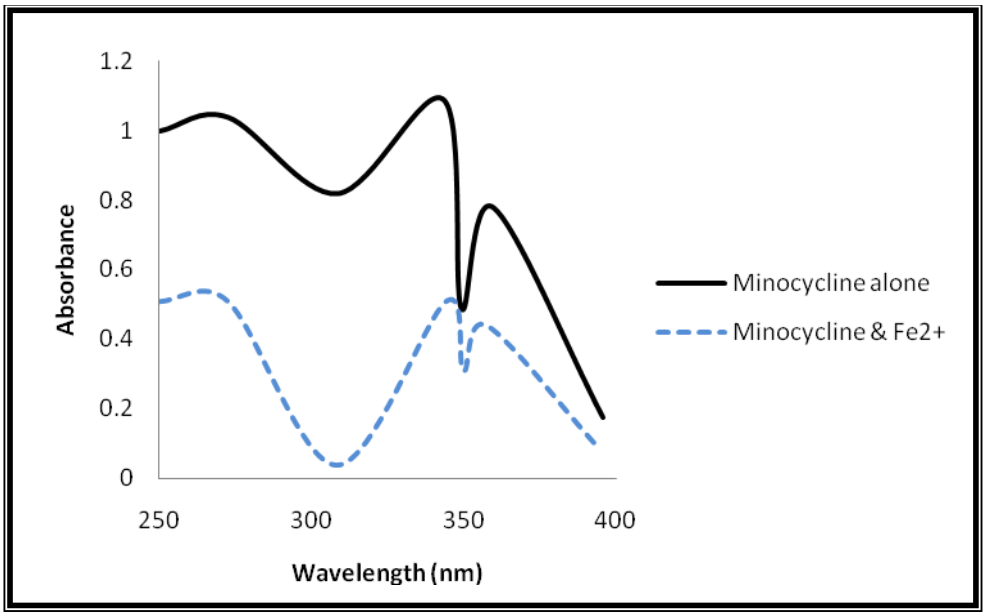

Figure 3.2: UV spectroscopic analysis of minocycline alone and in the presence of an equimolar concentration 1mM of Fe^{2+}.

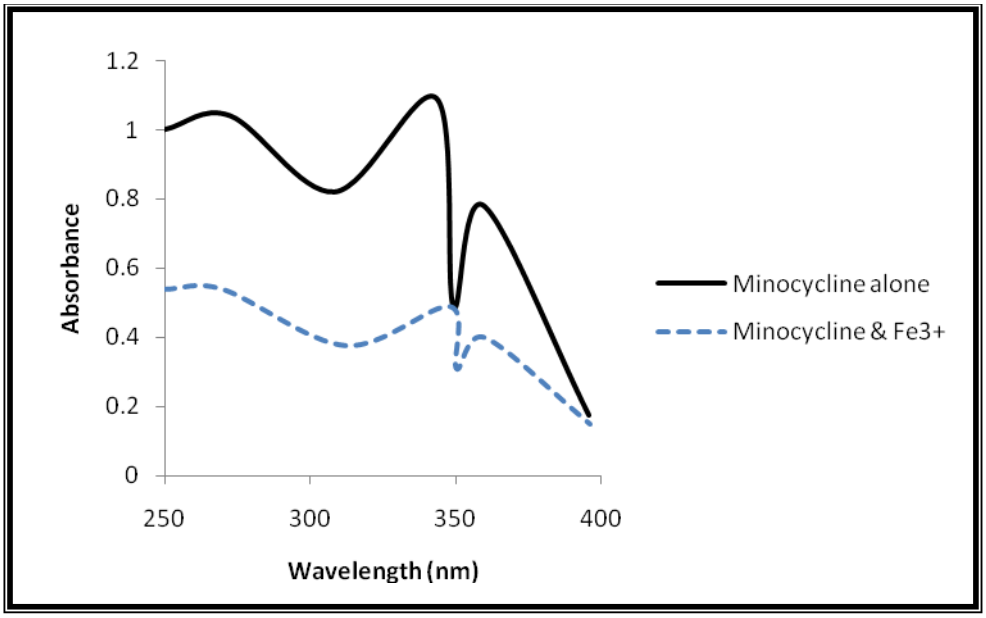

Figure 3.3: UV spectroscopic analysis of minocycline alone and in the presence of an equimolar concentration 1mM of Fe^{3+}.

3.2.4 DISCUSSION

It was expected that theanine would not produce a spectrum due to the fact that although theanine does possess atoms with lone pairs such as nitrogen (N) and oxygen (O), it is not highly delocalised which makes it more likely to produce a spectrum in the UV/VIS range. A study by Fu *et al.* (2007) shows that theanine can be detected in tea samples but with an indirect method of UV analysis.

Minocycline on the other hand, is a molecule with several types of delocalised systems within its structure. It contains benzene rings, carbon-oxygen double bonds and the lone pairs of electrons on the N and O within its structure (Figure 3.4). This makes it possible for the electrons in this structure to be excited to a π^* orbital. The minocycline is a yellow coloured solution. Yellow coloured solutions have wavelengths in the range 435 nm to 500 nm associated with them and the λ_{max} values of 349 nm and 359 nm obtained for minocycline correspond with this.

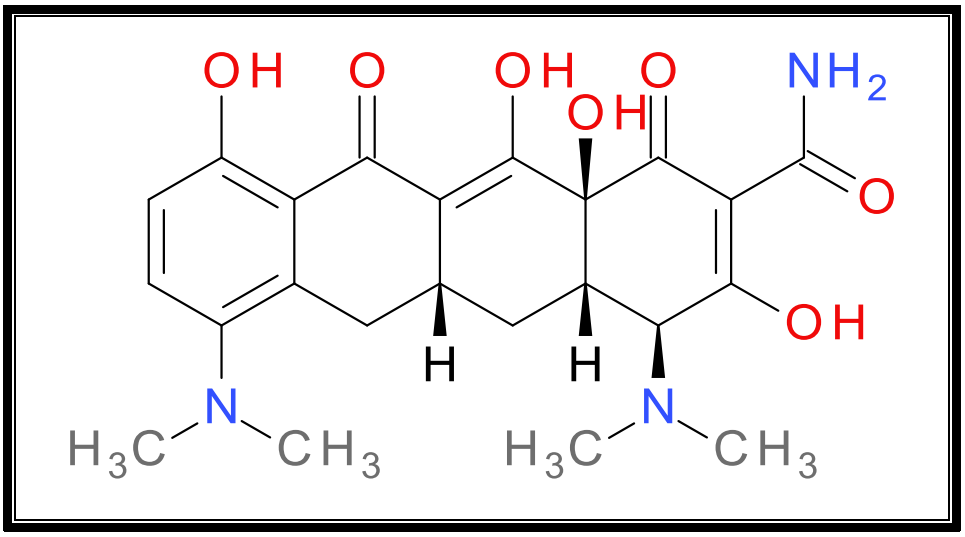

Figure 3.4: Chemical structure of minocycline (http://www.pharmgkb.org/images/drugs/PA450519-1.gif).

As previously mentioned, any change in the structure or composition of a known system will result in a change in the spectrum in terms of wavelength or intensity (Section 3.2.1). There was a decrease in the intensity of the minocycline absorption spectra on addition of Fe^{2+} and Fe^{3+}. This may be attributed to the fact that on mixing two compounds, there is a change in the intensity of the wavelength that may be attributed to the fact that there was interaction

between the minocycline and iron atoms or that the iron interferes with the absorption of minocycline.

The results then indicate that there is interaction between minocycline and iron. However, UV/VIS spectroscopy alone does not identify definite interactions therefore, electrochemistry and other experiments would have to be conducted to determine the nature of interaction if any, between the minocycline, theanine and iron.

3.3 ELECTROCHEMICAL ANALYSIS OF IRON (III) ALONE AND IN THE PRESENCE OF THEANINE OR MINOCYCLINE

3.3.1 INTRODUCTION

Electrochemistry is described as a scientific field that deals with the relation between electrical current or potential and a chemical system (Rubinstein, 1995). Cyclic voltammetry and adsorptive stripping voltammetry (AdSV) are interfacial electrochemical techniques that are classified as controlled potential techniques (Limson, 1998). Both methods make use of an electrical cell which consists of working, reference and auxiliary electrodes (Figure 3.5) (Bard and Faulkner, 1980; Limson, 1998).

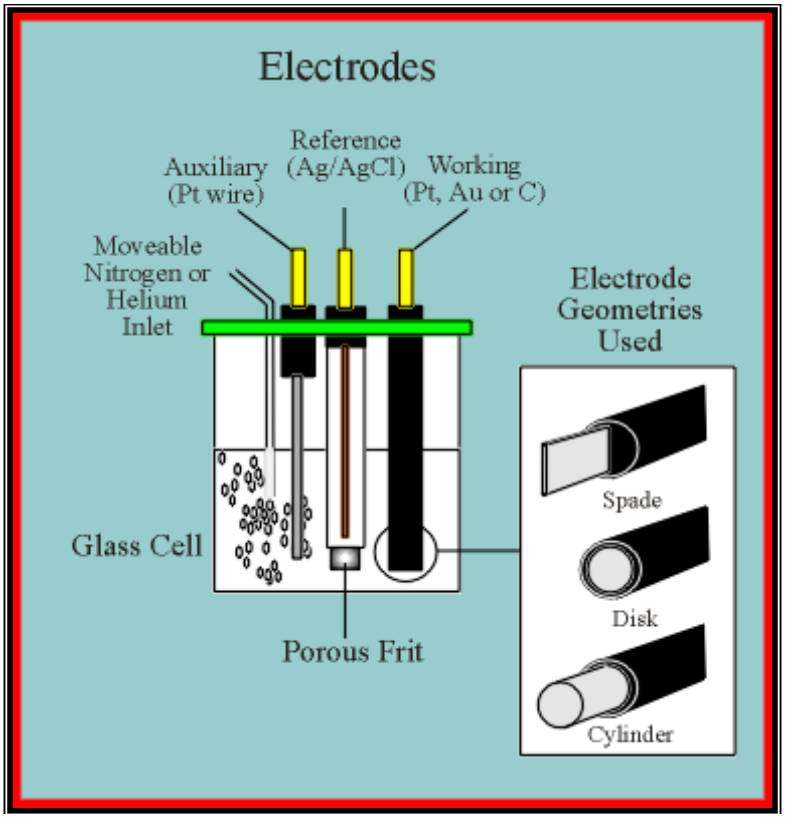

Figure 3.5: An electrochemical cell showing the three electrode system (**http://www.cce.paisley.ac.uk/marco/Enzyme_Electrode/Chapter1/Ferrocene_animated_CV1.htm**).

The chemical reactions take place on the surface of the electrode and involves the transfer of electrons, known as redox reactions (Rubinstein, 1995), where redox stands for reduction and oxidation. The current (i), that passes through the electrode is directly proportional to the flux

of redox active material on the surface of the electrode, which in turn is proportional to the concentration of the species at the electrolyte-electrode interface (Bard and Faulkner, 1980), thus, current is a direct measurement of the reaction rate (Rubinstein, 1995). Electron transfer that occurs at the electrolyte-electrode interface is observed as the current (Bard and Faulkner, 1980).

The working electrode imparts information such as a change in potential which may indicate a variation in electrode reaction (Bard and Faulkner, 1980). It is at this electrode that the analyte is either oxidised or reduced. The reference electrode maintains a constant potential and is isolated from the solution (Bard and Faulkner, 1980). The auxiliary electrode is in direct contact with the reaction solution and allows for the passage of electrons through it which results in a current through the cell that may be picked up externally (Bard and Faulkner, 1980).

Cyclic voltammetry and AdSV were the 2 electrochemical methods chosen to analyse the ability of theanine and minocycline to bind to Fe^{3+} under deaerated conditions. These studies were conducted in aqueous media and under biological pH conditions.

3.3.1.1 Cyclic voltammetry

Cyclic voltammetry is an electrochemical technique used to characterise the redox properties of organic compounds (Bard and Stratman, 1954; Limson, 1998). The technique of cyclic voltammetry involves the application of a linear electrical potential gradient (relative to the reference electrode) across the electrode–solution interface (at the working electrode) to oxidise or reduce the species in solution (Ligumsky *et al.,* 2005). The cyclic voltammogram produced of current versus potential where the current produced results from the applied potential and depends on a number of parameters such as the area of the electrode and the rate at which the potential is applied (Bard and Faulkner, 1980; Wang, 1994). The potential and current will give information about whether the molecules are able to donate electrons (Wang, 1994). The lower the current, the lower the reducing potential of the substrate. The voltammetric waves produced are in a peak-shaped or sigmoidal form (Wang, 1994). The voltammogram that is produced with this technique consists of a forward and reverse scan. The forward scan or positive potential show the oxidised species and the reverse scan or

negative potential show the reduced species (Limson, 1998). Peaks formed in the scan are caused by the formation of the diffusion layer near the electrode surface (Bard and Faulkner, 1980).

The shape of the current wave is determined by several factors and it will give information concerning the analyte. The interpretation of the cyclic voltammogram can be made based of the peak location on the current axis, the size of the anodic current and the peak potential (Ligumsky *et al.,* 2005). The peak location on the current axis reflects the ability of the compound to donate electrons, the size of the anodic current is proportional to the concentration of the substrate in the bulk solution and thus can be used to monitor the concentration and the peak potential is defined as the reducing power of the substrate (Ligumsky *et al.,* 2005).

Cyclic voltammetry is used to determine the reducing power of the substrate under investigation and is often conducted first in an electroanalytical study (Bard and Faulkner, 1980; Ligumsky *et al.,* 2005). Theanine and minocycline were analysed with this method to access their reducing power and AdSV was then carried out to access the interaction with Fe^{3+} with theanine and minocycline.

3.3.1.2 Adsorptive stripping voltammetry

This is a sensitive electrochemical technique that is employed in the measurement of trace metals (Limson, 1998). AdSV involves continuous stirring of the solution at a fixed rate allowing the convention of the analyte to the electrode and allows the metal to bind to the ligand (Limson, 1998). The metal-ligand complex is then adsorbed onto the electrode surface (adsorption or electrodeposition) and thereafter the stripping step takes place where the metal-ligand complex is released back into solution (Limson, 1998). This technique therefore relies on the formation of surface active complexes of the target metal in the presence of a complexing agent followed by the accumulation of the complex onto a film electrode (Bard and Stratman, 1954). This method is useful for the analysis of very dilute solutions down to concentrations of 10^{-10} to 10^{-11} molar solutions, as well as for trace metals that exhibit surface active properties (Wang, 1994).

3.3.2 MATERIALS AND METHODS

3.3.2.1 Chemicals and reagents

As described in section 3.2.2.1. Tris hydroxymethyl amino methane (tris-HCl) was purchased from Merck, Darmstadt, Germany.

3.3.2.2 Apparatus

Cyclic and adsorptive stripping voltammograms were recorded on an Autolab PGSTAT 30 voltammeter (Netherlands) equipped with a Metrohm VGA cell stand (Sweden). A 3mm glassy carbon electrode (GCE) was employed as the working electrode for the voltammetric experiments. A silver/silver chloride (Ag/AgCl) [(KCl = 3 M)] and a platinum wire were used as the reference and auxiliary electrodes respectively, in all the voltammetric experiments. Prior to use and between the scans, the GCE was cleaned by polishing with alumina on a Buehler pad, followed by immersion in dilute nitric acid solution and rinsing in milli Q water.

3.3.2.3 Cyclic voltammetry

For the cyclic voltammetry experiments, appropriate concentrations of theanine or minocycline were added to the electrochemical cell containing the electrolyte 0.2 M tris-HCl buffer, pH 7.4 which was then deaerated for 5 minutes. A potential window was then scanned to characterise and provide a fingerprint of the species in solution. The GCE was then cleaned and polished as described in section 3.3.2.2.

Changes in the current response and potential were observed in order to investigate whether theanine and minocycline were electrochemically active.

3.3.2.4 Adsorptive stripping voltammetry

An appropriate concentration of $Fe^{3+}SO_4$ was introduced into an electrochemical cell containing the electrolyte, 0.2 M tris-HCl buffer (pH 7.4) which was then deaerated for 5

minutes. Thereafter, an optimum deposition of potential for Fe^{3+} was identified and applied for 60 seconds to effect the formation and adsorption of the meat lino onto the GCE. A potential scan in the negative direction from the deposition potential to at least 0.20 V beyond the reduction of the metal was applied, at a scan rate of 0.10 Vs^{-1}, to strip the adsorbed metal species from the GCE.

During the stripping step, current responses due to the reduction of the metal species were measured as a function of potential to generate voltammograms. The GCE was then cleaned and polished as described in section 3.3.2.2. The procedure was then repeated between successive additions of appropriate concentrations of either theanine or minocycline (0-0.06 mM) to the electrolyte containing Fe^{3+} in the electrochemical cell. All reported potential values are referenced against the Ag/AgCl reference electrode. Current versus concentration plots were constructed to measure the extent of shifts in current response and reduction potential of metal with increasing concentrations of the ligand (either theanine or minocycline). Green tea is rich in polyphenols which have been shown to interfere with iron metabolism due to the formation of strong complexes (Hynes and Coinceanainn, 2002). This method of electrochemical analysis will help to determine if theanine, which is a component of green tea, has the potential to form complexes with iron.

3.3.3 RESULTS

The voltammograms have the current axis labelled i/A which represents recorded current (i) in amperes (A) and the potential axis labelled E/V which represents the potential (E) in volts (V). Figures 3.6 and 3.7 show the cyclic voltammograms obtained for theanine and minocycline respectively. A smooth shaped of the voltammogram for theanine was obtained (Figure 3.6). A voltammogram with peaks (shown by the arrows) was obtained for minocycline (Figure 3.7) with peaks at 0.4105 V, 0.1495 V and -0.1441V.

The adsorptive stripping voltammogram for Fe^{3+} (0.01 mM) alone and in the presence of increasing concentrations of theanine shown in Figure 3.7. There is a peak current response at -8.33 x 10^{-6} A at a reduction potential of -0.3603 V for Fe^{3+} in solution and a concentration dependent reduction shift in the reduction potential of Fe^{3+} towards less negative potentials. A similar voltammogram was obtained for minocycline.

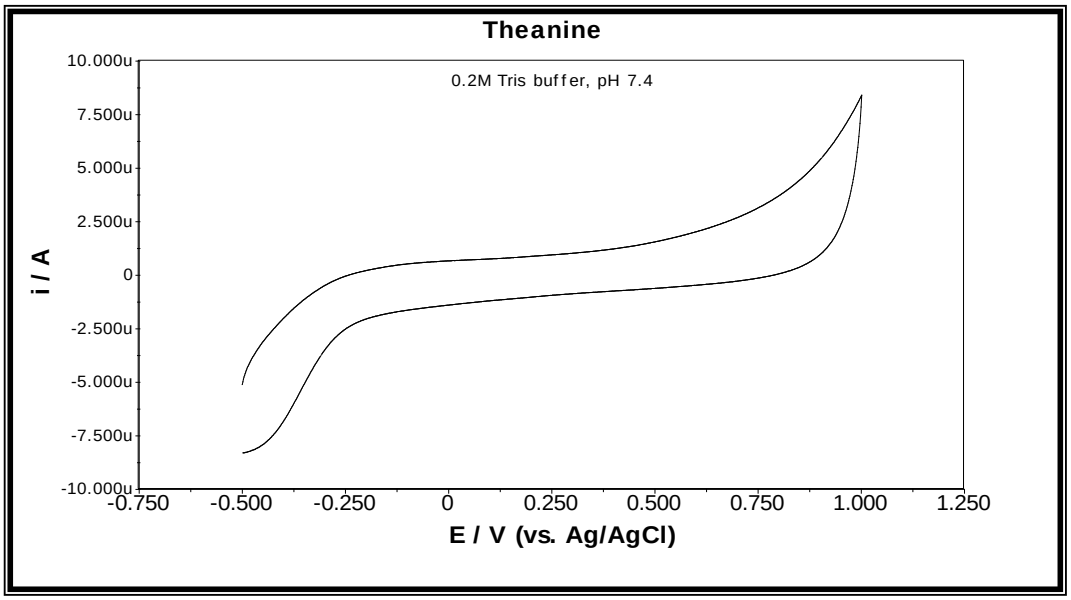

Figure 3.6: Cyclic voltammogram of theanine alone.

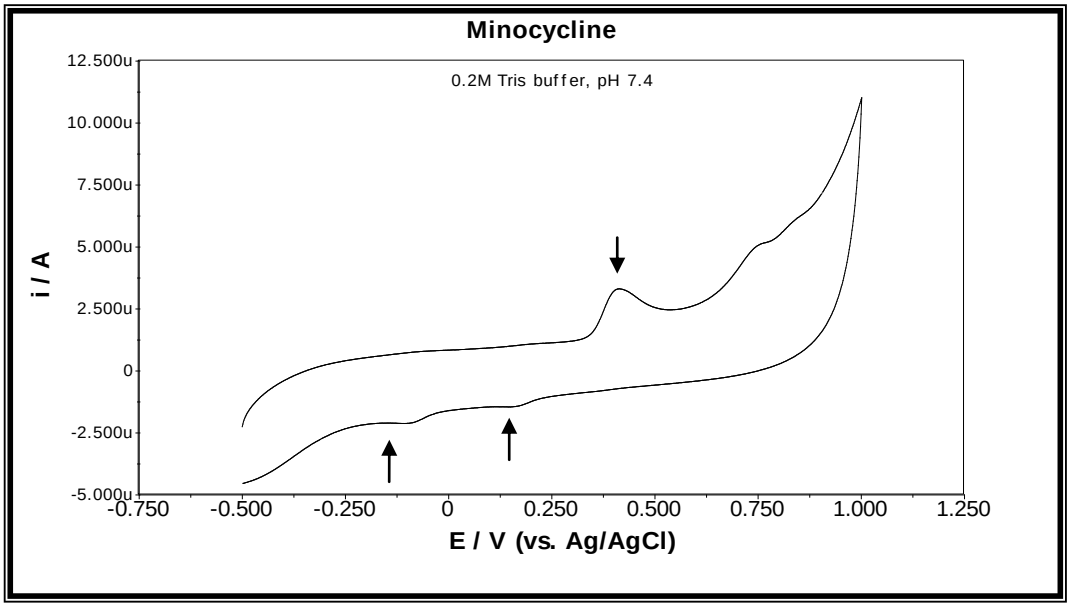

Figure 3.7: Cyclic voltammogram of minocycline alone.

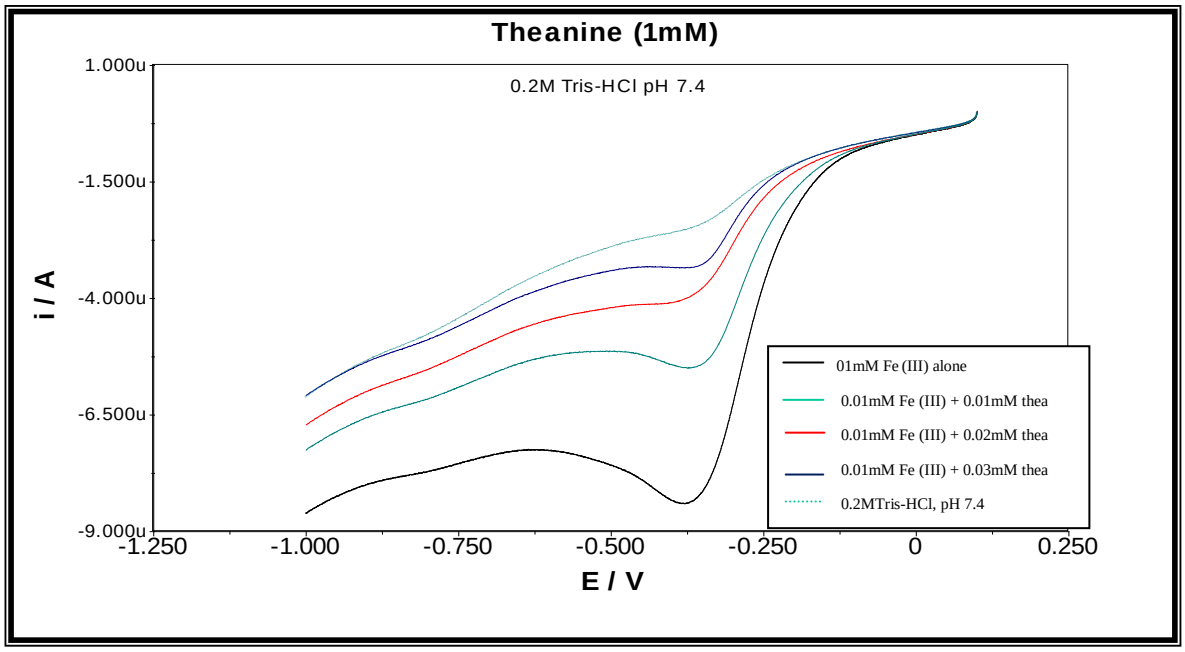

Figure 3.8: Adsorptive stripping voltammogram for Fe^{3+} (0.01 mM) alone and in the presence of increasing concentrations of theanine, abbreviated to thea (0.01 - 0.03 mM).

The percentage changes of increasing concentrations of theanine and minocycline on the peak current response of Fe^{3+} were calculated and presented in graphical means (Figures 3.9 and 3.10). A comparison of the ratio of added test compound to iron and the percentage increase in peak current response is represented in Table 3.1 in order to compare the relative difference in iron binding of both drugs as the concentrations of Fe^{3+} used to calculate the percentage increase in peak current response were 0.01 mM and 0.03 mM of Fe^{3+} for theanine and minocycline respectively.

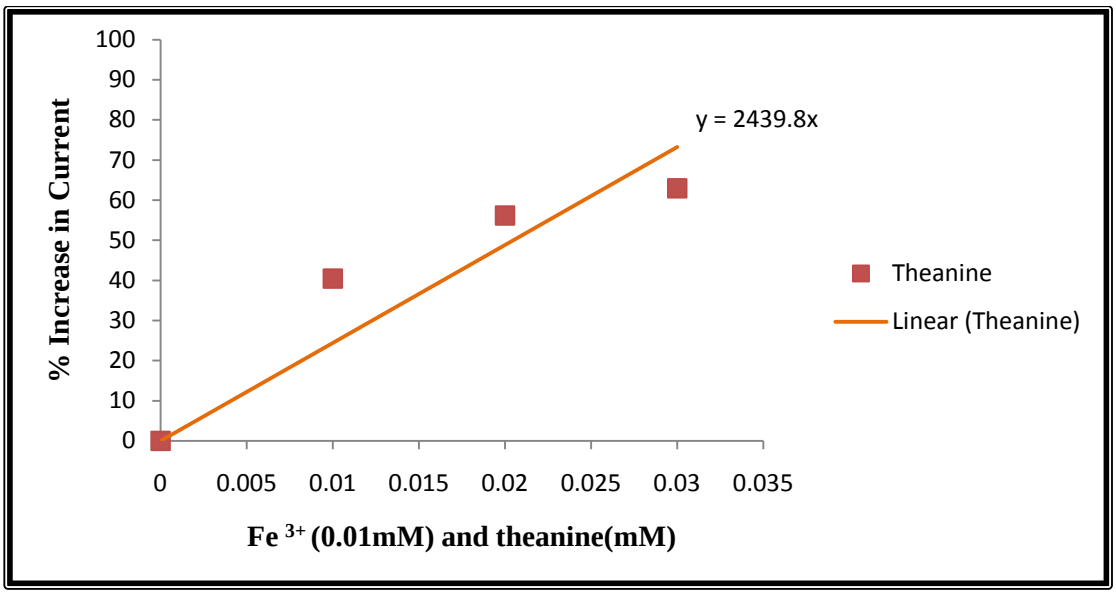

Figure 3.9: The percentage increase in the current of increasing concentrations of theanine on the peak current of 0.01 mM Fe^{3+}

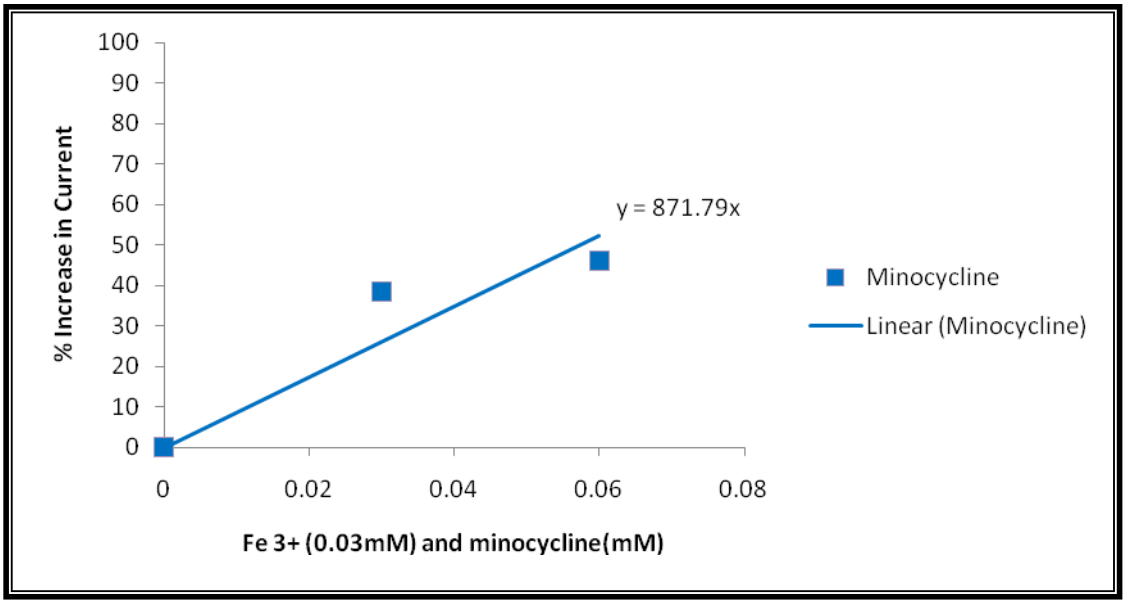

Figure 3.10: The percentage increase in the current of increasing concentrations of minocycline on the peak current of 0.03 mM Fe^{3+}.

Table 3.1: The ratios of the Fe^{3+} to test compound and the corresponding percentage increase in the current.

Ratio of Fe^{3+} to test compound	**% Increase in current**	
	Minocycline	**Theanine**
0:1	0	0
1:1	38	40
1:2	46	56

3.3.4 DISCUSSION

The smooth shape of the cyclic voltammogram for theanine implies that it may not be electrochemically active. This may be due to the fact that within the theanine molecule, there are very few electrons that could take part in an electrochemical reaction. The peaks (shown by the arrows) on the cyclic voltammogram for minocycline, indicate that it is electrochemically active due to the numerous electrons it contains within the benzene rings, carbon-oxygen double bonds and the lone pairs of electrons on the N and O within its structure.

Chelators affect the reduction potential of iron (the ease with which it is reduced) and thus its effectiveness as a catalyst (Winterbourn, 1995). In the Fenton reaction, Fe^{3+} is reduced to Fe^{2+} and it is the reduced form of iron that catalyses the Haber-Weiss reaction in which toxic hydroxyl radicals (•OH) are formed. Thus in the presence of a chelator, Fe^{3+} will either be reduced to form the ferrous ion or the oxidation of the Fe^{2+} iron to form the ferric ion. In this experiment, the formation of complexes of the test drug with Fe^{3+} was investigated because it is the ferric ion that is reduced in the Fenton reaction whose product (Fe^{2+}) ultimately catalyses the formation of •OH from the superoxide anion and hydrogen peroxide.

The increase in the reduction potential of iron when both theanine and minocycline is added to it shows that complexes are formed with the iron (Figure 3.8). Figure 3.9 further confirms this showing a concentration dependent formation of the theanine-Fe^{3+} and minocycline-Fe^{3+} complexes. In the UV/VIS study it was concluded that there was a reduction in the intensity of the wavelength that may be attributed to the fact that there was interaction between the minocycline and iron atoms or that the iron interferes with the absorption of minocycline

(section 3.2.4). The AdSV results indicate that there is definitely interaction between the minocycline and iron in terms of the formation of a complex.

The gradients of the percentage changes in current (Figures 3.9 and 3.10) are 2439.8 and 871.79 for theanine and minocycline respectively. The steeper gradient and higher value for the gradient of theanine implies that the complexes that it forms with iron are formed at a faster rate than the minocycline-iron complexes. However such a conclusion cannot be drawn due to the different concentrations of Fe^{3+} that were used to generate the respective AdSV voltammograms. Thus a comparison of the ratios of added test compound to iron and the percentage increase in peak current response was calculated and represented in Table 3.1 which shows that theanine has a higher percentage increase than minocycline in peak current response as there is an increase in theanine.

These results can be used to deduce that theanine produces complexes with ferric ions that are stronger than the complexes formed between minocycline and the ferric ions. The fact that both test compounds form complexes also implies that the complexes formed are strong enough to hinder the reduction of Fe^{3+} at the electrode and thus slow the cathodic current flow. However from the results above, it cannot be concluded how many ligands are required to completely hinder the iron from being reduced and how strong the chelating activity of the test compounds is. A further study will be carried out with ferrozine to determine strength of theanine and minocycline in inhibiting the formation of the iron (II)-ferrozine complex in the presence of ferritin.

3.4 THE COMPARATIVE EFFECTS OF THEANINE AND MINOCYCLINE ON THE INHIBITION OF THE IRON (II)-FERROZINE COMPLEX

3.4.1 INTRODUCTION

Ferrozine whose chemical name is 3-(2-Pyridyl)-5, 6-diphenyl-1, 2, 4 triazine 4'4''disulfonic acid sodium (Figure 3.11), is an effective chelator of Fe^{2+} and forms a magenta coloured solution. The Fe^{2+}-ferrozine complex (Figure 3.12) is made up of the metal and ligand in the ratio of 1 to 3, it has a λ_{max} of 562 nm and the Beer-Lambert law is obeyed up to 4 mg/L of iron (Stookey, 1970).

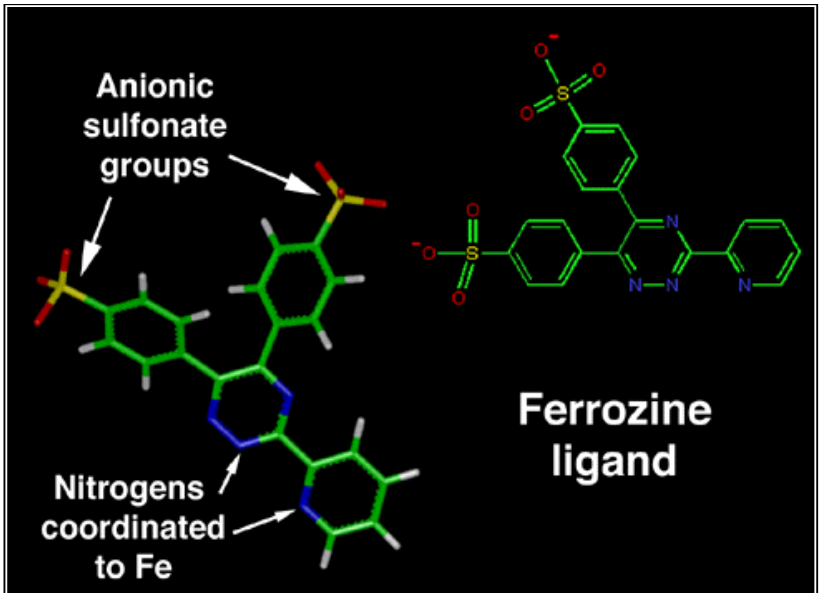

Figure 3.11: Illustration of the ferrozine ligand. The left figure is a stick representation of the indicator ligand ferrozine and the right figure is a ChemDraw representation of the ligand. *The nitrogen atoms on ferrozine form a coordinate bond with Fe (II). In this illustration, the colours of the carbon atoms are green, the hydrogens are white, the nitrogen atoms are blue, the oxygen atoms are red, and the sulphur atoms are yellow in this stick representation*. (http://www.chemistry.wustl.edu/~edudev/LabTutorials/Ferritin/ferrozine.html).

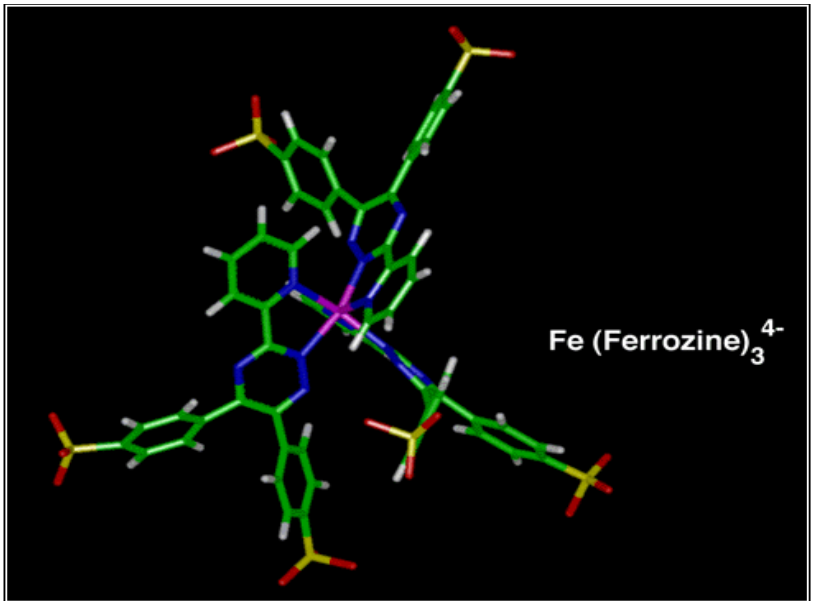

Figure 3.12: A stick representation of the complex ferrozine forms with iron. In this illustration, Fe (II) is complexed with three (3) ferrozine ligands. *The colours of the carbon atoms are green, the hydrogens are white, the iron atoms are magenta, the nitrogen atoms are blue, the oxygen atoms are red, and the sulphur atoms are yellow in this stick representation.* (http://www.chemistry.wustl.edu/~edudev/LabTutorials/Ferritin/feferr.html).

Ferrozine may be used for the direct determination of iron in aqueous solutions. The advantages of using ferrozine for the quantitative determination of iron are that it has minimum interferences, low cost and the high sensitivity of the experiment. In the presence of other chelating agents, the formation of the Fe^{2+}-ferrozine complex is inhibited or reduced depending on the other chelator's effectiveness and this results in a decrease in the colour of the complex. EDTA has been shown to be an effective inhibitor of the formation of the Fe^{2+}-ferrozine complex (Boyer and McCleary, 1987).

The measurement of the inhibition of the Fe^{2+}-ferrozine complex is important in determining whether a drug will have an affinity for $Fe^{2+}$and thus whether the drug will enhance or inhibit the Fenton reaction which involves the reduction of Fe^{3+} to Fe^{2+}. The purpose of this experiment is to investigate if theanine and minocycline inhibit the formation of Fe^{2+}-ferrozine complex and thus their potential as therapeutic agents in neurodegenerative diseases.

3.4.2 MATERIALS AND METHODS

3.4.2.1 Chemicals and reagents

Theanine, minocycline and ferrozine were purchased from Sigma Chemical Corporation, St Louis, Missouri, United States of America. EDTA and ferrous sulphate ($Fe^{2+}SO_4$) were purchased from Saarchem, Wadeville, Gauteng, South Africa. It is suggested that glassware is soaked in concentrated acid to ensure satisfactory and reproducible results (Stookey, 1970).

3.4.2.2 Chelating activity of theanine and minocycline

The chelating activity of theanine and minocycline was assessed by using a modified method of Decker and Welch (1990) is outlined below:

A 1 ml solution of the test compound (theanine or minocycline) in increasing volumes (0-100 µg/ml) was added to 3.7 ml milli-Q water. To this, 0.1 ml of $Fe^{2+}SO_4$ (2 mM) and 0.2 ml of ferrozine (5 mM) were added. The reaction mixture was then incubated at room temperature for 20 minutes. The absorbance of the reaction mixture was then measured at 562 nm using a Shimadzu UV mini 1240 UV/VIS spectrophotometer. A low absorbance value indicated a higher chelating activity of the test compound being analysed.

The percentage of the Fe-ferrozine complex formation was calculated using the following equation:

$$\%\ Inhibition\ of\ the\ Fe^{2+} - ferrozine\ complex\ = \left\{\frac{A_0 - A_1}{A_0}\right\} \times 100 \qquad \textbf{Equation 3.1}$$

Where,

A_o = absorbance of the control (reaction mixture without the test compound)

A_1= absorbance of the reaction mixture in containing the different concentrations of the test compound.

In this reaction, EDTA is at the same concentrations as the test compounds and was used as a positive control.

3.4.2.3 Statistical Analysis

The results were analysed using a one-way ANOVA followed by the Student Newman-Keuls Multiple Range Test using the Graph Pad 4 ® program. The level of significance used was $p < 0.05$ (Zar, 1974).

3.4.3 RESULTS

Figure 3.13 shows that both theanine and minocycline are ineffective in the chelation Fe^{2+} and the inhibition of the formation of Fe^{2+}-ferrozine complex despite an increase in concentration. There is a statistically significant difference between the percentage inhibition to the formation of the complex by EDTA in comparison to both theanine and minocycline.

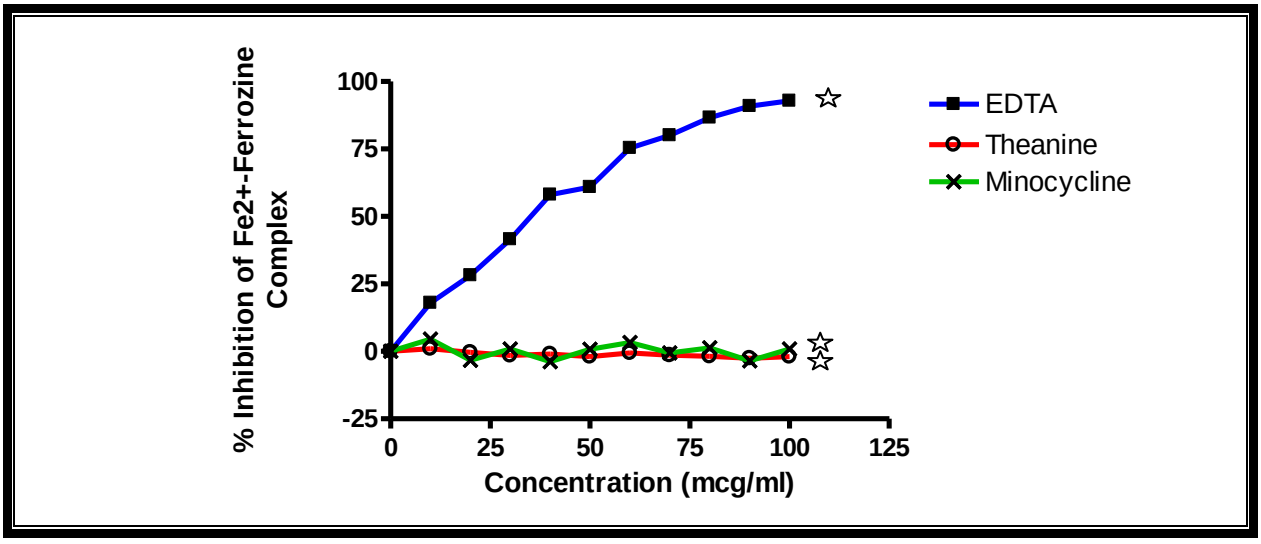

Figure 3.13: Percentage inhibition of the Fe^{2+}-ferrozine complex by theanine, minocycline and EDTA. Each point represents the mean ± SD (n=5). ☆($p < 0.001$) in comparison to EDTA (ANOVA followed Student Newman-Keuls Multiple Range Test).

3.4.4 DISCUSSION

As previously mentioned, ferrozine is a very strong iron chelator (section 3.4.1) and it forms a stable complex with iron that has a high extinction coefficient (Boyer *et al.*, 1988). Thus, addition of the $Fe^{2+}SO_4$ to the reaction mixture before adding the ferrozine would give the test compounds the opportunity to chelate with the ferrous sulphate before the stronger chelating agent ferrozine was added and once added there would be competition for the chelation of the iron. There would be evidence of this as there would most probably be a gradual colour change as the ferrozine binds to the iron during the incubation period. However, theanine and minocycline show very poor inhibition to the formation of the magenta coloured Fe^{2+}-ferrozine complex even at high concentrations of 100 µg/ml. There is a statistically significant difference between both theanine and minocycline's percentage inhibition of the Fe^{2+}-ferrozine complex and that of EDTA. EDTA has been shown to be an effective inhibitor of the formation of the Fe^{2+}-ferrozine complex (Boyer and McCleary, 1987) and the hyperbolic curve that is produced suggests that the concentration range results in saturation kinetics, implying that at concentrations greater than 100 µg/ml, it may be 100 % effective in inhibiting the formation of the complex.

Theanine and minocycline have exhibited poor iron (II) binding which would initiate even weaker iron (III) binding as the ferrous ion is more liable than the ferric ion.

3.5 THE COMPARATIVE EFFECTS OF THEANINE AND MINOCYCLINE ON THE RELEASE OF IRON (II) FROM FERRITIN

3.5.1 INTRODUCTION

Ferritin, as previously mentioned (Section 3.1) is the storage form of iron. *In vitro*, iron can be mobilised from ferritin by direct chelation of the ferric ion or by the presence of a chelating agent (Funk *et al.,* 1985). The channels within the ferritin molecule are narrow and are mechanically involved in the deposition and mobilisation of iron (Jameson *et al.,* 2004). A potential reductant must be able to penetrate the shell of the ferritin molecule in order to access the Fe^{3+} within its core and protonate the hydroxide ions that are coordinated to the surface of the ferric ion in hydrous ferric oxide (Funk *et al.,* 1985). Both a chelator and reductant are required for the effective release of ferrous iron from ferritin, the iron is released very slowly or ineffectively when only one is present (Jameson *et al.,* 2004). Ferritin has been shown to stimulate LP which leads to cell damage (Puntarulo, 2005). Green tea has been shown to lead to a decrease in ferritin concentrations in the plasma (Hynes and Coinceanainn, 2002) and as theanine is a component of green tea it could potentially contribute to this effect.

Ferrozine is an effective chelator, however, when mixed with ferritin, it forms the Fe^{2+}-ferrozine complex at a very slow rate. Ferrozine is unable to enter the ferritin protein shell due to its large size and its overall charge of -1 at physiological pH (Jameson *et al.,* 2004). Although reducing agents such as $O_2\bullet^-$ are effective in the removal of ferritin bound Fe, ferrozine is superior to these as a chelator (Boyer *et al.,* 1988).

Ferritin is principally found in the spleen, liver and bone marrow (Aisen, 1980), hence the use of horse spleen ferritin. In this experiment, the amount of iron released from ferritin will be measured as a percentage of control values. The iron released from ferritin will form a complex with ferrozine which will be detectable spectrophotometrically.

3.5.2 MATERIALS AND METHODS

3.5.2.1 Chemicals and reagents

Theanine, minocycline, ferrozine and horse spleen ferritin were purchased from Sigma Chemical Corporation, St Louis, Missouri, United States of America.

3.5.2.2 Iron release from ferritin assay

Iron release from ferritin was determined by the spectrophotometric measurement of the Fe^{2+}-ferrozine complex by a modified method of Babincová and Babinec (2005) described below:

In order to prevent auto oxidation from dissolved gases and any free metals that may be within the glassware, the milli Q water was deaerated with nitrogen gas (N_2) and all glassware was soaked in hydrochloric acid (Stookey, 1970). The reaction mixture containing 200 µg/ml of ferritin, 0.2 mM of ferrozine and the test compound in increasing concentrations (0.025, 0.050, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 mM) was incubated at 37 ^{o}C in phosphate buffered saline (PBS) (pH 7.4) for 20 minutes. The absorbance of the reaction mixture was then measured at 562 nm using a Shimadzu UV mini 1240 UV/VIS spectrophotometer.

The control mixtures contained no test compound. The amount of iron released from ferritin by the test compounds theanine and minocycline was expressed as a percentage of the control values and compared to EDTA a known inhibitor of the Fe^{2+}-ferrozine complex.

3.5.2.3 Statistical analysis

This was performed as described in Section 2.2.2.3.

3.5.3 RESULTS

Figure 3.14 illustrates the concentration dependent release of iron as Fe^{2+} from horse spleen ferritin. Theanine at a concentration of 0.8mM increases the release of iron by a mere 11 % in comparison to minocycline which increases the release of iron by 61 % in comparison to control values. There was a significant difference between all the compounds.

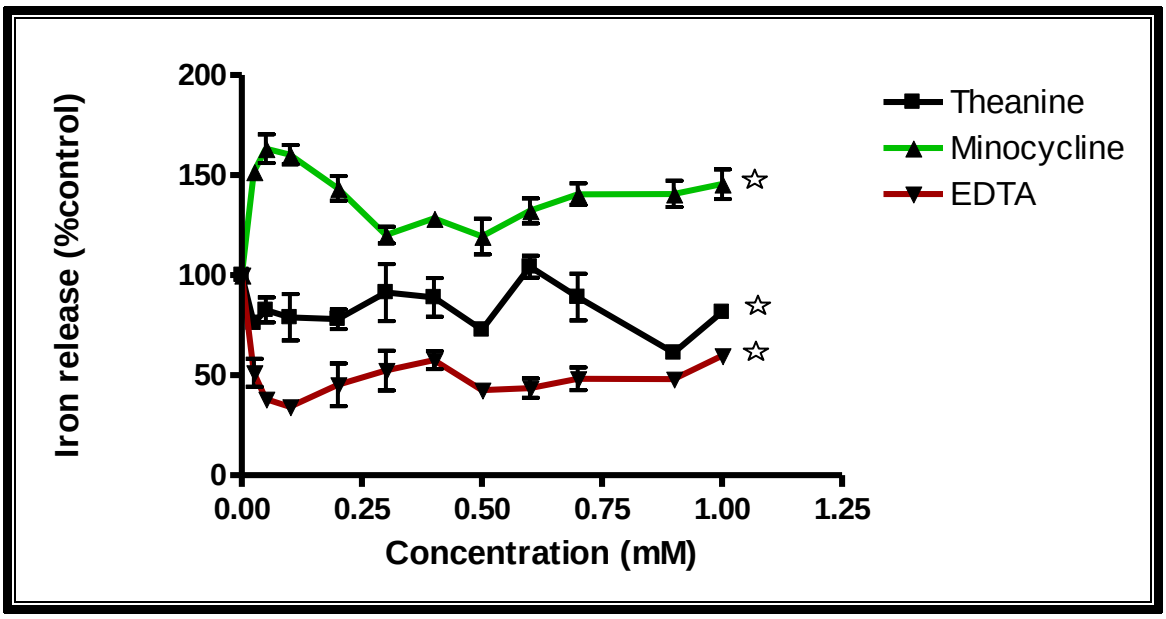

Figure 3.14: Release of iron from ferritin by theanine and minocycline expressed as a percentage of control values. Each point represents the mean ± SD (n=5). ☆($p < 0.001$) in comparison to other compounds (ANOVA followed by Student Newman-Keuls Multiple Range Test).

3.5.4 DISCUSSION

The ferritin assay is useful for testing the effectiveness of various reductants as ferritin iron releasing agents (Boyer *et al.*, 1987). The iron release is trapped and chelated by the ferrozine. The equations below (modified from Boyer *et al.*, 1987) explain how a drug participates in the mobilisation of ferritin iron:

$$Ferritin\left(Fe(II)\right)_n + drug = Ferritin\left(Fe(II)\right)_{n-2} + drug \qquad \textbf{Equation 3.2}$$

$$Fe\ (III) + 3Ferrozine \rightarrow Fe\ (Ferrozine)_3^{2+} \qquad \textbf{Equation 3.3}$$

An increase in percentage of iron release indicates that theanine and minocycline are able to mobilise Fe^{2+} from the ferritin core and make it available for the catalysis of the Haber-Weiss reaction (although theanine will do so to a lesser extent). Theanine only exhibits an increase of greater than 100 % in iron release at concentrations 0.3, 04 and 0.5mM releasing 120 %, 107 % and 115 % respectively. This implies that theanine is inefficient in reducing Fe^{3+} and is thus a poor reductant. However theanine is indeed statistically significantly more efficient than EDTA as a reductant. This is because EDTA is an efficient inhibitor of the iron (II)-ferrozine complex and thus causes a reduction in percentage iron released that is less than control conditions.

Fe^{3+} chelators have been shown to cause ferritin iron removal although the rates are very slow (Boyer *et al.,* 1987). Minocycline has been shown to form complexes with Fe^{3+} (Sections 3.2.4 and 3.3.3) and is most likely to bring about the reduction of iron (III) from within the ferritin core. It has shown to bring about a statistically significant increase in the iron mobilised from ferritin than theanine and EDTA. Because minocycline can complex with the Fe^{2+}, it will prevent it from participating in the formation of toxic •OH.

In oxidative stress conditions, the iron is made available for initiation of LP by two mechanisms: the release of iron from the ferritin core by theanine and minocycline and by the mobilisation of iron by $O_2^{\bullet -}$. This will lead to the synthesis of •OH which will ultimately lead to lipid peroxidation.

The brain is rich in PUFAs which make it particularly susceptible to attack by free radicals and it has the ability to readily accumulate iron. Zaleska and Floyd (1985) found that a linear relationship exists between the susceptibility of various rat brain regions to undergo LP *in vitro* and their content of endogenous iron. An agent that can prevent iron from catalysing the formation of free radicals and bind to iron and prevent it from accumulating in the brain will prove to be neuroprotective to a certain extent. Although both test compounds are poor inhibitors of the iron-ferrozine complex and thus prove to at least prevent iron chelate-mediated LP, these can promote the release of iron from ferritin thus making it available to take part as a catalyst in free radical reactions.

Effective and potential drug agents will be those that are able to form complexes with Fe^{2+} and react with bound Fe^{3+}. Overall, minocycline has been shown to be more effective than theanine in both these aspects. It will however need to be assessed in its role in other reactions such as lipid peroxidation to show its effectiveness as a potential neuroprotective agent.

CHAPTER 4

LIPID PEROXIDATION

4.1 INTRODUCTION

The major constituents of biological membranes are proteins and lipids. PUFAs are lipids that contain more than 2 carbon-carbon double covalent bonds. PUFAs are essential for normal cell functioning as these confer properties such as fluidity and permeability to the membrane that surrounds a cellular structure. Examples of PUFAs include arachidonic, docosaetraenoic and docosahexanoic acids. LP is an irreversible radical reaction that occurs when hydrogen is abstracted from PUFAs. PUFAs are especially vulnerable to attack by free radicals since the double bond within the fatty acid weakens the carbon-hydrogen bonds on the carbon adjacent to the double bond (Halliwell and Gutteridge, 1989). Saturated fatty acids do not have these double bonds and are therefore less susceptible to oxidation by free radicals (Etsuo *et al.*, 2005). LP results in the impairment of membrane function, decrease in fluidity, increase in non-specificity in permeability to ions such as calcium (Ca^{2+}) and the inactivation of membrane-bound receptors and enzymes (Gutteridge and Halliwell, 1990).

Endogenous LP occurs in response to oxidative stress. The LP radical chain reaction takes place in three stages: chain initiation, propagation and termination. The overall reaction is illustrated by the scheme shown in Figure 4.1. LP is initiated by the attack of the lipid by a chemical species that has sufficient reactivity to abstract a hydrogen from the lipid (LH) and thus cause the formation of a hydrogen radical (H•) and a carbon centred radical (L•). The latter which is highly reactive, becomes stable by the abstraction of hydrogen from another chemical species (Laguerre *et al.*, 2007). The propagation step takes place in aerobic conditions, during which the L• is converted into a lipid peroxyl radical (LOO•) (Equation 4.1) and this radical reacts in a 'self-sustained' reaction to form hydroperoxide (LOOH) and another L• radical (Equation 4.2).

$$L\bullet + O_2 \rightarrow LOO\bullet \qquad \textbf{Equation 4.1}$$

$$LOO\bullet + LH \rightarrow LOOH + L\bullet \qquad \textbf{Equation 4.2}$$

The final step of chain termination occurs when LOOH is converted into secondary nonradical oxidation compounds (Halliwell and Gutteridge, 1999; Laguerre *et al.*, 2007). A single initiation could lead to the conversion of several fatty acid chains into LOOH (Gutteridge and Halliwell, 1990).

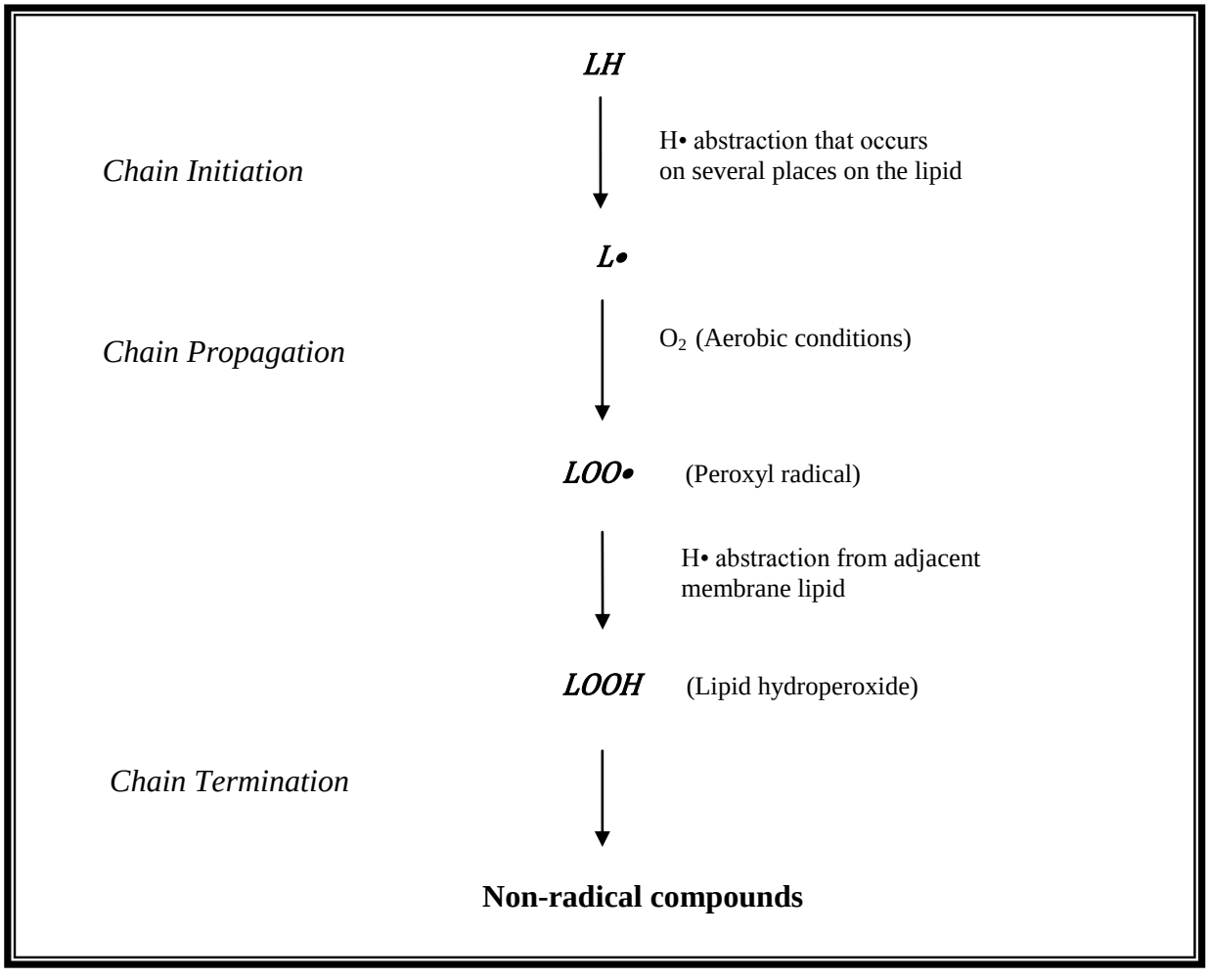

Figure 4.1: Mechanisms involved in LP (Gutteridge and Halliwell, 1990)

LOOHs are the primary product of LP and decompose rapidly in the presence of iron or copper ions, iron proteins such as haemoglobin and simple chelates of these ions (Gutteridge and Halliwell, 1990; Moore and Jackson-Roberts, 1998). The quantity of LOOHs produced depends on the number of double bonds present in the lipid molecule (Esterbauer, 1993). The mechanisms of the secondary and tertiary reaction of the LOOHs include chain cleavage, rearrangement and di- and polymerisation reactions (Esterbauer, 1993). Tissue or cell death leads to more LP due to the release of metal ions that are released by damaged cells and antioxidants within the tissue being diluted out (Gutteridge and Halliwell, 1990). LP is often a late event that accompanies rather than causes final cell death (Smith *et al.*, 1983; Gutteridge and Halliwell, 1990).

Transition metals such as copper and iron and their chelates are known to initiate LP by decomposing peroxides to radicals that are capable of abstracting hydrogen from the lipid and continuing the chain reaction (Bucher *et al.*, 1983; Gutteridge and Halliwell, 1990). Iron is the most important catalyst of LP as it takes part in electron transfer reaction with oxygen (Halliwell and Gutteridge, 1989). Iron catalyses the Haber-Weiss reaction (Equation 1.3) where $O_2^{\bullet-}$ is converted to •OH as well as the Fenton reaction (Equation 1.2) where H_2O_2 is also converted to •OH. *In vivo* animal studies have shown that treatment with iron complexes results in a significant increase in LP (Winterbourn *et al.*, 1991). The •OH does not to initiate LP as concomitant addition of •OH scavengers does not inhibit LP (Bucher *et al.*, 1983). Copper catalyses the formation of •OH from H_2O_2 (Halliwell and Gutteridge, 1984a) and decomposes lipid peroxides to form cytotoxic carbonyl compounds, hydrocarbon gases and free radicals that can continue the chain initiation reaction in LP (Gutteridge and Halliwell, 1990). Cells that have been injured by heat, mechanical trauma, infections and oxygen deprivation in the case of ischaemia, release metal ions which lead to a significant increase in LP in surrounding tissues (Gutteridge and Halliwell, 1990). Iron and copper also cause the acceleration of the decomposition of lipid peroxides by causing the fission of the O-O bond to form an alkoxyl radical (LO•) (Halliwell and Gutteridge, 1999). Heavy metals such as cadmium, cobalt, lead and nickel have been shown to increase the rate of lipid peroxidation (Kappus, 1987).

Antioxidant defences that protect the body from radical damage are divided into 4 categories according to their mechanism of action: antioxidants that prevent the formation of active

oxidants, free radical scavengers, those that quench and remove active oxidants and those that repair damage and excrete toxic oxidation products (Etsuo *et al.*, 2005). The inhibition of LP occurs by the action of low molecular weight antioxidants, protective enzymes and low oxygen tension (which is protective) and by chelation of metal ions (Barber and Bernhein, 1967; Esterbauer, 1993). Antioxidants act by providing hydrogen that can easily be abstracted by peroxyl radicals (Gutteridge and Halliwell, 1990). Intracellular antioxidants such as SOD and GSH peroxidise act by removing reactive oxygen species before they react with iron (Gutteridge and Smith, 1988). There are however no antioxidant enzymes in the extracellular fluid and the antioxidant mechanism employed is to limit radical reaction by converting pro-oxidant forms of iron and copper into less reactive forms by binding such ions to proteins (Gutteridge and Smith, 1988). The binding of metal ions to proteins prevents these from accelerating LP and other free radical reactions. Chelators such as EDTA inhibit both enzymatic and non enzymatic reactions that catalyse LP.

Cell damage by ROS and LP plays a crucial role in the pathogenesis of several chronic and acute diseases such as cancer, inflammation and ischaemia (Sies, 1991). Levels of malondialdehyde (MDA) which is an aldehyde formed from the decomposition of LOOH, or MDA-like products have been shown to be significantly higher in human serum in certain pathological conditions such as cardiovascular disorders, reperfusion injury and diabetes (Esterbauer, 1993). The extent of LP as a result of pathological conditions is considered an important parameter for oxidative stress. Reperfusion of ischaemic tissue causes the production of oxygen derived free radicals which in turn attack PUFAs and thus lead to LP (Cordis and Maulik, 1993).

The oxidation of lipids can be measured at the different stages of the LP reaction and the following can be measured to determine the extent of LP: the loss of PUFAs, the primary peroxidation products or the secondary carbonyls and hydrocarbon gases produced (Gutteridge and Halliwell, 1990). The most common assay used to assess the extent of LP is the thiobarbituric acid (TBA) assay which measures the secondary oxidation products of LP. In this assay the lipid sample and TBA are heated at low pH and this leads to the formation of a pink chromogen with a λ_{max} of 532 nm. The pink chromogen is formed by the reaction of 1 MDA molecule with 2 TBA molecules (Figure 4.2) (Laguerre *et al.*, 2007). MDA is a

recognisable marker for LP (Ottino and Duncan, 1997) and the TBA assay is suitable for both *in vivo* and *in vitro* assessments of LP.

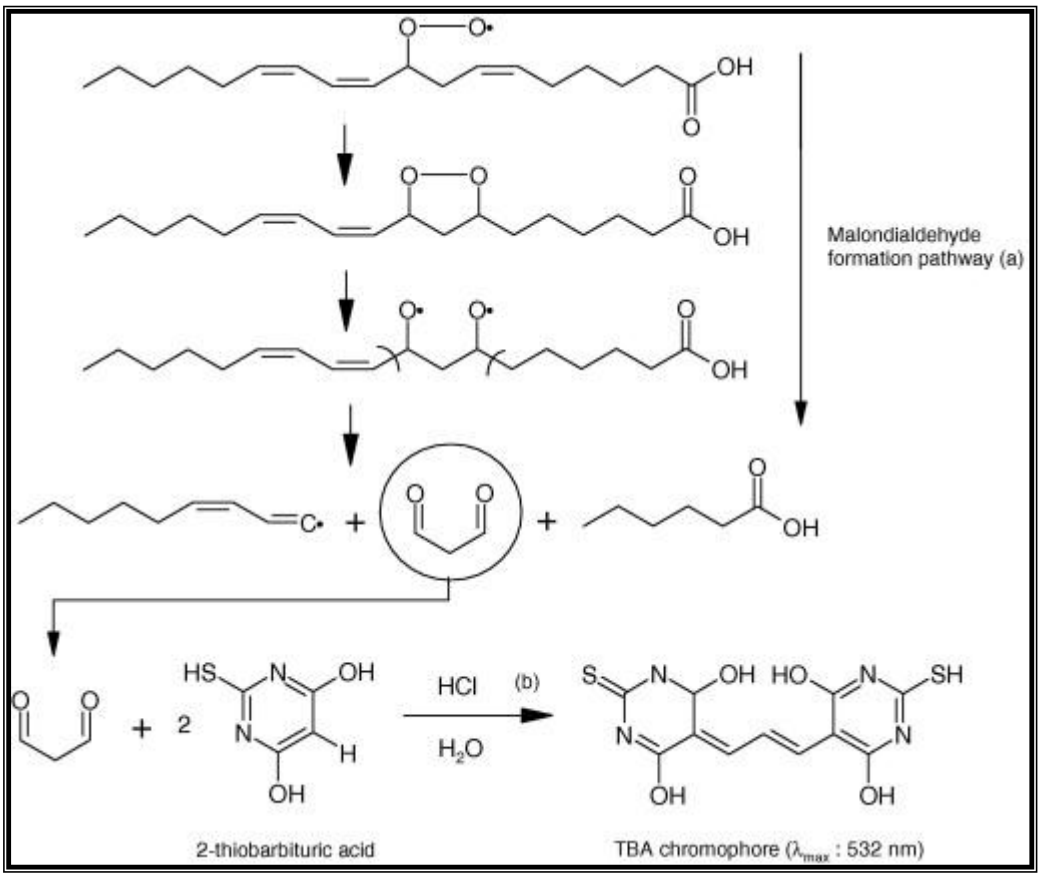

Figure 4.2: The formation of MDA (circled) from a peroxyl radical of tri-unsaturated fatty acid and the formation of TBA chromophore from TBA and MDA **(**Laguerre *et al.,* 2007)

The disadvantage of the TBA assay is that several other aldehydes are formed during the LP reaction to give other chromogens that are also detectable at a wavelength of 532 nm (Gutteridge and Halliwell, 1990). Despite this, the TBA assay may be employed to assess LP or the susceptibility of the tissues to peroxidation with some degree of reliability.

4.2 THE COMPARATIVE EFFECTS OF THEANINE AND MINOCYCLINE ON QA-INDUCED LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE *IN VITRO*

4.2.1 INTRODUCTION

Certain tissue homogenates such as rat brain and liver undergo LP more readily whereas others, such as intestinal homogenates, do not (Barber and Bernheim, 1967). The brain was homogenised as it has a high concentration of PUFAs and is easily oxidised during LP. LP can be induced in tissues by iron, organic hydroperoxides and the metabolism of carbon tetrachloride (CCl_4) which results in the production of free radicals among other chemicals (Kappus, 1987). Although LP may be induced by different chemicals, the degradation products are still the same.

QA which is a metabolite of the tryptophan-kynurenine pathway can be used to induce LP (Rios and Santamaria, 1991). It is released by activated microglia and macrophages in response to a variety of pathological diseases (Šťastný *et al.*, 2004) and its levels increase in inflammatory neurological diseases such as ischemia brought on by stroke (Halliwell and Gutteridge, 1989). QA changes the permeability of the BBB by activating the NMDA receptors and by inducing LP (Šťastný *et al.*, 2004). The average concentration of QA in the human brain is between 0.5 and 2 pmol/mg tissue (Wolfensberger *et al.*, 1983). QA is a useful toxin to study in systems that mimic what happens *in vivo* as it has been found that a mid to high micromolar concentration of QA could develop under pathological conditions owing to discrete subpopulations of QA producing cells secreting QA directly onto specific target sites that could potentially generate high local concentrations of QA (Šťastný *et al.*, 2004).

The brain is susceptible to LP because it is rich in PUFAs and relatively deficient in antioxidant protective mechanisms. Minocycline has shown more promising results as a neuroprotective agent than theanine as it is a potent free radical scavenger (Section 2.3.4) and is efficient in binding with both iron (III) (Fe^{3+}) and iron (II) (Fe^{2+}) ions. Their potential neuroprotective properties were further assessed by investigating their effects on QA-induced LP.

4.2.2 MATERIALS AND METHODS

4.2.2.1 Chemicals and reagents

Theanine, minocycline, 2, 3-pyridine dicarboxylic acid (QA), butylated hydroxytoluene (BHT), 2-thiobarbituric acid (TBA) and 1, 1, 3, 3-tetramethoxypropane (99 %) were purchased from Sigma Chemical Corporation, St Louis, Missouri, United States of America Trichloracetic acid (TCA), ethanol and butanol were purchased from Saarchem, Johannesburg, South Africa.

4.2.2.2 Animals

Adult male Wistar rats purchased from the South African Vaccine Producers, Johannesburg, South Africa were used throughout the study. The animals were housed under artificial illumination with a daily photoperiod of 12 hours (lights were switched on at 0600 hours and switched off at 1800 hours). The animal-house temperature was maintained at a temperature range of between 20 ºC to 24 ºC, while an extractor fan ensured the constant removal of stale air. The rats that were between 200 g and 250 g were housed 5 per cage with food and water *ad libitum*. The Rhodes University Animal Ethics Committee approved all the protocols for the experiments that were conducted.

4.2.2.3 Brain removal

The rats were sacrificed by cervical dislocation followed by decapitation. The brain was exposed by making an incision through the bone on either side of the parietal structure, from the foramen magnum to near the orbit. The calvarium was removed, exposing the brain which was them removed and either used immediately or frozen in liquid nitrogen and stored at -70ºC for future use.

4.2.2.4 Homogenate preparation

Once the brain was removed it was weighed and homogenised in a glass Teflon hand held homogeniser in ice cold 0.1 M phosphate buffered saline (PBS), at pH 7.4 to yield a 10 %

w/v homogenate. This is necessary to prevent lyosomal damage of the tissue. The homogenate was then used immediately for the assay.

4.2.2.5 Preparation of MDA standard curve

The construction of a calibration curve for the TBA assay is complicated by the fact that MDA is very unstable thus, one of its derivatives, either 1, 1, 3, 3-tetramethoxypropane or 1, 1, 3, 3-tetraethoxypropane is used instead (Halliwell and Gutteridge, 1989). 1, 1, 3, 3-Tetramethoxypropane was used in the preparation of the standard curve. A series of reaction tubes, each containing varying concentrations of 1, 1, 3, 3-tetramethoxypropane standards (in the range 0-50 nmoles/ml) diluted in 0.1 M PBS was prepared to give a total volume of 1 ml. To these tubes, 0.5 ml of BHT (0.5 % in ethanol) and 1 ml of TCA (15 % in milli-Q water) were added, the reaction tubes were vortexed. A 2 ml aliquot was removed and to this 0.5ml of TBA (0.33 % in milli-Q) was added. This mixture was vortexed and incubated at 95 ºC for an hour. A standard curve was generated by measuring the absorbance at 532 nm using a Shimadzu UV mini 1240 UV/VIS spectrophotometer and plotting these against the molar equivalent of MDA in the complexes formed (Appendix III).

4.2.2.6 Lipid peroxidation assay

A modification of the TBA assay as described by Placer *et al.,* (1966) was used in this experiment and is described below:

Triplicate samples of rat brain homogenate (1 ml) contained 1mM QA (100 µl) in the absence and presence of increasing concentrations (0-2 mM) of theanine and minocycline (100 µl). The samples were incubated in an oscillating water bath for an hour at 37 ºC. Control samples did not contain QA or drugs. The reaction was terminated at the end of the incubation period by the addition of 1 ml TCA (15 % in milli-Q water) and 0.5 ml BHT (0.5 % in ethanol) to each sample. The samples were vortexed and heated at 95 ºC for 15 minutes in an oscillating water bath to release protein-bound MDA. Following this, samples were cooled and centrifuged at 2000 x g for 20 minutes to yield a protein-free supernatant. The supernatant (2 ml) was transferred to a clean set of test tubes and 0.5 ml TBA (0.33 % in milli-Q water) was added. All samples were vortexed and heated at 95 ºC for an hour in an oscillating water

bath to allow for the formation of the MDA-TBA complex. After rapidly cooling the test tubes in ice, 2 ml of butanol was added to the samples to extract the pink complex. The samples were then vortexed and centrifuged at 2000 x g for 15 minutes. An aliquot of the extracted complex in butanol (the top layer) was read at 532 nm using a Shimadzu UV mini 1240 UV/VIS spectrophotometer. MDA levels were then determined from a standard curve generated from 1, 1, 3, 3-tetramethoxypropane as described in Section 4.2.2.5. Final results were expressed as MDA (nmoles/mg tissue).

4.2.2.7 Statistical analysis

This was performed as described in Section 2.2.2.3.

4.2.3 RESULTS

As shown in Figure 4.3, 1 mM QA increases the amount of MDA in comparison to control ($p < 0.05$). Minocycline reduces the QA-induced LP in a concentration dependent manner. The suppression of MDA is highly significant for each concentration used ($p < 0.01$) in comparison to samples containing QA alone. In contrast there is no significant difference between samples with QA alone and any of those containing QA and theanine.

A comparison between the two drugs at the same concentration shows that minocycline decreased QA-induced LP significantly ($p < 0.01$) for 0.5, 1 and 2 mM and ($p < 0.05$) for 0.25 mM when compared to theanine.

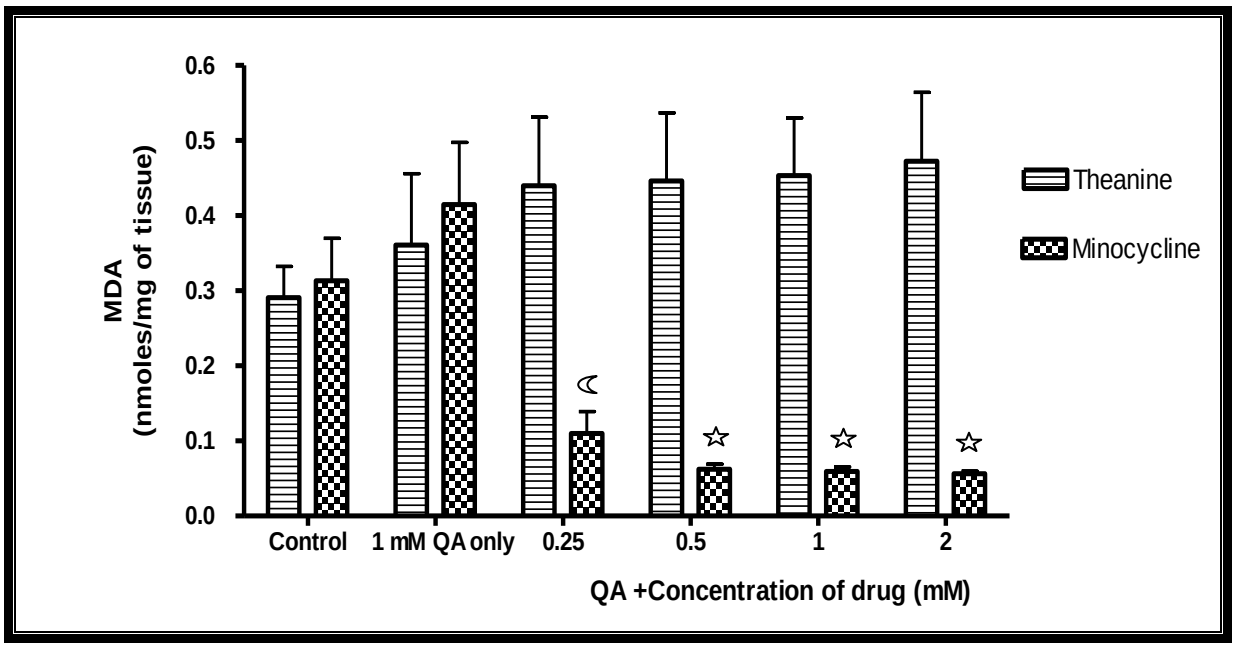

Figure 4.3: Effect of 1 mM QA alone and in combination with either theanine or minocycline on LP in rat brain homogenate *in vitro*. Each bar represents the mean ± SD; (n=5). ☆ ($p < 0.01$) in comparison to QA and ☾ ($p < 0.05$) in comparison to QA (ANOVA followed by Student Newman-Keuls Multiple Range Test).

4.2.4 DISCUSSION

Štípek *et al.* (1997) reported that the oxidising activity of QA on LP in the rat brain *in vitro* as investigated by Rios and Santamaria (1991) requires the presence of Fe^{2+} ions and that the mechanism is likely to involve Fe^{2+} chelation by QA. Homogenising tissue results in the release of metal ions especially iron from storage sites within cells (Barber, 1963; Gutteridge and Stocks, 1976). In the TBA assay, QA was added to the homogenised tissue and thus was able to form a complex with Fe^{2+}. UV absorption spectra have shown that 2-pyridinecarboxylic acid containing compounds such as QA are able to chelate Fe^{2+} ions (Figure 4.4) and that a UV spectral wave generated disappeared on addition of hydrogen peroxide (H_20_2) indicating that the complex may have been oxidised by H_20_2 to Fe^{3+} with the subsequent formation of the potent •OH radical by the Fenton reaction (Iwahashi *et al.*, 1999). Furthermore, on addition of the iron chelator, desferoxamine, the increase in QA-induced LP was abolished (Štípek *et al.*, 1997).

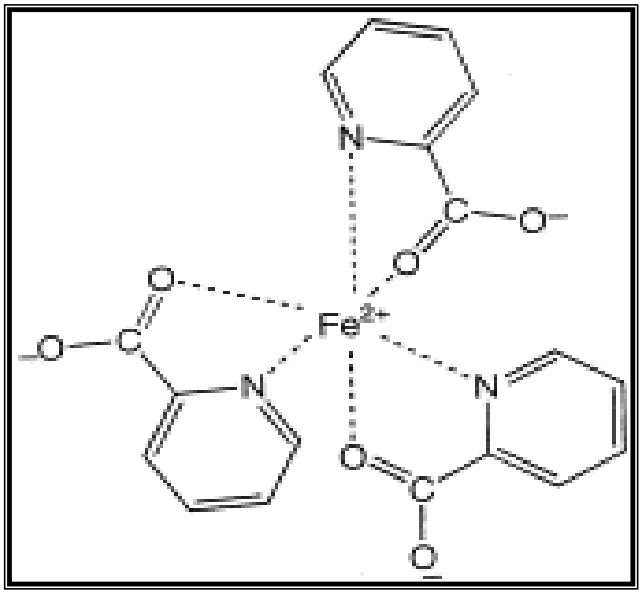

Figure 4.4: Binding of Fe^{2+} by the 2-pyridine carboxylic acid moiety (Iwahashi *et al.*, 1999)

Metals with two valency states with suitable redox potential generally cause an increase in LP rate (Barber and Bernheim, 1967) by the decomposition of LOOH into the alkoxyl (LO•) and peroxyl (LOO•) radicals as shown in Equation 4.3 (Gutteridge and Smith, 1988). The radicals produced from this reaction can continue the LP reaction within the lipid. Iron exists in two valency states and can thus cause an increase in the LP rate.

$$LOOH \xrightarrow{Fe^{2+}} LO\bullet + LOO\bullet$$ **Equation 4.3**

Antioxidants are categorised according to their mechanism of action in protecting the body from damage by free radicals (Section 1.3.2.1). Minocycline has shown to be a highly effective free radical scavenger (Section 2.3.4) and a strong chelator of Fe^{2+} and Fe^{3+} (Section 3.5.4). From the results above (Section 4.2.3), minocycline has been shown to statistically significantly inhibit QA-induced LP in comparison to QA alone samples. The mechanism that could be proposed for such a reduction in LP could be that it acts as chelator of the Fe^{2+} ions and thus prevent the formation of the Fe^{2+}-QA complex which is required for the oxidising activity of QA in LP. Theanine is a weak chelator of iron and thus it does not prevent the QA from chelating the Fe^{2+} that is in the homogenate and thus allows for the progression of QA-induced LP.

The TBA assay relies on the formation the TBA-MDA chromophore, therefore it follows that less MDA was produced in the presence of minocycline and hence, less LOOH decomposed to MDA. Minocycline prevents the decomposition of LOOH into other radical forms (minocycline exhibited antioxidant activity that was shown in Section 2.3.4) and can be categorised into the group of antioxidants that act by preventing the formation of active oxidants by donating a hydrogen to LOO• and thus prevent the chain propagation reactions of LP from continuing the generation of free radicals. Phenol compounds such as minocycline are able donate to a hydrogen as it has the capacity to stabilise unpaired electrons by delocalisation within its aromatic structure and the presence of other cyclic structures within it lead to the extension of the delocalisation and thus increase the stability of the phenol radical formed when the hydrogen is abstracted by the LOO• (Laguerre *et al.,* 2007).

Iron chelates have been shown to induce LP *in vitro* by catalysing the conversion of superoxide $O_2^{\bullet-}$ and H_2O_2 into •OH radicals. Fe^{2+} chelates are more effective at promoting the formation of MDA from LOOH than free Fe^{3+} ions (Bucher *et al.*, 1983). Although LP may be induced by different toxins, the degradation products are still the same. The effects of the iron-minocycline chelates are less effective than the iron-QA chelates in causing lipid peroxidation. This is evidenced by the fact that there is a statistically significant difference in the MDA produced in the presence of QA and in the presence of minocycline in concentrations even as low as 0.25 mM. Minocycline also exhibits a concentration dependent decrease in the formation of MDA. However as stated in Section 4.2.3, above the concentration of 0.5 mM, there is no statistically significant change in the inhibition of LP. Kraus and colleagues (2005) reported a decrease in iron-induced LP *in vitro* and that this effect was due to minocycline's radical scavenging activity.

Minocycline could also prove to be superior to theanine as it is lipophilic and theanine is not which could increase its effectiveness in inhibiting QA from interacting with iron in lipid homogenates. Minocycline has been proven to be an effective chelator of Fe^{2+} (Section 3.5.4) and this physiochemical property allows it to be an effective inhibitor of QA-induced LP and can thus be further investigated for more potentially neuroprotective properties.

4.3 THE EFFECT OF THEANINE ON QA-INDUCED LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE *IN VIVO*

4.3.1 INTRODUCTION

Theanine showed very poor inhibition of QA-induced LP *in vitro* (Section 4.2.4). It was however decided to explore its potential as a neuroprotectant in rats that had been administered a daily dose of theanine for 5 and 10 days.

4.3.2 MATERIALS AND METHODS

4.3.2.1 Chemicals and reagents

The drugs used and purchased are the same as those described in Section 4.2.2.1.

4.3.2.2 Animals

Adult male Wistar rats were purchased from the South African Vaccine Producers, Johannesburg, South Africa and cared for as described in Section 4.2.2.2.

4.3.2.3 Drug treatment

The rats were divided into 2 groups: control and theanine. Each group consisted of 5 rats. The control groups were administered daily doses of normal saline and the remaining groups of rats were administered 5 mg/kg/day (0.5 ml) theanine i.p for 5 and 10 days.

4.3.2.4 Brain removal

The brain was removed as described in Section 4.2.2.3

4.3.2.5 Homogenate preparation

The homogenate was prepared as described as in Section 4.2.2.4.

4.3.2.6 Lipid peroxidation assay

The LP assay was performed as described in Section 4.2.2.6. However, no exogenous theanine was added. The assay resumed after 1 ml QA (100 µM) was added to the homogenate and the samples were incubated.

4.3.2.7 Statistical analysis

This was performed as described in Section 2.2.2.3.

4.3.3 RESULTS

Figure 4.4 illustrates that treatment with theanine over a period of 5 and 10 days resulted in a statistically significant decrease ($p < 0.01$) in QA-induced LP in comparison with brains treated with QA only. Treatment with theanine also reduced the MDA produced in the control. However there is no statistically significant difference treating the rats for 5 or 10 days.

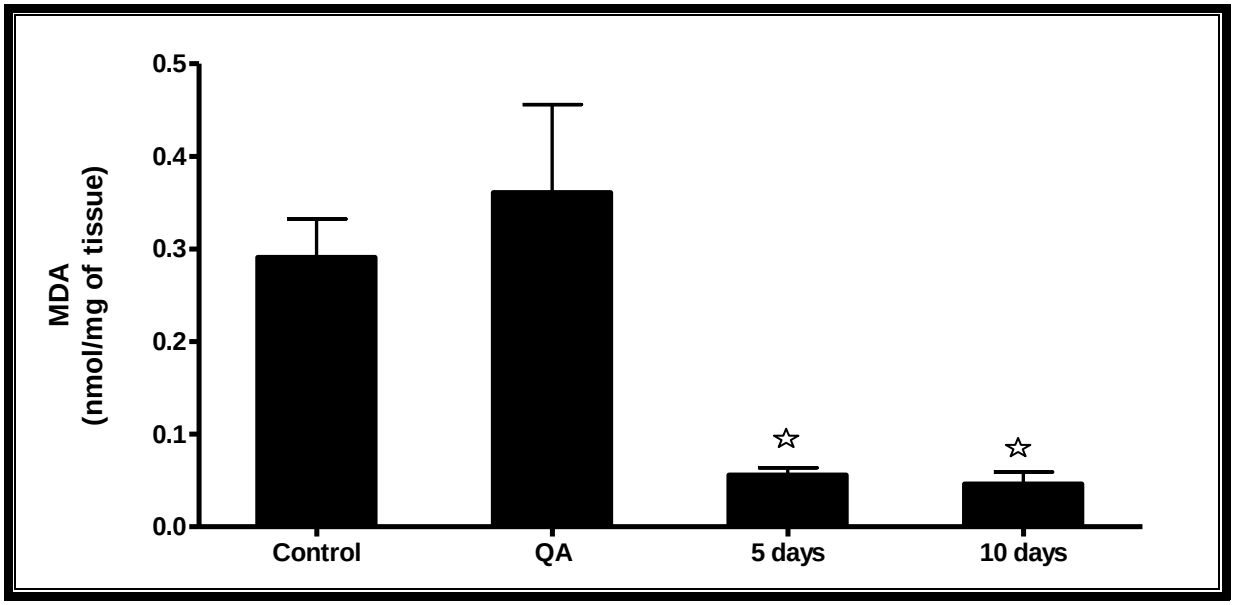

Figure 4.5: Effect of 1 mM QA alone and in combination with either theanine on LP in rat brain homogenate *in vivo*. Each bar represents the mean ± SD; (n=5). ☆ ($p < 0.01$) in comparison to QA (ANOVA followed by Student Newman-Keuls Multiple Range Test).

4.3.4 DISCUSSION

There is a significant change in theanine's ability to prevent QA-induced LP in pre-treated brains in comparison to the *in vitro* results (Section 4.2.3) where theanine displayed no statistically significant change in QA-induced LP. This suggests that treatment with theanine results in some form of neuroprotection against QA-induced LP.

Alpha-tocopherol (vitamin E), a major antioxidant found in all membranes (Parker, 1991) inhibits lipid peroxidation both *in vivo* and *in vitro* (Burton and Ingold, 1989; Murphy *et al.*, 1989). Pre-treatment of animals with vitamin E has been found to reduce secondary damage in several models of ischaemic traumatic injury to the CNS (Halliwell and Gutteridge, 1989). This suggests that pre-treatment may have a degree of neuroprotection depending on the drug analysed.

Long term supplementation (of about 50 days) with green tea has been shown to decrease LP in the rat liver (Sano *et al.*, 1995) and decrease lipid peroxide levels in hamsters (Vinson and Dabbash, 1998). Green tea was found to be efficient in trapping radicals after a single dose (Serafini *et al.*, 1996). These findings suggest that pre-incubation with green tea has desirable

effects in the mammalian body. As a component of green tea, theanine may be better as a free radical scavenger and iron chelator after pre-treatment.

The results (Section 4.3.3) suggest that pre-treating the brain with theanine improved its ability to protect it from LP induced by QA. In homogenising the tissue, antioxidants present in the tissue are diluted thus rendering these less effective than when these are at normal intracellular concentration. Both theanine's free radical scavenging ability (Section 2.3.4) and its ability to chelate iron (Section 3.5.4) *in vitro* are both shown to be poor. The observed reduction in MDA levels in theanine-treated brains implies that these characteristics must have improved when theanine was administered over 5 days or more.

Theanine may be one of the components which contribute to the lowering of LP and lipid peroxide levels and free radical scavenging properties exhibited by green tea. A study in rats with ischaemic brain injury by Wang *et al.* (2008), showed that rats treated with theanine exhibited significantly higher levels of SOD activity and decreased levels of MDA contents as compared to the ischaemia-reperfusion group. These findings were attributed to theanine's regulatory effect on the imbalance of free radical metabolism due to ischaemia. The findings by Wang *et al.* (2008) as well as those that have been investigated in this *in vivo* study, imply that dosing with theanine improves its QA-induced LP lowering and antiradical properties. The mechanism in which theanine lowers LP could be by increasing the SOD activity and thus reducing the amount of $O_2^{\bullet-}$ that can participate in the progression of LP. Theanine thus significantly reduces QA-induced LP. The effect of theanine on QA biosynthesis will be investigated in Chapter 5. If theanine is shown to reduce QA biosynthesis this could contribute to the ways in which theanine is neuroprotective.

CHAPTER 5

THE BIOSYNTHESIS OF QUINOLINIC ACID

5.1 INTRODUCTION

TRP is an essential amino acid that is required for physiological processes such as the synthesis of proteins and as a biochemical precursor of 5HT, melatonin and niacin (Saito *et al.,* 1993a). It is metabolised by the KP into 3 main neuroactive chemicals namely QA, KYNA and 3-OH-KYN. The KP is the predominant route by which TRP is metabolised in the brain and in the periphery (Bender, 1983) (Figure 5.1). Along the KP, TRP is oxidised to N-formylkynurenine and rapidly catabolised by the action of TDO or IDO to KYN (Schwarcz and Pellicciari, 2002). There is a functional connection between the KP and cholinergic system (Sas *et al.,* 2007).

An imbalance in the KP metabolites may interfere with the normal functioning of the brain that may lead to the pathology of some brain disorders (Sas *et al.,* 2007a). After transient cerebral ischaemia, there is a delayed increase in KP metabolism and an increase in the concentration of L-KYN, L-TRP and neurotoxic QA in the forebrain (Saito *et al.,* 1993b). QA leads to excitotoxic neuronal death. In HD there is an increase in 3-OH-KYN levels in the brain (Reynold and Pearson, 1989). QA an excitotoxin produced in the KP, is involved in the pathology of AD. QA-induced LP and the ultimate generation of free radicals and its agonistic effect on NMDA receptors have a significant impact on the pathogenesis of AD (Guillemin and Braw, 2002). In addition to this, the KP is upregulated in the AD brain (Sas *et al.,* 2007).

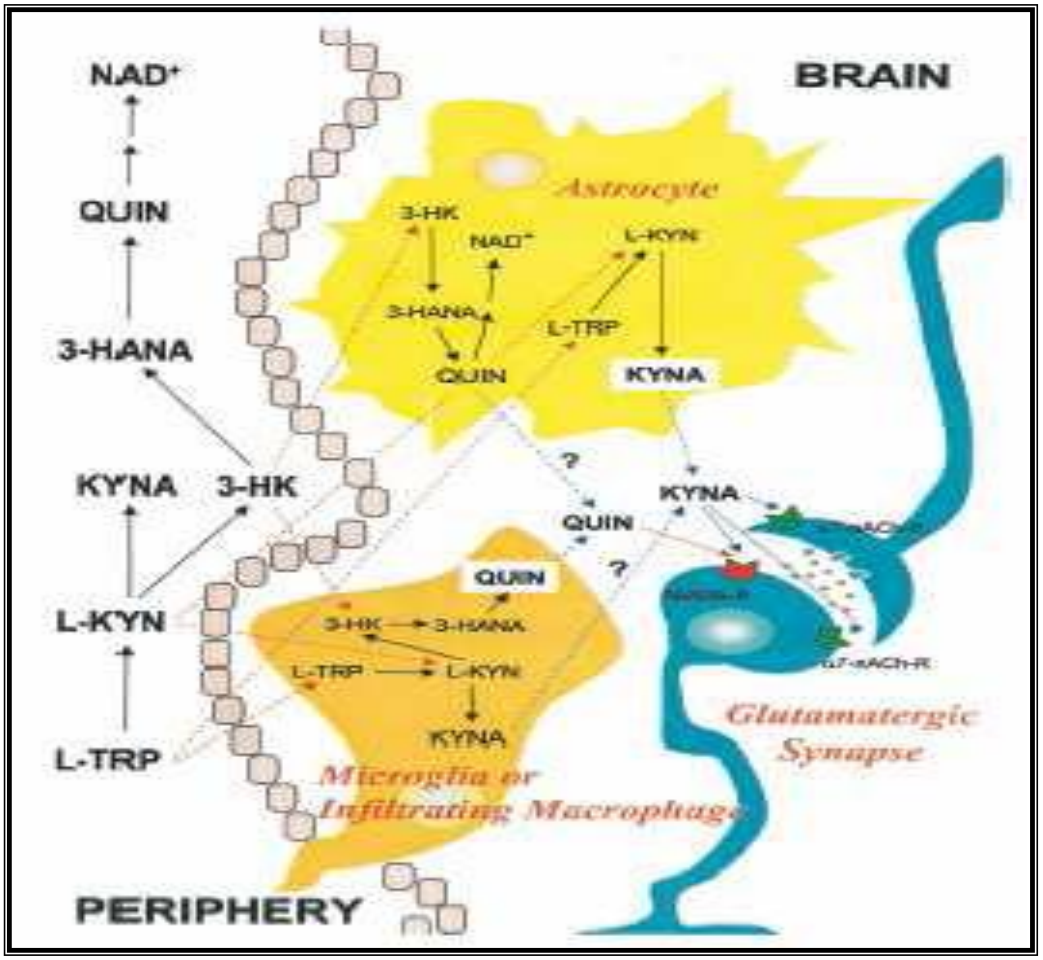

Figure 5.1: Dynamics of kynurenine pathway metabolism in the CNS and periphery. *The abbreviations in the illustration above in brackets are: L-tryptophan (L-TRP); L-kynurenine (L-KYN); kynurenic acid (KYNA); 3-hydroxy kynurenine (3-HK); 3-hydroxyanthranilic acid (3-HANA), Quinolinic acid (QUIN); NMDA receptor (NMDA-R); 7-nicotinic acetylcholine receptor (7-nACh-R). Broken arrows: brain entry/cellular uptake (red), release (blue). Solid arrows: enzymatic conversion (black), receptor agonist (red), receptor antagonist (blue)* (Schwarcz and Pellicciari, 2002),

TDO is the hepatic enzyme that converts TRP to N-formylkynurenine by irreversibly inserting oxygen into the pyrrole portion of TRP (Hayaishi *et al.*, 1957). In the liver, TDO is associated with a "free" haeme pool, which usually provides sufficient haeme to saturate half the available TDO. As a result, TDO exists in two forms, namely, the already active holoenzyme form and the apoenzyme form which requires haeme or haematin for its activation *in vivo* and *in vitro* respectively (Badawy and Evans, 1975). Chapter 4 has shown the deleterious effects of QA-induced LP on the rat brain and the neuroprotective effect of minocycline *in vitro* and that of theanine *in vivo*. Thus, the aim of this chapter is to assess the effect of theanine and minocycline on the activity of TDO which catalyses the conversion of TRP to QA.

5.2 THE COMPARATIVE EFFECT OF THEANINE AND MINOCYCLINE ON TDO ACTIVITY IN RAT LIVER HOMOGENATE *IN VITRO*

5.2.1 INTRODUCTION

Changes in the KP have been shown to play a role in the pathology of various diseases. TRP, a substrate of TDO and IDO, is required for the synthesis proteins and 5HT. Metabolites of the KP that are of interest are QA, which is neurotoxic and KYNA, which is neuroprotective. The changes in KP metabolite levels may be attributed to changes in the TRP levels and in enzymes that participate in the KP.

Serum levels of TRP may be attributed to dietary TRP levels or changes in KP enzymatic activity. TRP plasma levels are lower in AD and in HD. This is due to the up-regulation of the KP in these disease states (Sas *et al.*, 2007). Saito *et al.*, (1993) reported an increase in kynureninase, IDO and 3-hydroxylase activity after 10 minutes of ischaemia and that there was an increase in the concentration of QA associated with the increase in enzymatic activity. Macrophages infiltrate regions of neuronal necrosis after ischaemia in the rat (Smith *et al.*, 1983) and this causes an increase in the production of $O_2^{\bullet-}$ (Saito *et al.*, 1993b).

TDO exists as the active holoenzyme and inactive apoenzyme and its activity in the liver is responsible for the conversion of TRP to N-formylkynurenine (Section 1.2.6.2.2.1). Haeme is a cofactor that plays a role in rat liver TDO and increases the activity of hepatic TDO as it activates the inactive apoenzyme (Badawy and Evans, 1975). The ability of haeme to saturate the apoenzyme is decreased by agents that destroy haeme or inhibit haeme synthesis and increase by agents that increase the utilisation or synthesis of haeme (Badawy and Evans, 1975). The effect of theanine and minocycline on TDO activity will be assessed in the TDO assay (Section 5.2.2.5) where their effects on the active holoenzyme and apoenzyme will be determined.

5.2.2 MATERIALS AND METHODS

5.2.2.1 Chemicals and reagents

Theanine, minocycline, haematin, L-TRP, Bradford's reagent and bovine serum albumin (BSA) were purchased from Sigma Chemical Corporation, St Louis, Missouri, United States of America. Trichloracetic acid (TCA), sodium hydroxide (NaOH) and sodium chloride (NaCl) were purchased from Saarchem, Johannesburg, South Africa.

5.2.2.2 Animals

Adult male Wistar rats were purchased from the South African Vaccine Producers, Johannesburg, South Africa and cared for as described in Section 4.2.2.2.

5.2.2.3 Removal of the liver

The rats were sacrificed by cervical dislocation followed by decapitation. To remove the liver, a mid-ventrial incision was made through the abdominal musculature from the pelvic region to the posterior edge of the sternum. A transverse cut was made anteriorly to expose the liver which was then removed. The liver was immediately perfused with ice cold 0.9 % NaCl to remove all endogenous haeme and either used immediately or frozen in liquid nitrogen and stored at -70 ºC for future use.

5.2.2.4 Preparation of the tissue

The livers were thawed, weighed and chopped into fine pieces and rapidly homogenised with a glass-teflon hand held homogeniser in 0.01 M PBS (pH 7.4), to give a final concentration of 10 % w/v. The homogenate was then sonicated for a period of 2 minutes at 30 second intervals for complete removal of the enzyme from the cells. The entire procedure where possible, was conducted in ice.

5.2.2.5 Determination of TDO activity

A modification of the method described by Badawy and Evans (1975) was used and is outlined below:

An aliquot of 15 ml of homogenate was added to 2 flasks containing 12.5 ml water and vortexed. To one of the 2 flasks, haematin (100 µl) was added to make a final concentration of 2 µM. This flask was then stirred and allowed to stand for 1 minute to allow for the activation of the enzyme by haematin. Thereafter, 2.5 ml of 0.03 M L-TRP was added to each flask. The flasks were stirred before aliquots of 2 ml were transferred to test tubes in triplicate. Thereafter, either theanine or minocycline was added to each of the test tubes such that incubation concentrations of 10 µM and 100 µM of each drug was achieved. Neither theanine nor minocycline was added to the control samples. The test tubes were then stoppered with carbogen and incubated for 60 minutes at 37ºC in an oscillating water bath. To stop the reaction, 0.9 M TCA (2 ml) was added to each test tube. The resulting precipitate was then centrifuged at 4500 x g for 15 minutes. The supernatant (2.5 ml) was transferred to a new set of test tubes and 0.6 M NaOH (1.5 ml) was added to each test tube. An aliquot from each test tube was removed for analysis. The blank consisted of 2 ml of TCA and 1.5 ml of NaOH. The absorbance was read at 365 nm using a Shimadzu UV mini 1240 UV/VIS spectrophotometer. 365 nm is the λ_{max} of N-formylkynurenine. The extinction coefficient of N-formylkynurenine, $\varepsilon = 4540\ M^{-1}.cm^{-1}$ was used to determine the concentration of N-formylkynurenine. Activity of TDO was expressed as nmol N-formylkynurenine/mg of protein/min. The results from test tubes that did not contain haematin represented total holoenzyme activity. Apoenzyme activity was calculated as the difference between the total enzyme activity and holoenzyme activity.

5.2.2.6 Protein assay

Protein estimation was performed using the principles described by Bradford (1976).

A calibration curve was generated using BSA which was prepared in 0.01 M PBS within a concentration range of 0.1 to 1.4 mg/ml. The assay was performed in test tubes. Protein samples (0.1 ml) of each concentration was added to the test tubes. To each test tube,

Bradford reagent (3 ml) was added and gently vortexed. The total liquid volume in each tube was 3.1 ml. The samples were left at room temperature to incubate for a period of 15 minutes. The absorbance was then read at 595 nm using a Shimadzu UV mini 1240 UV/VIS spectrophotometer. The test tube of zero concentration of protein consisted of the PBS only. The protein-dye complex is stable for up to 60 minutes and care was taken to record the absorbance of the samples before the 60 minute time limit and within 10 minutes of each other. A standard curve was generated by plotting concentration of protein against net absorbance (Appendix IV).

The concentration of the protein was determined in each of the liver samples by using the standard curve generated.

5.2.2.7 Statistical analysis

This was performed as described in Section 2.2.2.3.

5.2.3 RESULTS

As shown in Figure 5.2, it is evident that at a concentration of 100 µM, minocycline significantly increases the total enzyme and holoenzyme activity of TDO ($p < 0.001$), whilst 10 µM minocycline and both concentrations of theanine had no significant effect on either form of the enzyme.

There is also a statistically significant concentration dependent increase in the activity of both total and holoenzyme activity of TDO with minocycline at 10 µM and 100 µM ($p < 0.001$). As evidenced by the results, minocycline only has an effect on the holoenzyme of TDO and not on apoenzyme. The difference between the effect on the holoenzyme between minocycline and theanine when both are used at a concentration of 100 µM is also statistically significant ($p < 0.001$).

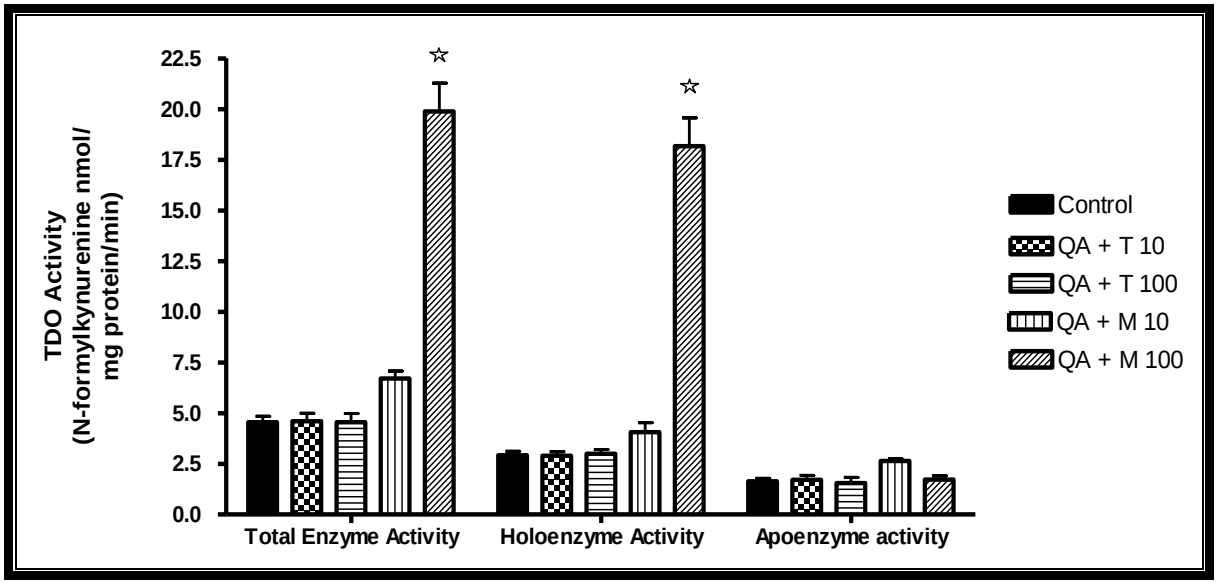

Figure 5.2: Effect of theanine and minocycline on TDO enzyme activity in rat liver *in vitro*. *T 10 and T 100 represent theanine at concentrations of 10 µM and 100 µM respectively; M 10 and M 100 represent minocycline at concentrations of 10 µM and 100 µM respectively.* Each bar represents the mean ± SD (n=5). ☆ ($p < 0.001$) in comparison to controls (ANOVA followed by Student Newman-Keuls Multiple Range Test).

5.2.4 DISCUSSION

TDO exists in two forms: the active holoenzyme that does not require the addition of exogenous haematin for demonstration of its activity *in vitro* and the inactive apoenzyme that requires exogenous haematin to activate its activity (Greengard, 1964; Badawy and Evans, 1975). TDO activity is stimulated by the presence of TRP. The manner in which TRP reacts with TDO has been discussed (Section 1.2.6.2.2.1) and Equation 5.1 adapted from Knox (1966) will be used to briefly describe the reaction between TDO and TRP:

$$Apoenzyme \xrightarrow{L-TRP+\ Haematin} Oxidised\ holoenzyme \xrightarrow{L-TRP\ or\ Reducing\ agent} Reduced\ holoenzyme$$

Equation 5.1

The equation above shows that the activation of TDO occurs in a series of steps starting with the inactive apoenzyme which is conjugated with haematin and requires small concentrations of L-TRP. The oxidised ferric form of the holoenzyme is then reduced to the ferrous form in the presence of L-TRP and a reducing agent such as vitamin C. The reduced holoenzyme is fully active and can participate in the conversion of L-TRP to N-formylkynurenine in aerobic conditions. Oxygen has a role in the reaction of the active holoenzyme and L-TRP (not shown in the equation). It is responsible for the oxidation of the ferrous form of the holoenzyme into the ferric form resulting in it being reduced to $O_2^{\bullet-}$ and the formation of a TRP peroxide (Equation 1.7) which will undergo further reaction independent of TDO to form N-formylkynureine. It is apparent therefore that TRP brings about its own oxidative removal by activating TDO which is responsible for its metabolism (Knox, 1966).

From Figure 5.2 it is apparent that theanine has no significant effect on TDO activity *in vitro* and that minocycline does. Addition of haematin to the minocycline treated livers does not bring about a significant change in the activity of the apoenzyme activity *in vitro* and the fact that there is only a significant change in holoenzyme activity, shows that minocycline affects only the activity of the holoenzyme and that it does not require the presence of haematin in its mechanism of increasing total TDO activity.

There are two mechanisms by which minocycline may bring about the increase in TDO activity and TRP metabolism and these are:

- Minocycline can act as a reducing agent and facilitate the conversion of oxidised holoenzyme into the reduced holoenzyme or
- Minocycline can facilitate the reduction of oxygen to $O_2^{\bullet-}$ thereby facilitating the formation of the TRP peroxide which will undergo further reactions independent of TDO to form N-formylkynureine.

Minocycline has been shown to form complexes with Fe^{3+} (Sections 3.2.4 and 3.3.3) and to bring about the reduction of ferric iron to the ferrous ion (Section 3.5.4). Knox (1966) demonstrated that for maximal TDO enzyme activity, the enzyme preparation requires the addition of a reducing agent such as vitamin C. Minocycline reducing ability *in vitro* is

comparable to that of vitamin C (Section 2.3.4) and thus it is able to reduce the oxidised ferric holoenzyme TDO to the active ferrous holoenzyme. This means that more holoenzyme is available for the metabolism of L-TRP.

The other possible mechanism for its increase in the levels of holoenzyme levels could be attributed to the possibility that minocycline is able to increase the levels of $O_2^{\bullet-}$ by the reduction of oxygen. Its potential as a generator of $O_2^{\bullet-}$ will be further investigated in Chapter 7. If minocycline is proved to be a generator of $O_2^{\bullet-}$, this could explain the drastic increase in TDO activity when compared to control liver samples as the increase in the TDO activity is approximately 400 % that of the control.

5.3 THE COMPARATIVE EFFECT OF THEANINE ON TDO ACTIVITY IN RAT LIVER HOMOGENATE *IN VIVO*

5.3.1 INTRODUCTION

The concentration of hepatic TDO changes according to the dietary and hormonal state of the mammal (Muñoz-Clares *et al.,* 1980). Theanine showed no significant change in TDO activity (Section 5.2.4). It was decided to explore its effect on TDO in rats that had been administered a daily dose of theanine for 5 and 10 days.

5.3.2 MATERIALS AND METHODS

5.3.2.1 Chemicals and reagents

The drugs used were the same as those described in Section 5.2.2.1.

5.3.2.2 Animals

Adult male Wistar rats were purchased from the South African Vaccine Producers, Johannesburg, South Africa and cared for as described in Section 4.2.2.2.

5.3.2.3 Drug treatment

The rats were treated in a same manner as described in Section 4.3.2.3.

5.3.2.4 Removal of the liver

The animals were sacrificed and the liver was removed as described in Section 5.2.2.3.

5.3.2.5 Preparation of the tissue

The liver homogenate was prepared as described in Section 5.2.2.4.

5.3.2.6 Determination of TDO activity

This was conducted as described in Section 5.2.2.5. However, after the addition of 0.03 M L-TRP (2.5 ml) to each flask, aliquots of 3 ml instead of 2 ml were transferred in triplicate to test tubes. Theanine was not added to any of the test tubes.

5.3.2.7 Protein assay

The determination of protein was performed as described in section 5.2.2.6.

5.3.2.8 Statistical analysis

This was performed as described in Section 2.2.2.3.

5.3.3 RESULTS

The experimental results obtained indicate a reduction in total TDO activity on treatment with theanine for 10 days however the only significant decrease of total TDO enzyme activity occurs when theanine was administered for 10 days ($p < 0.05$). There is however no significant change in the apoenzyme and holoenzyme activities after treatment with theanine for 10 days although there is an increase in total enzyme activity. There is a statistically significant decrease in the total TDO activity between treating the rats for 5 and 10 days ($p < 0.01$). There is an increase in the total and holoenzyme TDO after a 5 day treatment with theanine however this is not statistically significant.

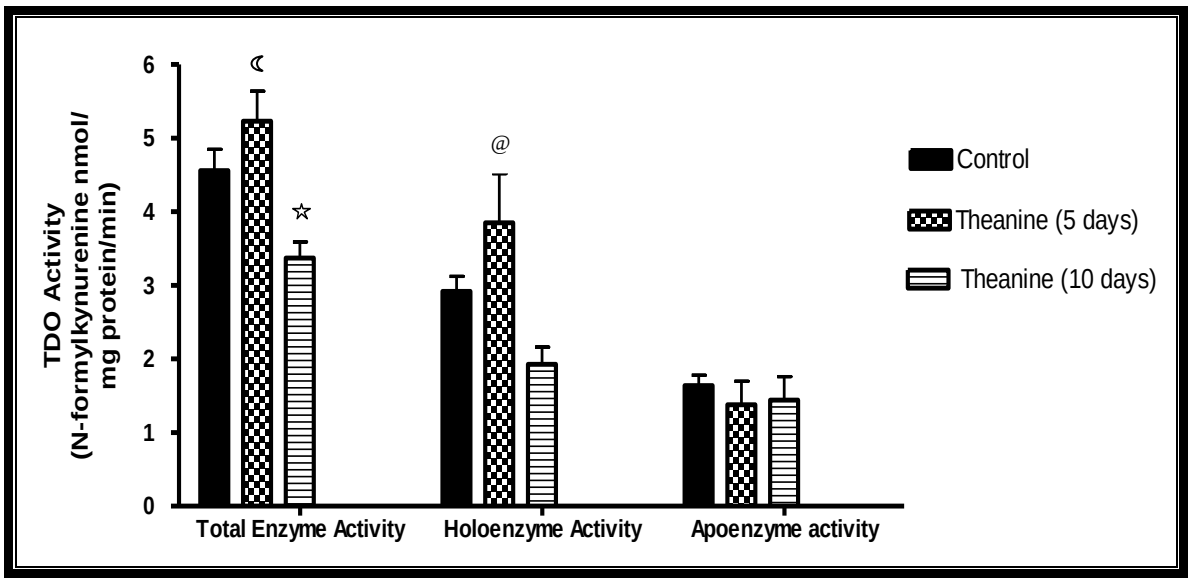

Figure 5.3: Effect of theanine on TDO enzyme activity in rat liver *in vivo*. Each bar represents the mean ± SD (n=5). ☆ ($p < 0.05$) in comparison to control, ☾ ($p < 0.01$) and @ ($p < 0.05$) in comparison to theanine (10 days) (ANOVA followed by Student Newman-Keuls Multiple Range Test).

5.3.4 DISCUSSION

TRP catabolism is facilitated by 2 enzymes, TDO and IDO, which oxidise TRP (Knox and Mehler, 1950). The KP occurs differently in the brain and in the periphery organs, as TDO is limited to the liver and IDO to various organs for example, the small and large intestines, kidney spleen and brain (Takikawa, 2005).

Research has been conducted on the relationship between KP metabolites in the periphery and in the brain. Badawy *et al.,* (1981) reported that liver TDO plays a role in determining the levels of circulating TRP in the blood. For instance, Weekley *et al.,* (1985) discovered that the levels of KP metabolites 5HT and 5-hydroxyindole-3-acetic acid (5HIAA) were reduced in the pineal, pituitary, various brain regions and duodenum of aflatoxin-treated rats. The authors concluded that the decreased levels of 5HT and 5HIAA in all tissues may be attributed to decrease in free tryptophan or alterations in 5-hydroxyindole biosynthesis.

A decrease in the levels of TRP corresponds to lower levels of KP metabolite formation. However, because the level of TRP remains constant in this experiment (Section 5.3.2.6), the theanine-induced decrease in TDO activity could be attributed to changes in the enzyme environment. There is no significant change in TDO activity on addition of haematin which

suggests that the apoenzyme activity is not affected by the administration of theanine. The variability in the holoenzyme activity for both treatment with theanine of 5 days and 10 days suggests that the holoenzyme activity is affected by theanine administration.

The theanine-induced decrease in holoenzyme activity may be attributed to the following:

- Changes in the manner in which it binds to TRP or
- Scavenging $O_2^{\bullet-}$ or inhibition of the reduction of oxygen to $O_2^{\bullet-}$.

A chemical that can bind at the TDO catalytic site is required to have an indole-NH group within its structure (Uchida *et al.,* 1992). A chemical that possesses this indole-NH group will act as a competitive inhibitor to the binding of TRP to the inactive form of the holoenzyme, thereby inhibiting the activity of the holoenzyme (Uchida *et al.,* 1992). Chemicals that do not possess the indole-NH group but still inhibit holoenzyme activity are non-competitive inhibitors of the reaction between TRP and the holoenzyme (Uchida *et al.,* 1992). Theanine does not possess an indole-NH group and can thus be termed a non-competitive inhibitor of TDO by interfering with the binding of TRP to TDO, resulting in a decrease in TDO activity. The mechanism by which theanine may do this is yet to be investigated. As previously mentioned another way in which theanine may inhibit holoenzyme activity is by preventing the reduction of oxygen to $O_2^{\bullet-}$ or by scavenging the $O_2^{\bullet-}$ produced. The effect of theanine on $O_2^{\bullet-}$ generation will be investigated in Chapter 7.

Yokogoshi *et al.* (1998) reported that administration of theanine resulted in an increase in TRP concentration in the rat brain. This may mean that theanine reduces TDO activity in the liver in order to increase the brain levels of TRP which is a precursor of 5HT. Administration of theanine significantly increases 5HT levels in the hippocampus, striatum and hypothalamus (Yokogoshi *et al.,* 1998) and this contributes to its anxiolytic effect (Lu *et al.,* 2004a). The decrease in the TDO enzymatic activity and the inhibition of QA-induced LP *in vivo* (Section 4.3.4) prove that theanine possesses neuroprotective properties as it inhibits the synthesis and effects of the neurotoxin QA *in vivo*.

CHAPTER 6

STROKE STUDIES

6.1 INTRODUCTION

Several animal models of ischaemia exist (Section 1.1.5). The animal model chosen in any particular study must be relevant to the human and closely resemble what happens in the human body during ischaemia. The findings of the experiment should be useful in understanding the pathophysiological consequences of ischaemia and drug development studies of stroke (Durukan and Tatlisumak, 2007). The focal ischaemic model is superior to the other models of ischaemic insult (Section 1.1.1.4).

The focal ischaemic model is more widely studied than global ischaemic models for 2 important reasons. Firstly, even at the core of the lesion formed during the stroke, the blood flow is usually higher than that in global ischaemia (Lipton, 1999). Secondly, the significant progression of ischaemia from the core of the lesion to the boundary of the lesion produce different metabolic conditions within the affected site which makes the focal ischaemic model more complex than global ischaemia (Lipton, 1999).

The most common stroke experienced by humans is caused by the obstruction of the MCA or one of its branches (Durukan *et al.,* 2008). Thus the focal ischaemic model involving the occlusion of the MCA is an appropriate model for investigating the potential neuroprotective properties of certain drugs. The occlusion of the MCA can be performed surgically or by introducing a suture into the MCA.

Surgical MCA occlusion includes direct surgical occlusion of the MCA or combining the occlusion of the MCA and other vessels. The disadvantage of surgical models are that these are invasive, excellent surgical skills are required for optimum induction of ischaemia and the technique requires a craniotomy which affects BBB function due to the exposure of the brain to the atmosphere (Durukan *et al.,* 2008). The main advantage of surgical occlusion of the MCA is that the site of occlusion and size of the infarct can be controlled (Durukan *et al.,* 2008).

The most widely used approach to MCA occlusion is to insert a suture into the CCA past the point at which the MCA branches so that the MCA is occluded at its origin (Figure 6.1) (Lipton, 1999). The size of the lesion in the thread occlusion technique depends on the size and shape of the thread as well as the site of thread occlusion (Woizik *et al.*, 2006). The advantages of this method are that it is relatively simple to perform and that there is a large and reproducible lesion size (Durukan *et al.*, 2008).

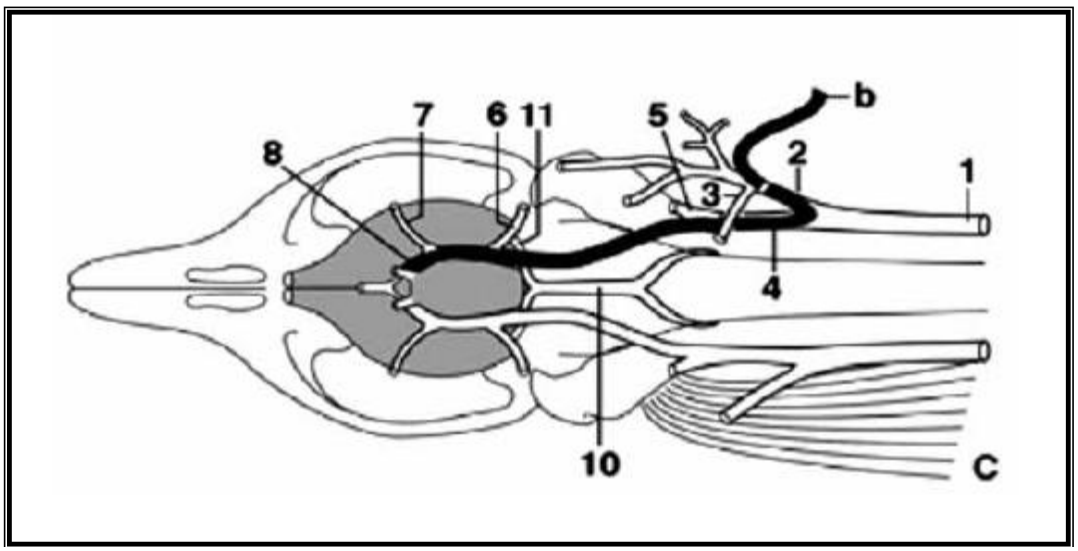

Figure 6.1: Intraluminal occlusion of the MCA via the external carotid artery. *Showing the suture (b); 1: Common carotid artery, 2: external carotid artery; 3: occipital artery, 4: internal carotid artery; 5: pterygopalatine artery; 6: posterior communicating artery; 7: Middle cerebral artery; 8: anterior cerebral artery (rostral cerebral artery); 10 Basilar artery; 11 Posterior cerebral artery* (Durukan *et al.*, 2008).

6.2 THE COMPARATIVE EFFECT OF THEANINE AND MINOCYCLINE ON A MCA MODEL OF CEREBRAL ISCHAEMIA

6.2.1 INTRODUCTION

The chosen method to induce focal ischaemia involved the introduction of a suture intravascularly to occlude the MCA as this type of stroke model represents the most common type of stroke experienced clinically. Rats were the animals chosen to model the stroke. Rats are suitable for such a model as their anatomy, physiology and intracranial vasculature is well known and is similar to that of humans and they are easy to breed, maintain and handle especially when it comes to anaesthetising and operating on them (Gupta and Briyal, 2004; Durukan *et al.,* 2008). It is recommended by Gupta and Briyal (2004) that anaesthetics such as barbiturates that have proven to be neuroprotective, should not to be used during any course of the procedure.

The length of the suture affects the size of the lesion and deeper suture insertion is associated with an increase in lesion size. The suture inserted from the bifurcation of the CCA is usually between 17 and 22 mm depending on the size of the rat (Shimamura *et al.,* 2006) as shorter insertion distances from the CCA have been shown to produce small subcortical infarcts (He *et al.,* 1999). The hippocampus was chosen as the area of the brain to study after ischaemia as it is rich in NMDA receptors and thus vulnerable to ischaemia (Kakuda, 2002).

6.2.2 MATERIALS AND METHODS

6.2.2.1 Chemicals and reagents

Theanine and minocycline were purchased from Sigma Chemical Corporation, St Louis, Missouri, United States of America. Sodium chloride (NaCl) was purchased from Saarchem, Johannesburg, South Africa. Chloral hydrate was purchased from Farmitalia Carlo Erba, Milan, Italy.

6.2.2.2 Animals

Adult male Wistar rats were purchased from the South African Vaccine Producers, Johannesburg, South Africa and cared for as described in Section 4.2.2.2.

6.2.2.3 Drug treatment

The rats were divided into 4 groups: control, stroke alone, theanine and minocycline. Each group consisted of 1 rat. The control and stroke alone groups were administered a dose of normal saline and the remaining groups of rats were administered 5 mg/kg (0.5 ml) drug i.p. an hour before the MCA occlusion was performed. The rat in the control group was not subjected to MCA occlusion.

6.2.2.4 Stroke study

A modification of the MCA occlusion as described by Longa *et al.*, (1989) was used in this experiment and is outlined below:

An hour after the rat was administered the drug (5 mg/kg) or normal saline, the rat was administered with chloral hydrate (450 mg/kg) to anaesthetise it for the procedure. A midline incision was made on the rat and by careful dissection from the surrounding tissue, both the left and right CCA were exposed. After CCA exposure, the bifurcation of the CCA was located and the blood flow into the MCA was blocked by introducing a 4-0 nylon intraluminal suture through the right extracranial internal carotid artery (ICA). The left ICA and the right external carotid artery (ECA) were temporarily clipped. The suture was gently advanced approximately 20 mm until resistance was felt. The suture was left in place for 3 minutes and then withdrawn and the brain was immediately formalinised.

6.2.2.5 Removal of brain

After the procedure the rats were decapitated. The brain was exposed by making an incision through the bone on either side of the parietal structure, from the foramen magnum to near the orbit. The calvarium was removed, exposing the brain which was then removed.

6.2.2.6 Histological Technique

The brain was cut transversely into slices and mounted onto glass slides. The brains were then photographed under high magnification (10 X). The areas of the hippocampus on both the ischaemic and non-ischaemic side were measured in mm^2. Figure 6.2 illustrates the position of the hippocampus on such a brain slice. Infarct areas for each slice were calculated by summing the total area on the non-ischaemic side and subtracting them from the total area on the ischaemic side. The difference in the area was then calculated as a percentage of the area of the non-ischaemic side of the hippocampus.

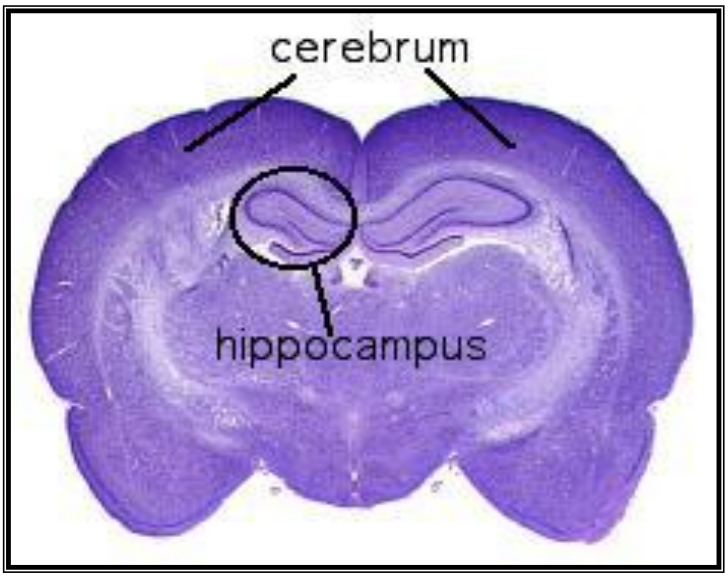

Figure 6.2: A Nissl-stained section of rat brain, showing the hippocampus (http://www.instruct1.cit.cornell.edu/courses/bionb424/students/cth5/images/brain_cross_section_showing_hipp.jpg.).

6.2.3 RESULTS

The image Figure 6.3 is a photograph of the control brain in which no MCA occlusion was performed. Both sides of the hippocampus are the same size as no stroke was induced. The images (Figures 6.4 to 6.6) below are the photographs of all the brains post-ischaemia at a final magnification of 10 X. The arrow shows the hippocampus on the side in which the ischaemia was induced (on the image it is on the right hand side but it is on the animals left side). On the side on which the MCA was blocked, the infarct on the affected side of the

hippocampus, is visible and the vasculature was more visible in than the rest of the brain tissue. Due to the lack of suitable morphologic assessment equipment, it was not possible to evaluate the extent of necrosis despite the fact that this was observed. However, this can be assessed in an extension of the present study.

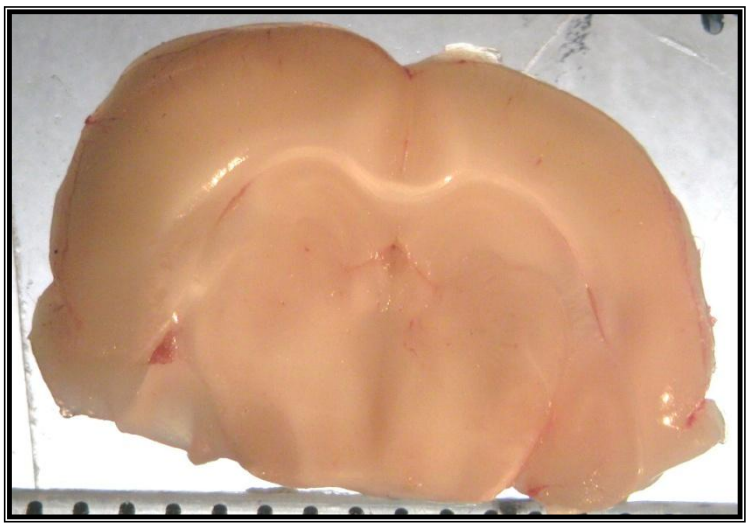

Figure 6.3: Image of the control brain in which no stroke was induced.

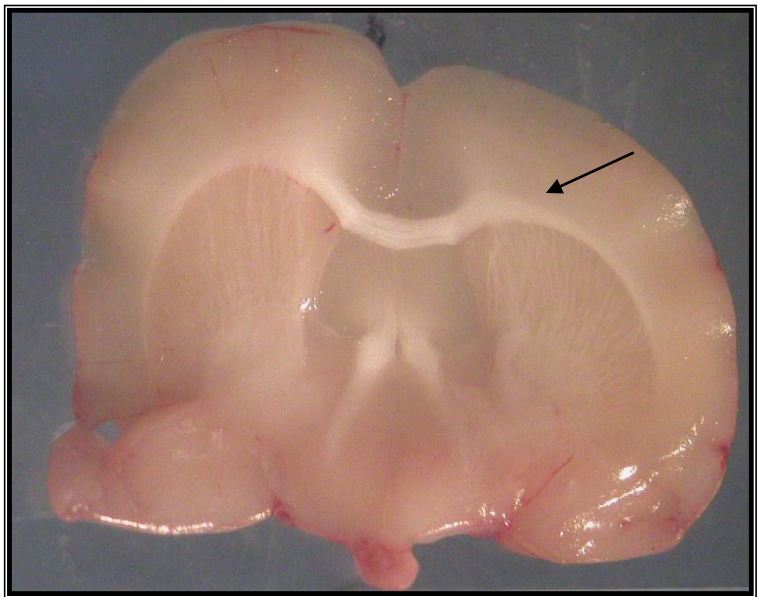

Figure 6.4: Image of a brain in which the stroke was performed but no drug was administered**.**

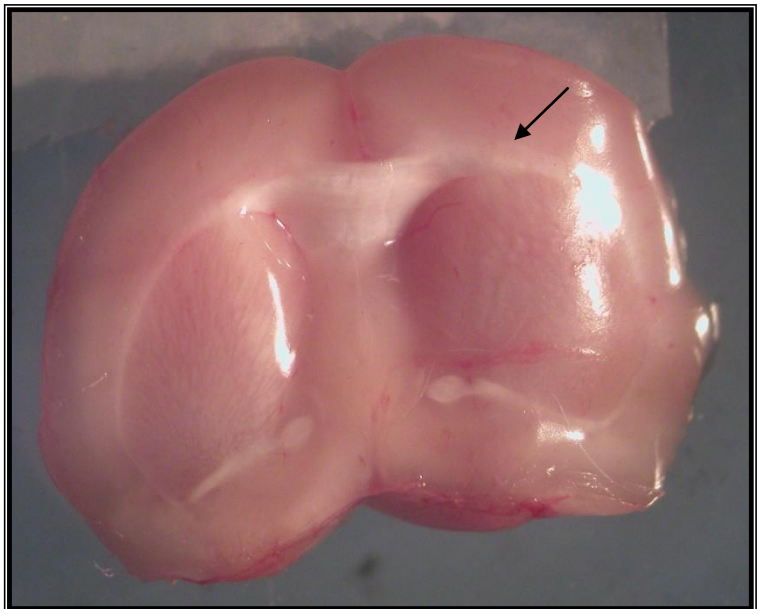

Figure 6.5: Image of the minocycline-treated post ischaemic brain.

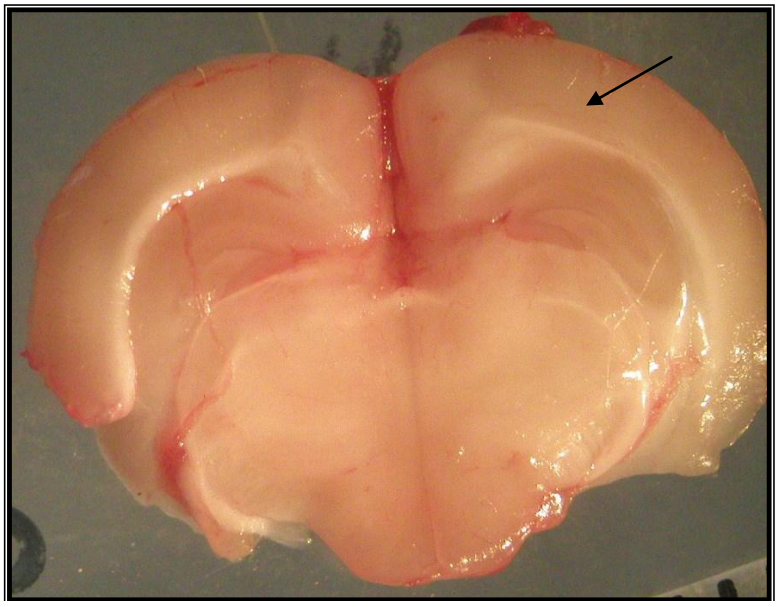

Figure 6.6: Image of the theanine-treated post ischaemic brain.

The potential of the drug in reducing the size of the infarct produced in the MCA occlusion was assessed by calculating the difference in size between the ischaemic side of the hippocampus from the non-ischaemic side and calculating as it as percentage growth of the non-ischaemic side of the hippocampus. Table 6.1 shows the percentage growth of the hippocampus of the control brain, the post ischaemic brain that had not been treated and each of the treated post-ischaemic brains. Minocycline showed least growth in the hippocampus

post-ischaemia which implies that it is more neuroprotective of the effects of the stroke than theanine.

Table 6.1: Showing the percentage growth of the hippocampus in each of the experimental groups.

Treatment	Percentage Growth in Hippocampus
Control	0
Stroke only	39
Stroke & Theanine	44
Stroke & Minocycline	7

6.2.4 DISCUSSION

Rats between 2-3 years old are reported to be clinically ideal as this age range would mimic that of humans above the age of 50 years in which stroke usually occurs (Gupta and Briyal, 2004). The rats used in this focal ischaemic model were 4 weeks old at the time of the experiment. Although not clinically relevant with regard to age, this model still serves to compare the potential neuroprotection shown of each drug in a model of ischaemia.

Comparison of the control brain to the post-ischaemic brains shows that ischaemia produces an infarct in the hippocampus. This infarct is due to blockage of the MCA which causes oxygen and nutrient deprivation to the side of the hippocampus on which the MCA was blocked. The hippocampus is rich in NMDA receptors as it has an important function in the glutamatergic system (Cotman *et al.,* 1987). There is a significant increase in the levels of QA in the hippocampus after ischaemia secondary to the induction of IDO and other enzymes in the KP (Saito *et al.,* 1993b). It is for these reasons it was the main part of the brain examined after ischaemia as it is the most vulnerable to oxidative stress leading to activation of the glutamate receptors such as the NMDA receptors.

Currently, thrombolytics are used to reduce neuronal damage and improve recovery post-ischaemia (Gupta and Briyal, 2004). A tissue plasminogen activator which is a type of

thrombolytic, is an agent used for the treatment of stroke. However, its main limitation is that only a small percentage (5-8 %) of patients benefit from treatment with it as its administration is restricted to 3 hours after stroke (Yrjänheikki *et al.,* 1999; Gupta and Briyal, 2004). The mechanism responsible for cell death in ischaemic stroke is yet to be determined but it is however known that 3 major factors contribute to cell death and these are acidosis, the production of free radicals and a significant increase in intracellular concentrations of Ca^{2+} (Siesjo, 1992). Various experimental stroke studies using glutamic acid and Ca^{2+} antagonists in rodents have shown promise in reducing the size of infarcts resulting from MCA occlusion (Deshpande and Wieloch, 1986; Nuglisch *et al.,* 1990; Hoffman and Boast, 1995) but have failed in the clinical setting (Onténiente *et al.,* 2003). Failure of clinical trials is due to either toxic side effects or lack of efficacy in the clinical setting. Therefore there is a need to search for neuroprotective agents that will prove to be effective in the clinical setting. Both theanine and minocycline readily cross the BBB and can thus be used as test compounds for an ischaemic model (Kakuda, 2002; Dormercq and Matute, 2004).

Pre-treatment with theanine has a neuroprotective effect in ischaemia-induced hippocampal neuronal death by reducing its occurrence in a dose-dependent manner (Kakuda *et al.,* 2000) and it significantly decreased the contents of water, Ca^{2+} and MDA in cerebral tissues after ischaemia (Wang et al, 2008). Administration of theanine decreases the size of infarcts in a MCA occlusion model in mice (Egashira *et al.,* 2004). The proposed mechanism of theanine was due to its regulatory effect on the imbalance of free radicals generated during ischaemia as it increases SOD enzyme activity in the brain (Wang *et al.,* 2008). Theanine is an antagonist at AMPA and NMDA receptors (Kakuda *et al.,* 2002) and thus provides neuroprotection. AMPA receptor antagonists provide protection against ischaemia induced by MCA occlusion (Umemura *et al.,* 1997). GABA, an inhibitory neurotransmitter plays a role in ischaemia as the levels of its synthesis and receptor expression decrease following MCA occlusion (Sieghart, 1995). Egashira *et al.,* (2007) showed that the neuroprotective effect of theanine involves its binding to GABA receptors.

Although the dose of theanine administered before inducing the stroke was in a similar range to other studies which showed theanine as a neuroprotective in focal ischaemic, the theanine did not reduce brain damage in comparison to the control. The explanation of this could be that epidemiological studies on green tea and stroke have shown that long term tea

consumption is necessary to confer the effects of green tea on stroke (Fraser *et al.,* 2007) and that theanine is more effective when it is administered before and after the stroke (Egashira *et al.*, 2004). In addition to this, most studies on theanine in ischaemic models involve reperfusion for several hours or days. The reperfusion in this model was very brief before decapitation took place. Reperfusion allows for an increase in blood flow through the penumbra allows for brain cells to recover and regain their functionality (Hossman, 1994). Other reasons for the difference in theanine showing little neuroprotection in comparison to previous stroke studies could also be because the theanine was administered directly into the brain via the lateral ventricle before ischaemia (Kakuda *et al.,* 2000) and that although it takes 30 minutes for theanine to be taken up by the brain after intraperitoneal administration (Kimura and Murata, 1971), it only reaches peak concentrations in 5 hours (Terashima *et al.,* 1999) thus by the time the MCA occlusion was carried out, the theanine was still in low concentrations in the brain.

Minocycline has been shown to be neuroprotective in several stroke models such as intracerebral haemorrhagic stroke (Wasserman and Schlichter, 2007) and focal ischaemia (Xu *et al.,* 2004; Li and McCullough, 2009). Yrjänheikki and colleagues (1999) showed that minocycline reduces infarct volume by 76 % and by 63 % when it is administered 12 hours and 4 hours before the onset of ischaemia respectively. The results of this study (Section 6.2.3) agree with the previous studies and show that minocycline is neuroprotective in ischaemia. Minocycline provides neuroprotection in ischaemia by enhancing neurogenesis, reducing NMDA excitotoxicity by inhibiting microglia activity (Tikka and Koistinaho, 2001; Koistinaho and Koistinaho, 2007; Liu *et al.,* 2007). Inhibiting microglial activity is an important characteristic of minocycline's neuroprotection post-ischaemia, as activation of microglia contributes to neuronal death. Other ways in which minocycline is neuroprotective post-ischaemia is that it protects neurons via an anti-apoptotic mechanism which prevents neuronal apoptotic cell death (Matsukawa *et al.,* 2006) and it inhibits the inflammatory cascade (Yrjänheikki *et al.,* 1999; Dormercq, 2004). A study by Fagan and colleagues (2004) show that absorption after i.p. administration of minocycline is erratic and incomplete and that the T_{max} levels are reached after 2.5 hours. Minocycline's superior neuroprotective properties are exhibited in this model of stroke despite documented evidence of poor absorption after i.p. administration.

Most drugs are found to not be efficacious in the clinical setting after showing great promise in experimental models. Theanine administered in doses up to 4000 mg/kg in rats was found not to have any adverse effects (Borzelleca *et al.,* 2006). An intravenous dose of 3 mg/kg of minocycline is required to obtain serum levels in rats similar to that obtained in humans after a standard therapeutic dose of 200 mg of minocycline (Xu *et al.,* 2004). Matsukawa *et al.,* (2006) reported that a high dose of minocycline (100 μM) which is approximately 50 mg/kg of minocycline is toxic and markedly reduces cell survival. The doses of both theanine and minocycline (5 mg/kg) that were administered to the rats are within the non-toxic window of both drugs and within the therapeutic window for minocycline. Further studies would have to be performed to investigate chronic administration of theanine before and after a stroke in order to confirm its potential neuroprotective properties. The results of this study show that minocycline is neuroprotective in a clinically appropriate stroke model, within its therapeutic range.

CHAPTER 7

OXIDATIVE STRESS

7.1 INTRODUCTION

Oxidative stress occurs when there is an imbalance between radical species produced and the endogenous antioxidant defences (Halliwell, 2007). The brain is particularly susceptible to oxidative damage as it consumes about 20 % of the O_2 inspired despite accounting for only 2 % of the total body weight. In addition to this, the brain is rich in PUFAs and has relatively low levels of antioxidant defences stress (Papa, 1996; Butterfield, 2006). The free radicals that are in abundance in a state of oxidative stress are ROS and RNS. The mitochondria is responsible for energy production and disturbances to its function due to diseases or injury result in the disturbance of mitochondrial function. Diseases that result in mitochondrial dysfunction include cancer, heart failure, diabetes, cerebral ischaemia and atherosclerosis, among other diseases (Sas *et al.,* 2007).

The results of mitochondrial dysfunction include enhanced radical production, oxidative damage resulting from radical production leads to the mtPTPs opening and thus inducing apoptosis (Halliwell and Gutteridge, 1989). In addition to this, there are disturbances of the mitochondrial Ca^{2+}, decreases in ATP synthesis and ROS metabolism (Brookes *et al.,* 2004). The escaping electrons from the respiratory chain interact with O_2 to form the $O_2^{\bullet-}$ radical. The $O_2^{\bullet-}$ can also be generated by other means such as the oxidation of xanthine, hypoxanthine, NADPH and the auto-oxidation of monoamines (Fridovich, 1999). The mitochondria are sensitive to $ONOO^-$ which induces the opening of mtPTPs and activates caspase dependent and caspase independent pathways (Sas *et al.,* 2007). The major targets for ROS are microglia, neutrophils and macrophages (Sas *et al.,* 2007). The mechanism of cell death involves apoptosis and excitotoxicity in which ROS plays a role in both processes (Sas *et al.,* 2007).

Neuronal injury leads to the release of glutamic acid into the synaptic space. The excess glutamic acid causes an overstimulation of glutamate receptors and ultimately this leads to excessive Ca^{2+} influx into the neuronal cell (Arundine and Tymianski, 2004). Ca^{2+} overload

ultimately leads to an increase in the production of ROS and RNS, activation of genetic signals leading to apoptosis and mitochondrial dysfunction. Overall, apoptosis is mediated by the production of ROS, disturbances in Ca^{2+} homeostasis, caspase activation and mitochondrial dysfunction (Mattson *et al.,* 2000). It is not only elevations of extracellular glutamic acid that mediate ischaemic brain damage but alteration of glutamate receptor functioning has also been implicated as a potential mechanism in the mediation of toxicity to neurons (Arundine and Tymianski, 2004).

Free radicals generated as a result of oxidative stress are removed by enzymatic and/or non-enzymatic means. The main defence systems against radical damage are SOD, GSH, GSH peroxidise, CAT, GSH reductase and nutrients such as vitamins E and C (Figure 7.1) (Fang *et al.,* 2002). SOD is responsible for the removal of $O_2^{\bullet-}$ by a dismutation reaction in which H_2O_2 and O_2 are formed (Fridovich, 1975). CAT removes the H_2O_2 that is produced. GSH peroxidise and GSH-S-transferase are enzymes that also remove H_2O_2 as well as lipid peroxides (Halliwell and Gutteridge, 1986).

Aging is characterised by a reduction in the ability of the body to combat environmental stress and declined antioxidant defences. There is an increase in the amount of ROS produced and because of the reduced ability of an aged body to cope with ROS, there tends to be an accumulation of toxic radicals which leads to oxidative damage. Oxidative damage and Ca^{2+} release is an important factor in neuronal injury that takes place as a result of various neurodegenerative disorders.

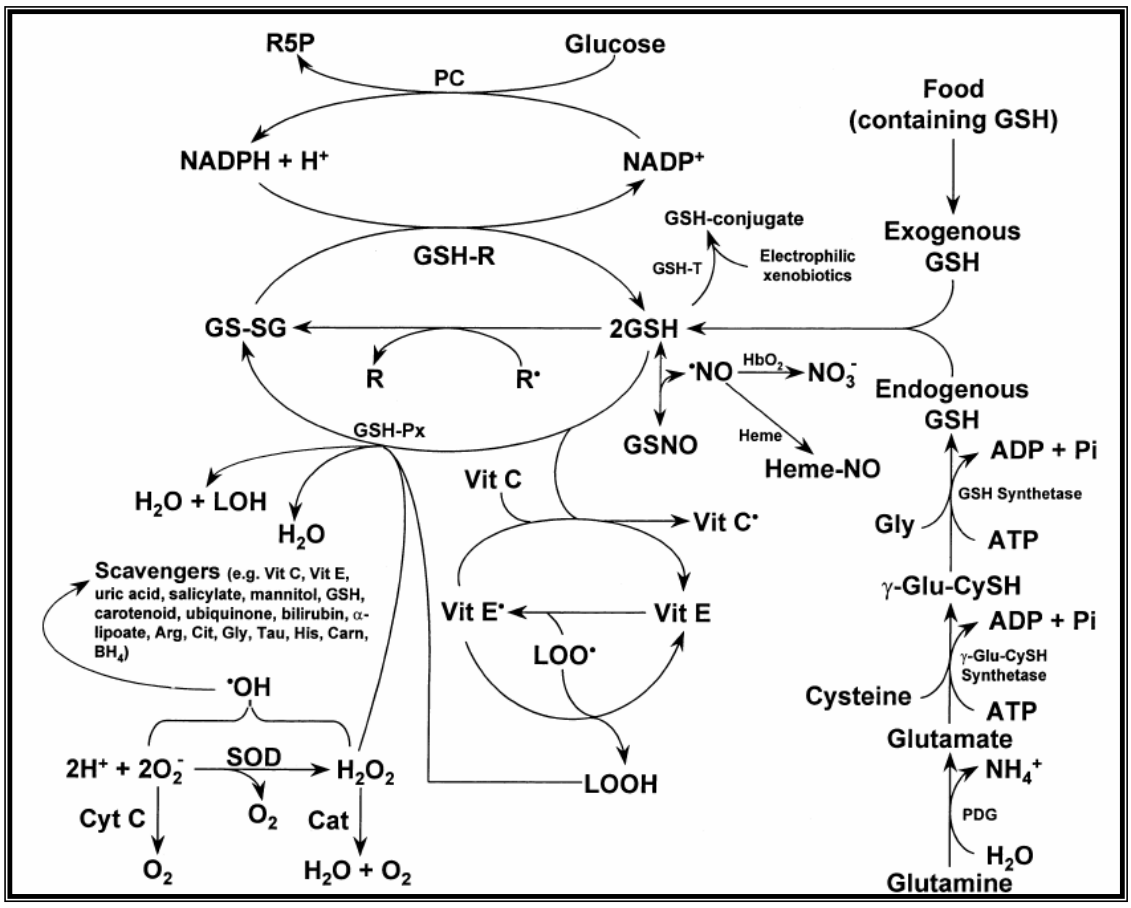

Figure 7.1: The mechanisms by which ROS and RNS are removed from the body. *Adenosine diphosphate (ADP); arginine (Arg); (6R)-5,6,7,8,-tetrahydro-L-biopterin (BH4); carnosine (Carn); catalase (Cat); citrulline (Cit); cytochrome C (Cyt C); L-glutamate (Glu); glycine (Gly); glutamyl-cysteine (Glu-CySH); oxidized glutathione, (GS-SG); reduced glutathione (GSH); glutathione peroxidases (GSH-Px); glutathione reductase (GSHR);glutathione S-transferase (GSH-T); nitrosylated glutathione (GSNO);oxyhemoglobin (HbO2); heme-nitric oxide (Heme-NO); histidine, (His); lipid alcohol (LOH); lipid peroxyl radical (LOO.); lipid hydroperoxide (LOOH); nitric oxide (.NO); nitrate (NO3-); superoxide anion radical, (O_2^-); pentose cycle (PC); radicals (R.); non radicals (R); ribulose 5-phosphate (R5P); superoxide dismutase (SOD); taurine (Tau);vitamin C, (Vit C); vitamin C radical, (Vit C•); vitamin E, (Vit E); vitamin E radical, (Vit E•)* (Fang *et al.*, 2002).

RNS and ROS generated intracellularly and extracellularly play a role in the neurodegeneration that occurs in AD (Multhaup *et al.*, 1997). Decreased levels of antioxidants play a role in neurodegenerative diseases. In PD there are decreased levels of the antioxidant GSH in the substantia nigra (Pearce *et al.*, 1997). Oxidative stress also plays a role in ischaemia. An ideal stroke model must truly represent the pathophysiological consequences of ischaemia such as excitotoxicity and a state of oxidative stress (Figure 7.2). The effect of theanine and minocycline were investigated *in vitro* and *in vivo* post ischaemia.

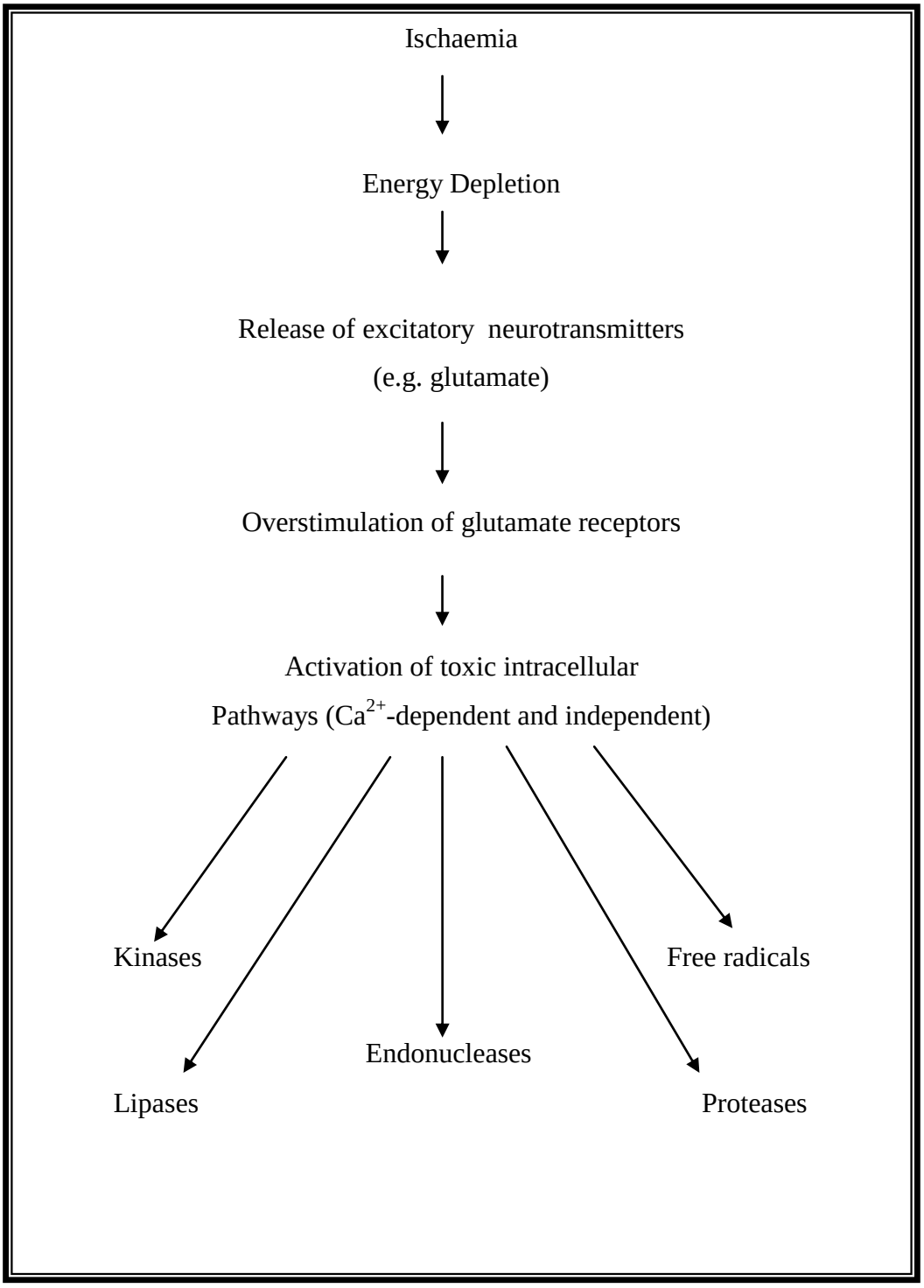

Figure 7.2: Diagram showing the multiple processes involved in damaging and killing neuronal cells ischaemia or stroke. *Involved in these processes are free radicals* (Reiter, 1998).

7.2 THE COMPARATIVE EFFECTS OF THEANINE AND MINOCYCLINE ON CYANIDE-INDUCED ON SUPEROXIDE ANION GENERATION IN THE RAT BRAIN *IN VITRO*

7.2.1 INTRODUCTION

Although the mitochondria converts most of the O_2 it consumes to water, about 1-2 % of the O_2 is reduced to the $O_2^{\bullet-}$ (Papa, 1996). The endoplasmic reticulum (ER) in the mitochondria is responsible for the storage of Ca^{2+}, cell signalling and the folding of newly synthesised proteins (Sas *et al.,* 2007). ER dysfunction induces oxidative stress, mitochondrial dysfunction and the activation of caspases which leads to apoptosis (Sas *et al.,* 2007).

$O_2^{\bullet-}$ is produced by a variety of enzymatic reactions in the tissues or by the auto-oxidation reactions of tissue components. Intracellular production of $O_2^{\bullet-}$ occurs during processes such as the oxidation of PUFAs, auto-oxidation of small molecules, haemoglobin, mitochondrial components and oxidative enzymes such as xanthine oxidase and NADPH oxidase in phagocytic cells (Kontos, 2001). Equation 7.1 shows how O_2 is converted to $O_2^{\bullet-}$ (Fridovich, 1978).

$$O_2 \xrightarrow{e^-} O_2^{\bullet-} \xrightarrow{e^-+2H^+} H_2O_2 \xrightarrow{e^-+2H^+} \bullet OH \xrightarrow{e^-+H^+} H_2O$$ **Equation 7.1**

The enzymatic defence against $O_2^{\bullet-}$ is SOD which converts $O_2^{\bullet-}$ to H_2O_2. There are 2 types of SOD which lead to the dismutation of $O_2^{\bullet-}$ and these are manganese SOD (MnSOD) in the mitochondria and copper and zinc SOD (CuZnSOD) in the cytosol (Figure 7.3) (Fridovich, 1989; Evans *et al.,* 2002). The brain is susceptible to damage by $O_2^{\bullet-}$ because it has low levels of the antioxidant SOD which scavenges $O_2^{\bullet-}$ (Gariballa, 2000).

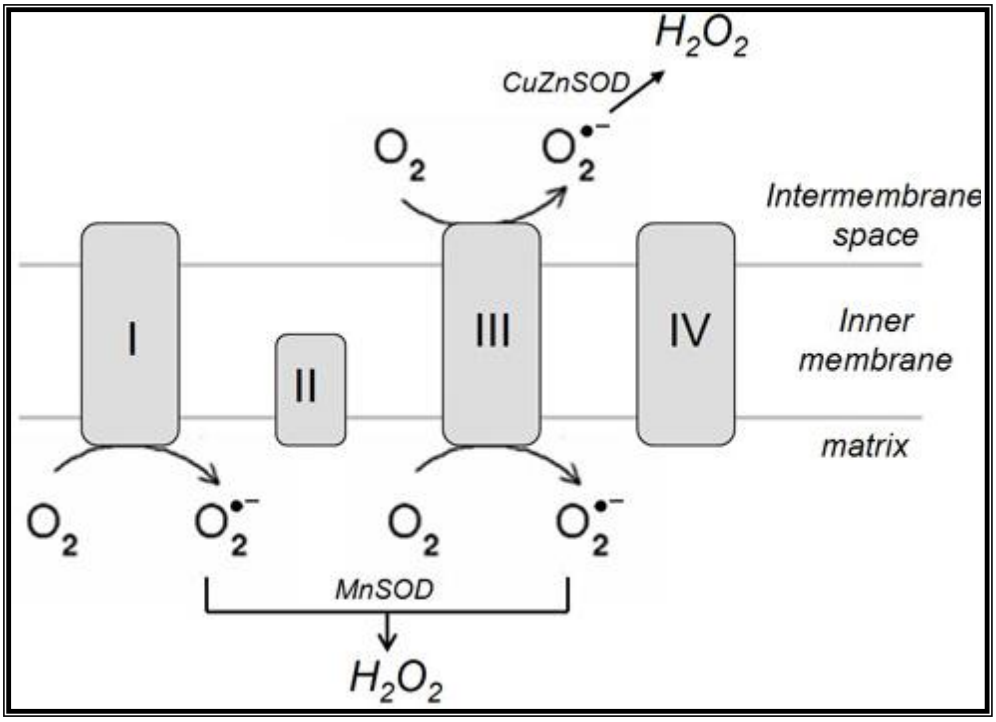

Figure 7.3: The dismutation of $O_2^{\bullet-}$ by copper and zinc superoxide dismutase (CuZnSOD) and manganese superoxide dismutase (MnSOD) (Szeto, 2006).

The (nitro-blue tetrazolium) NBT assay is a reliable method of assaying $O_2^{\bullet-}$ (Das *et al.*, 1990; Gariballa, 2000). The principle of the assay relies on $O_2^{\bullet-}$ generated in the tissues to reduce the yellow NBT dye into the water insoluble nitro-blue formazan (NBD) which can be extracted by glacial acetic acid (GAA) and quantitatively analysed spectrophotometrically. The $O_2^{\bullet-}$ in the assay may be generated by chemicals such as potassium cyanide (KCN) or QA. Any molecule capable of scavenging $O_2^{\bullet-}$ will lead to there being less NBD formed.

Cyanide is a respiratory toxin whose mechanism of action involves the inhibition of cytochrome oxidase aa_3 which is a terminal oxidative enzyme of the electron transport chain (Section 1.2.6) and this later leads to radical-induced neuronal damage.

7.2.2 MATERIALS AND METHODS

7.2.2.1 Animals

Adult male Wistar rats were purchased from the South African Vaccine Producers, Johannesburg, South Africa and cared for as described in Section 4.2.2.2.

7.2.2.2 Chemicals and reagents

Theanine, minocycline, KCN, NBD and NBT were purchased from Sigma Chemical Corporation, St Louis, Missouri, United States of America. Ethanol and GAA were purchased from Saarchem, Johannesburg, South Africa.

7.2.2.3 Brain removal

Rats were sacrificed and the brains removed as described in Section 4.2.2.3.

7.2.2.4 Homogenate preparation

The brain homogenate was prepared as described in Section 4.2.2.4.

7.2.2.5 Preparation of the NBD standard curve

NBD was used to prepare the standard curve. A series of NBD standards (0 – 400 nmols/ml) were prepared in triplicate using glacial acetic acid as a diluent to give a final volume of 1 ml. A standard curve was then generated by measuring the absorbance 560 nm on a Shimadzu UV mini 1240 UV/VIS spectrophotometer and plotting these against the molar equivalent weight of NBD (Appendix V).

7.2.2.6 Nitro-blue Tetrazolium Assay

A modified method by Das *et al.*, (1990) was used for this assay and is outlined below:

Rat brain homogenate (1 ml) was prepared in triplicate containing 1 mM KCN (250 µL) in the absence and presence of increasing concentrations (0 – 2 mM) of either theanine or minocycline (250 µL) and 0.4 ml NBT (0.1 % in ethanol and then made up to the required volume with milli-Q water). The samples were then incubated in an oscillating water bath for 1 hour at 37 ºC. Control samples did not contain KCN, theanine or minocycline.

The reaction was terminated and the NBD formed was extracted by centrifuging the samples for 10 minutes at 2000 x g. The reaction was terminated by the resuspension of the pellets with 2 ml of GAA. To remove insoluble debris, the samples were centrifuged for 5 minutes at 2000 x g. An aliquot of the supernatant (extracted NBD in GAA) was read at 560 nm using a Shimadzu UV mini 1240 UV/VIS spectrophotometer. NBD levels were then determined from the standard curve generated as described in Section 7.2.2.5. Final results were expressed as diformazan (nmol/mg tissue).

7.2.2.7 Statistical Analysis

This was performed as described in Section 2.2.2.3.

7.2.3 RESULTS

Figure 7.4 shows that 1 mM KCN has a significant effect ($p < 0.05$) in increasing the levels of $O_2^{\bullet-}$ *in vitro* in comparison to control. Co-incubation of rat brain with KCN and either theanine or minocycline produced no significant change in the levels of $O_2^{\bullet-}$ in comparison to the brains treated with KCN.

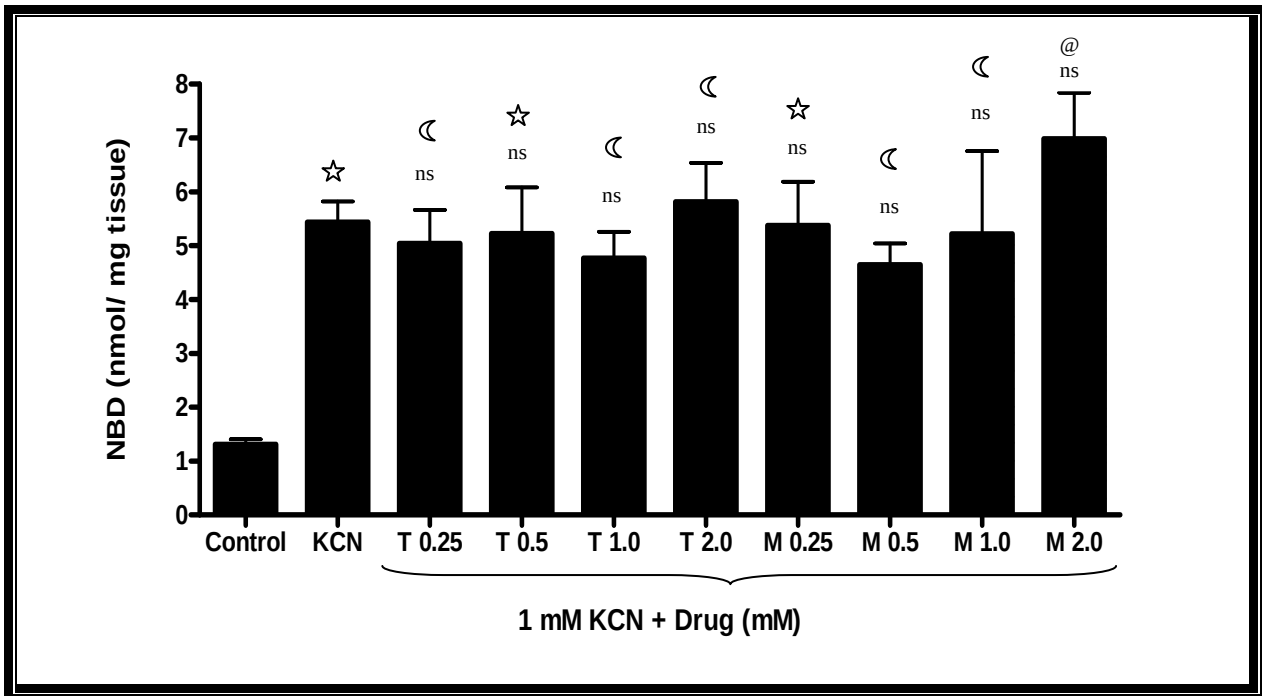

Figure 7.4: Effect of theanine and minocycline on the generation of $O_2^{\bullet-}$ in rat brain homogenate *in vitro*. *T and M represents co-incubation with theanine and minocycline respectively with the concentration of the drug in mM next to the letter representing the drug.* Each bar represents the mean ± SD; (n= 5). ☆ ($p < 0.05$) in comparison to control; ns ($p < 0.01$) in comparison to KCN alone; @ ($p < 0.001$) in comparison to control (ANOVA followed by Student Newman-Keuls Multiple Range Test).

7.2.4 DISCUSSION

The results of this study demonstrate that KCN is a potent generator of $O_2^{\bullet-}$ *in vitro* as evident from Figure 7.4. As previously mentioned (Section 1.2.6), cyanide inhibits cytochrome oxidase aa_3 which is a terminal oxidative enzyme of the electron transport chain and consequently the homeostatic ATP-dependent Na^+/K^+ pumps. This results in an influx of Ca^{2+} and elevated levels of Ca^{2+} within the cell which ultimately leads to neuronal death.

There is statistically no significant difference between the $O_2^{\bullet-}$ generated by KCN and that by both theanine and minocycline co-incubated with KCN. This implies that the KCN generates the $O_2^{\bullet-}$ and that the drugs have no effect on the generation of $O_2^{\bullet-}$. According to Equation 7.1, an electron is to be donated to O_2 in order to generate $O_2^{\bullet-}$. Looking at the chemical structure of theanine, it is unable to donate an electron to O_2 and thus it is unable to lead to the increase in the generation of $O_2^{\bullet-}$. From the results, it is clear that theanine is also unable to scavenge the $O_2^{\bullet-}$.

The same reasoning that the drug has no effect on the generation of $O_2^{\bullet-}$ could possibly apply to minocycline. Although the increase in $O_2^{\bullet-}$ generation with the combination of minocycline and KCN was not statistically significant, minocycline may be able to donate an electron to O_2 and become resonance stabilised. An increase in the concentration of minocycline increases the availability of electrons to donate electrons to O_2 and account for the slight increase in the generation of $O_2^{\bullet-}$. A study by Hu *et al.* (1996) showed minocycline to be an effective $O_2^{\bullet-}$ scavenger. The reason for this discrepancy could be because the study carried out by Hu and colleagues was in a cell free medium.

7.3 THE COMPARATIVE EFFECT OF THEANINE AND MINOCYCLINE ON CYANIDE-INDUCED SUPEROXIDE ANION GENERATION IN THE RAT BRAIN *IN VIVO* POST-ISCHAEMIA

7.3.1 INTRODUCTION

Free radicals produced during ischaemia have negative effects on cells. These can cause LP, denaturation of proteins, inactivation of enzymes, release of Ca^{2+} from intracellular stores (Gariballa, 2000; Kontos, 2001; Ullegaddi *et al.,* 2005). The reactive species formed during cardiovascular injury are shown in Figure 7.5. $O_2^{\bullet-}$ has effects on cerebral vascular system. $O_2^{\bullet-}$ causes the dilation of cerebral arterioles by the opening of Ca^{2+}-activated K^+ channels (Wei *et al.,* 1996) which means that $O_2^{\bullet-}$ has an important role in cerebral blood flow in ischaemia. Increases in the levels of $O_2^{\bullet-}$ during ischaemic insult have been reported (Arundine and Tymianski, 2004) and the levels of $O_2^{\bullet-}$ are dependent on the duration of the ischaemic insult (Kontos, 2001).

The dramatic increase in the extracellular glutamic acid in the brain following ischaemia (Roettger and Lipton, 1996) results in a drastic increase in Ca^{2+} influx into the cell due to overstimulation of glutamate receptors by glutamic acid. The generation of ROS due to Ca^{2+} overload contributes to the disruption of cellular homeostasis and cell death (Camacho and Massieu, 2006). Neurotoxicity mediated by NMDA receptor over-stimulation results from over-production of NO• and $O_2^{\bullet-}$ (Halliwell and Gutteridge, 1989; Arundine and Tymianski, 2004). Keller and colleagues (1998) proved that $O_2^{\bullet-}$ production contributes to neuronal death after MCA occlusion model as there was a decrease in the size of infarcts and neuronal apoptosis by suppression of peroxynitrite production and mitochondrial dysfunction when the animal overproduced SOD. In ischaemic-reperfusion models, it was found that there was an increase in $O_2^{\bullet-}$ production due to the accumulation of macrophages in the brain (Saito *et al.,* 1993).

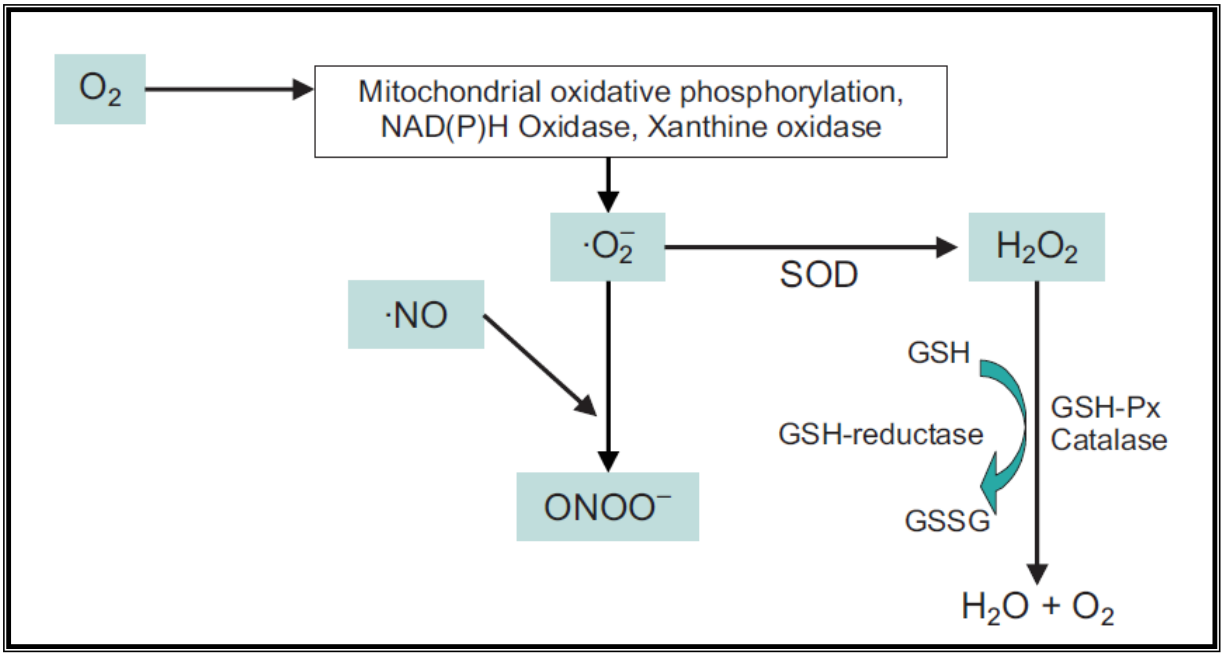

Figure 7.5: Generation of reactive species (Sachidanandam *et al.*, 2005).

Kontos (2001) found that stimulation of AMPA receptors by glutamic acid leads to the generation of $O_2^{\bullet-}$ which leads to vasodilation of cerebral vasculature. The $O_2^{\bullet-}$ generated can react with nitric oxide within the cell and produce peroxynitrite which can further lead to neuronal damage (Kontos, 2001). $O_2^{\bullet-}$ participates in the death of neurons directly and indirectly by the formation of reactive species (Section 1.2.2.3).

Although several methods such as inhibiting AMPA receptors have been suggested as a preventative measure to reduce the effects of radicals, it is more effective to prevent neuronal death by the scavenging of free radicals rather than to target a specific site of radical reaction. Scavenging radicals produced is a useful approach in minimising damage in cerebral ischaemia (Kontos, 2001). There is an increase in •OH production during ischaemia leading to neuronal damage (Negishi *et al.*, 2001). Because the •OH radical is the most reactive ROS and thus the most likely to cause a great amount of radical-mediated damage following ischaemic insult, it would be practical to target this radical with scavengers (Kontos, 2001). However, due to its high reactivity, the •OH would require great amounts of scavenger in order to neutralise its effects (Beckman *et al.*, 1990). It would be very impractical to achieve such high concentrations of scavengers, thus it would be more worthwhile to target the less reactive $O_2^{\bullet-}$ (Kontos, 2001) which is responsible for its formation by the Haber-Weiss reaction (Equation 1.3).

The following study was carried out in order to ascertain whether theanine and minocycline are able to scavenge $O_2^{\bullet-}$ and thus possess this radical scavenging properties as one of its neuroprotective properties.

7.3.2 MATERIALS AND METHODS

7.3.2.1 Animals

Adult male Wistar rats were purchased from the South African Vaccine Producers, Johannesburg, South Africa and cared for as described in Section 4.2.2.2.

7.3.2.2 Chemicals and reagents

The drugs used and purchased are the same as the ones described in Section 7.2.2.2.

7.3.2.3 Stroke Study

This was performed as described in Section 6.2.2.4.

7.3.2.4 Brain removal

Rats were sacrificed and the brains removed as described in Section 4.2.2.3 except in this case, the cerebellum was removed and the rest of the brain was used for the preparation of the homogenate for the assay. The cerebellum was removed based on a study by Saito *et al.* (1993) which showed that there is no significant change in the levels of neurotransmitters in the cerebellum post-ischaemia compared to other parts of the brain, namely the cerebral cortex, thalamus, striatum and hippocampus.

7.3.2.5 Preparation of tissue

The brain homogenate was prepared as described in Section 4.2.2.4.

7.3.2.6 Preparation of the NBD standard curve

The standard curve was prepared as described in Section 7.2.2.5.

7.3.2.7 Nitro-blue Tetrazolium Assay

The assay was carried out as described in Section 7.2.2.6. However, no exogenous theanine and minocycline were added to triplicate samples of rat brain homogenate (1 ml).

7.3.2.8 Statistical Analysis

This was performed as described in Section 2.2.2.3.

7.3.3 RESULTS

Administration of minocycline i.p resulted in a statistically significant ($p < 0.01$) increase in $O_2^{\bullet-}$ generation in comparison to both control and in the model where stroke alone was induced without the pre-treatment with drugs (Figure 7.6). On comparing treatment of the animals with and without theanine after inducing a stroke, there was no statistically significant change ($p > 0.05$) in the generation of $O_2^{\bullet-}$.

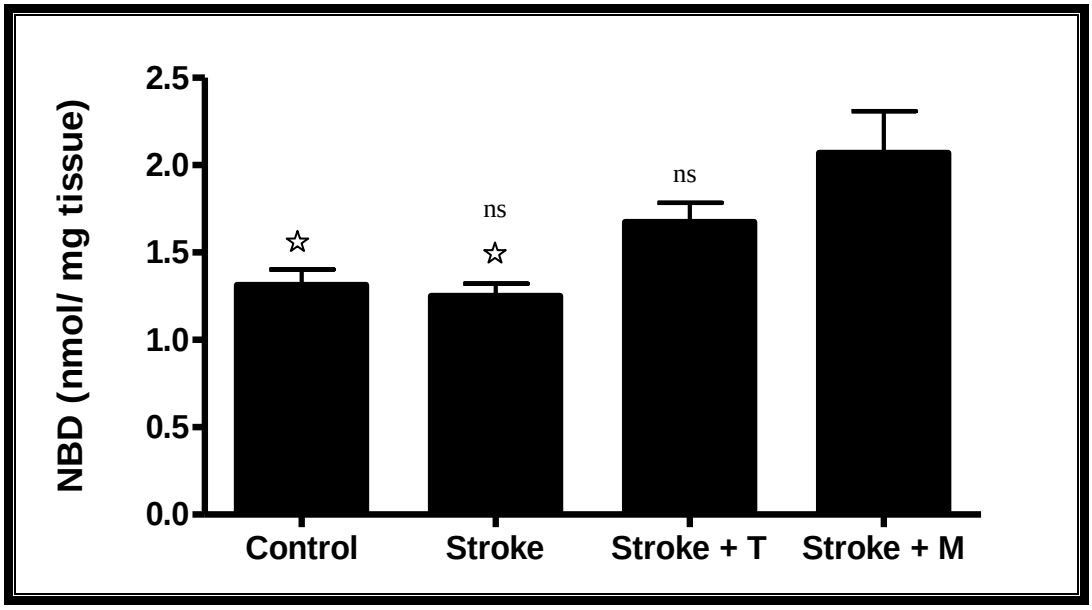

Figure 7.6: Effect of pre-treatment with theanine and minocycline on the generation of $O_2^{\bullet-}$ in the rat brain homogenate *in vivo*. *Stroke + T and Stroke + M represents stroke after pre-treatment with theanine and minocycline respectively.* Each bar represents the mean ± SD; (n=5). ns ($p > 0.05$) in comparison to control; ☆ ($p < 0.01$) in comparison to Stroke + M. (ANOVA followed by Student Newman-Keuls Multiple Range Test).

7.3.4 DISCUSSION

ROS particularly the $O_2^{\bullet-}$ has been shown to cause extensive damage to brain cells (Sections 1.2.3.3 and 7.2.1). Neuronal damage can also occur due to overstimulation by glutamic acid causing excitotoxicity (Section 1.2.3.11).

Drugs must be able to cross the BBB and accumulate in it in order to be considered potentially neuroprotective (Zhang *et al.*, 2005). High levels of antioxidants in the blood is associated with a reduced risk of ischaemic risk (Gariballa, 2000) which implies that antioxidants have an important role in preventing stroke related oxidative damage.

The stroke only model of ischaemia produced no significant increase in $O_2^{\bullet-}$ generation in comparison to control which does not agree with previous studies which show that ischaemia induces the production of $O_2^{\bullet-}$ (Halliwell and Gutteridge, 1989; Lipton, 1999). This discrepancy may be due to the short period of reperfusion after inducing the stroke followed by rapid decapitation. McCord (1985) reported that there is increased generation of $O_2^{\bullet-}$ after reperfusion when the O_2 supply is restored to the tissue. Kontos (2001) reported that the

concentration of $O_2^{\bullet-}$ in the tissues depends on the duration of the preceding ischaemia and also on the availability of O_2. This ischaemic model had a short duration of ischaemia compared to other models of MCA occlusion in which the MCA was occluded for over 1 hour and tissues reperfused for days (Takagi *et al.*, 1993; Zhang *et al.*, 2005; Xu *et al.*, 2006). This implies that with this short duration of ischaemia (3 minutes) and reperfusion may have influenced the amount of $O_2^{\bullet-}$ generated within the tissues.

In this study, both theanine and minocycline had no free radical scavenging activity. Minocycline caused a significant increase in $O_2^{\bullet-}$ generation in comparison to control and stroke alone. This agrees with the findings in Section 7.2.4 in which minocycline caused a significant increase in $O_2^{\bullet-}$ levels in a dose dependent manner.

Kontos (2001) studied the effects of effective O_2 radical scavengers during ischaemia and found that in order to achieve maximal effectiveness of radical scavengers these would have to be administered at the time of reperfusion. Supplementation with antioxidants within 12 hours post-ischaemia increased the antioxidant capacity of the plasma and reduces oxidative damage (Ullegaddi *et al.*, 2005). Minocycline was shown to be an effective radical scavenger *in vitro* (Section 2.3.4), therefore there may be a possibility that on administering it both before and after ischaemia that it may be able to scavenge $O_2^{\bullet-}$. This may hold true for theanine as well, since it was shown to be an effective radical scavenger (Section 2.4) and it has been shown by Egashira (2004) that administering it before and after a stroke has proven it to be neuroprotective.

Melatonin, an endogenous hormone (Section 1.3.2.5) has poor interaction with $O_2^{\bullet-}$, but it stimulates the production of SOD (Reiter, 1998; Maharaj *et al.*, 2007). This implies that melatonin may lower intracellular levels of $O_2^{\bullet-}$ by increasing the levels of the enzyme that scavenges it. It can also be hypothesised that the increase in the levels of $O_2^{\bullet-}$ in this *in vivo* study could be caused minocycline which shows significant increases in $O_2^{\bullet-}$ production, may be due to a reduction in the levels of SOD in the brain after pre-treatment.

Irrespective of the mechanism by which increased levels of $O_2^{\bullet-}$ are observed, evidence from the *in vitro* and *in vivo* studies are that theanine and minocycline's neuroprotective properties do not include the scavenging of $O_2^{\bullet-}$.

7.4 THE COMPARATIVE EFFECTS OF THEANINE AND MINOCYCLINE ON TOTAL GLUTATHIONE LEVELS IN THE RAT BRAIN *IN VIVO* POST-ISCHAEMIA

7.4.1 INTRODUCTION

The principle antioxidant defence systems in the body include SOD, GSH, reduced GSH, CAT, GSH peroxidases and nutrient antioxidants (Section 1.3.2; Figure 7.1). GSH is a thiol containing substance found in cells. It is synthesised from glutamic acid, cysteine and glycine (Fang *et al.,* 2002). GSH is classified as a free radical scavenger and it acts by donating electrons to radical species (Ligumsky *et al.,* 2005). As a component of the intracellular antioxidant system, it has the following characteristics:

- GSH is both an exogenous and endogenous antioxidant as it can be partly absorbed from the small intestine and synthesised within the body,
- The GSH radical (GS•) is produced when GSH is oxidised and can react with another GS• to form GS-SG which can be reduced by GSH,
- GSH is able to react with various xenobiotic electrophilic compounds in the catalytic reaction of GSH-S-transferase,
- GSH acts as a free radical scavenger of ROS,
- NO reacts with GSH forming the S-nitroso-GSH adduct which is then cleaved by thioredoxin system to re-generate NO and GSH and
- GSH indirectly plays a role in the regulation of cellular redox homeostasis by interacting with thioredoxin and glutaredoxin (Fang *et al.,* 2002).

GSH is a substrate of GSH peroxidase which removes H_2O_2 and converts GSH to GSSG (Section 1.3.2.2). Damage to DNA usually occurs with a decrease in the GSH: GSSH ratio (Halliwell and Gutteridge, 1989). Astrocytes are more resistant than neurons to damage by ONOO- due to their high GSH content which is able to neutralise the effects of the ONOO- (Sas *et al.,* 2007).

GSH levels can be determined spectrophotometrically. The principle on which the assay depends is the reduction of 5' 5' di-thiobis-(2-nitrobenzoic acid) (DTNB) by SH groups into the yellow coloured 2-nitro-5-mercaptobenzoic acid.

In Sections 7.2.4 and 7.3.4 theanine showed no significant change in $O_2^{\bullet-}$ and minocycline was shown to increase levels of $O_2^{\bullet-}$ *in vitro* and post ischaemia. It was therefore decided to investigate how minocycline mediates its neuroprotective property in stroke (Section 6.2.4) by investigating whether it changes the levels of GSH and if theanine is able to have neuroprotective properties by increasing GSH levels after a stroke.

7.4.2 MATERIALS AND METHODS

7.4.2.1 Animals

Adult male Wistar rats were purchased from the South African Vaccine Producers, Johannesburg, South Africa and cared for as described in Section 4.2.2.2.

7.4.2.2 Chemicals and reagents

Theanine, minocycline, reduced GSH and DTNB were purchased from Sigma Chemical Corporation, St Louis, Missouri, United States of America. Methanol was purchased from Saarchem, Johannesburg, South Africa. Tris hydroxymethyl amino methane (tris-HCl) was purchased from Merck, Darmstadt, Germany.

7.4.2.3 Stroke Study

This was performed as described in Section 6.2.2.4.

7.4.2.4 Brain removal

Rats were sacrificed and the brains removed as described in Section 4.2.2.3 and the cerebellum was removed and the rest of the brain used for homogenate preparation for reasons stated in Section 7.3.2.4.

7.4.2.5 Preparation of tissue

The brain homogenate was prepared as described in Section 4.2.2.4.

7.4.2.6 Preparation of the GSH standard curve

Reduced GSH was used to prepare the standard curve. A series of GSH standards (0-10 nmols/ml) were prepared in triplicate using PBS (pH 7.4) as the diluents to give a total volume of 0.5 ml. To this, 1.5 ml of 0.2 M tris-HCl buffer (pH 8.2) and 0.1 ml of 0.01 M DTNB were added to the test tubes. The mixture was then made up to 10 ml by the addition of 7.9 ml of methanol and then left to stand in the dark for 30 minutes. A standard curve was then generated by measuring the absorbance at 412 nm on a Shimadzu UV mini 1240 UV/VIS spectrophotometer and plotting these against the molar equivalent weight of GSH (Appendix VI).

7.4.2.7 Glutathione Assay

The levels of GSH were estimated using a modified method of Sedlak and Lindsay (1968) and is described below:

To precipitate the proteins, 0.5 ml of TCA (25 % in milli-Q water) was added to rat brain homogenate (1 ml). In so doing, the protein-bound thiol containing compounds were separated from the non-protein bound thiol-containing compounds (Sedlak and Lindsay). The samples were then centrifuged at 2000 x g for 15 minutes. Aliquots of the supernatant were transferred to a clean set of test tubes to which 1.5 ml of 0.2 M of tris-HCl (pH 8.2) and 0.1 ml DTNB (0.01 M in methanol) were added and made up to 10 ml by adding methanol. The samples were incubated at room temperature for 30 minutes. The incubation mixture was then centrifuged at 2000 x g for 15 minutes and the supernatant was read at 412 nm using a Shimadzu UV mini 1240 UV/VIS spectrophotometer. The levels of GSH were determined using the GSH standard curve generated (Section 7.4.2.6). Final results were expressed as GSH (nmol/mg tissue).

7.4.2.8 Statistical Analysis

This was performed as described in Section 2.2.2.3.

7.4.3 RESULTS

Figure 7.7 illustrates that there was no statistically significant ($p > 0.05$) change in the levels of GSH after the stroke was induced in comparison to control. There was also no statistically significant change in the levels of GSH in the rats that were pre-treated with theanine and minocycline, in comparison to both control and stroke alone.

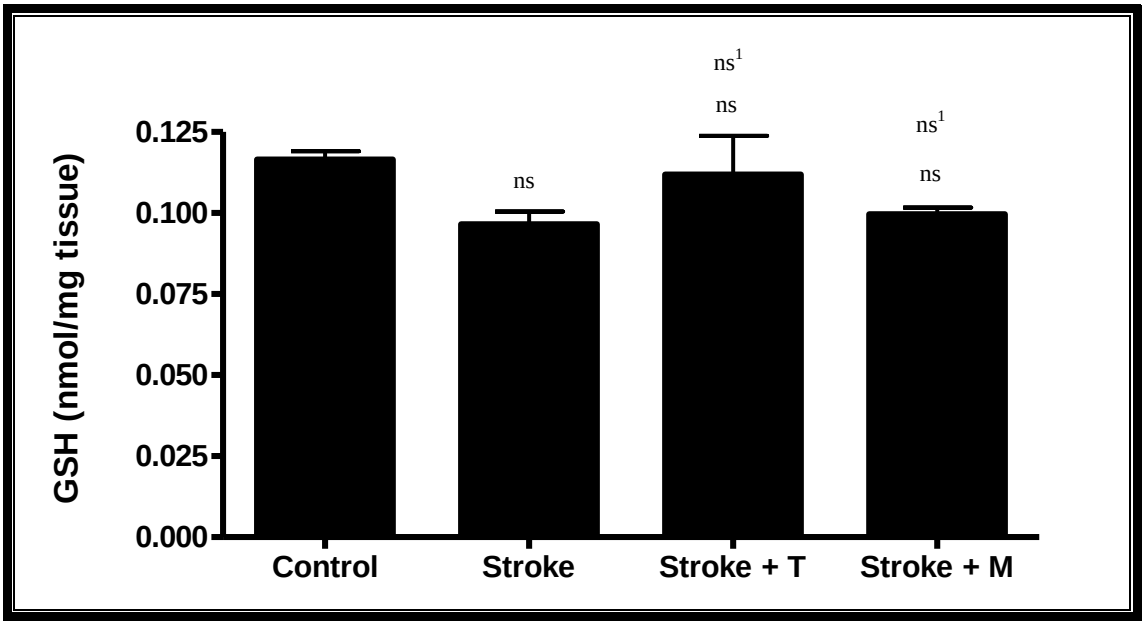

Figure 7.7: Effect of pre-treatment with theanine and minocycline on GSH levels in rat brain homogenate *in vivo*. *Stroke + T and Stroke + M represents stroke after pre-treatment with theanine and minocycline respectively*. Each bar represents the mean ± SD; (n=5). ns ($p > 0.05$) in comparison to control; ns[1] ($p > 0.05$) in comparison to Stroke (ANOVA followed by Student Newman-Keuls Multiple Range Test).

7.4.4 DISCUSSION

The brain contains low concentrations of GSH peroxidase (Halliwell and Gutteridge, 1985) which implies that there are also low levels of its substrate GSH. The low levels of protective antioxidant enzymes is one of the reasons that the brain is extremely vulnerable to injury after ischaemia.

The results of this study show that neither stroke nor pre-treatment with theanine and minocycline influenced the levels of GSH in the brain. This implies that both drugs do not stimulate the production of GSH and thus allow for the GSH to mediate in the removal of H_2O_2.

CHAPTER 8

SUMMARY OF RESULTS AND GENERAL CONCLUSIONS

8.1 SUMMARY OF RESULTS

The experiments that were conducted in this study shed light on the neuroprotective properties of both theanine and minocycline and the mechanisms in which they act. Oxidative stress that arises after a stroke leads to neuronal damage. Agents that will limit free radical damage will cause and enhance the endogenous capacity of the body to combat neuronal death and will prove to be of therapeutic potential.

During oxidative stress the antioxidant system within the body is unable to cope with free radicals generated during a tissue injury. The free radicals cause extensive damage to biomolecules and thus it is important that a potentially neuroprotective drug possesses antiradical properties. Chapter 2 explored the potential of each drug in scavenging the DPPH radical *in vitro* in comparison to vitamin C, a known radical scavenger with rapid reaction kinetics. The results showed that minocycline was a highly effective radical scavenger with intermediate reaction kinetics whose scavenging activity was comparable to that of vitamin C. Theanine on the other hand has slow reaction kinetics and its scavenging activity was found to be very low compared to both minocycline and vitamin C. Minocycline is thought to be a highly effective radical scavenger due to its ability to donate a hydrogen to the DPPH• and thus it may prevent chain initiation and propagation reactions in radical-mediated membrane damage.

Iron is an essential element in the body and it is abundant in the brain. When damage occurs in the brain, excess iron is released from intracellular 'pools' and catalyses the Fenton and Haber-Weiss reactions (Equations 1.1, 1.2 and 1.3) in which ROS are generated. The purpose of Chapter 3 was to investigate the iron binding properties of both drugs. UV/VIS spectroscopy, electrochemical analysis, ferrozine and ferritin assays were employed to assess the iron binding properties of the drugs. A UV/VIS spectrum was not generated by theanine due to the lack of delocalised electrons within its structure, however minocycline was able to as it has several delocalised electrons in its structure. Further UV/VIS studies confirmed that

minocycline interacts with Fe^{2+} and Fe^{3+} as exhibited by the decrease in the absorptive intensity of the minocycline spectrum when both ions were added to it. Further electrochemical analysis using AdSV and cyclic voltammetry demonstrated that both theanine and minocycline formed complexes with Fe^{3+}. This complexing ability implies that these drugs would prevent the reduction of Fe^{3+} to Fe^{2+} by forming a complex with it.

As chelation therapy has been regarded a suitable approach to the removal of toxic iron (Goyer, 1995), the chelating ability of theanine and minocycline was further investigated by assessing their inhibition of the formation of the Fe^{2+}-ferrozine complex. Ferrozine is a strong chelator of Fe^{2+} and EDTA was used as a positive control as it is a powerful inhibitor of the formation of the Fe^{2+}-ferrozine complex. Both test compounds showed poor inhibition of the formation of the complex and this shows that the complexes they form with iron may be disrupted easily in the presence of a strong chelator. The iron binding properties of the drugs was further investigated using ferritin, the storage protein of iron, to assess whether they are able to complex with the Fe^{2+} released from its core. This experiment was conducted as increased levels of ferritin in the blood have been associated with the severity of a stroke (Erdemoglu and Ozbakir, 2002). In this experiment, EDTA was again used as a positive control. Minocycline exhibited the ability to complex with the released Fe^{2+} whilst theanine was unable to do so. This chapter shows that minocycline is able to bind to both forms of iron whereas theanine does not.

LP is one of the undesirable effects of a stroke and the reperfusion of blood into hypoxic tissues after a stroke. LP is initiated by free radicals and its rate increases with an increase in iron in the tissues (Zaleska and Floyd, 1985). Previous studies have shown that antioxidants that prevent radical-mediated damage to lipids and chelators of metals are able to prevent LP (Halliwell and Gutteridge, 1989). Theanine has shown poor free radical scavenging and iron binding properties in comparison to minocycline. The objective of Chapter 4 was to explore the extent of theanine and minocycline's protection against QA-induced LP. Minocycline's ability to significantly reduce LP *in vitro* was speculated to be due to its iron binding ability. Theanine displayed poor inhibition of LP *in vitro* but on further *in vivo* studies in which the rats were treated with theanine for 5 and for 10 days, theanine significantly reduced LP. It was hypothesised that theanine's neuroprotective effects are improved when it is administered chronically and thus its radical scavenging and iron binding characteristics were

improved making it effective in protecting the brain against LP. Theanine has been shown to increase levels of SOD (Wang *et al.*, 2008) and therefore theanine may mediate its protective property against LP by decreasing the levels of $O_2^{\bullet-}$ in the brain. The effects of both theanine and minocycline on $O_2^{\bullet-}$ levels was investigated in chapter 7.

QA is released by activated microglia and macrophages in response to injury and its levels increase during a cerebral ischaemic episode (Halliwell and Gutteridge, 1989). The liver enzyme TDO is important in the catalysis of TRP to QA. *In vitro* studies demonstrated that co-incubation of liver homogenates with minocycline at a concentration 100 µM produces an increase in the holoenzyme and total enzyme activity. This effect was speculated to be due to minocycline either reducing the inactive form of the holoenzyme to the active form or reducing O_2 to $O_2^{\bullet-}$. Theanine demonstrated no effect on co-incubation with liver homogenates in vitro. However, pre-treatment with theanine for 10 days exhibited an inhibitory effect on the total enzymatic activity. This effect was postulated to be due to either theanine affecting the way in which TRP binds to the enzyme or to it preventing the reduction of O_2 to $O_2^{\bullet-}$.

Focal ischaemia is a result of the occlusion of a cerebral blood vessel, is the most clinically encountered stroke and an animal model of this type of stroke was studied. Ischaemia results in oxidative stress which leads to tissue injury. Thus far minocycline has shown the most promise as a neuroprotective agent as it decreases LP, chelates iron and scavenges free radicals. Theanine has shown improved neuroprotection on pre-treatment. The purpose of Chapter 6 was to investigate whether these agents are able to reduce the size of the infarct in the hippocampus after the occlusion of the MCA when administered 1 hour before the stroke. It was shown that the stroke model resulted in 39 % expansion of the hippocampus in the side of occlusion. Treatment before the stroke resulted in a 44 % and 7 % increase in hippocampal size with theanine and minocycline respectively. Thus theanine was not as effective as minocycline in decreasing the size of the infarct.

The conclusions in Chapters 4 and 5 found that the reduction of O_2 into $O_2^{\bullet-}$ played a role in the mechanisms of action of the drugs. Since $O_2^{\bullet-}$ has a role in LP and both the Fenton and Haber-Weiss reactions, it was decided to investigate the effects of both drugs on cyanide-induced $O_2^{\bullet-}$ generation. The drugs showed no significant effect on $O_2^{\bullet-}$ levels generated by

the toxic KCN. Their effects on $O_2^{\bullet-}$ generation after ischaemia were also investigated. It was found that stroke and treating the rat with theanine before inducing the stroke caused no significant effect in the generation of $O_2^{\bullet-}$ in comparison to the control brain. However, minocycline significantly reduced $O_2^{\bullet-}$ generation in the brain.

According to Equation 1.3, the Haber-Weiss reaction depends on a metal catalyst to drive the formation of •OH from H_2O_2 and $O_2^{\bullet-}$. This means that in ischaemic models, the release of iron together with the ischaemia-induced increase in $O_2^{\bullet-}$ and H_2O_2, the Haber Weiss reaction must be considerably favoured. However, minocycline is a chelator of iron and although it may result in an increase in the levels of $O_2^{\bullet-}$ will probably not facilitate the fast progression of the Haber-Weiss reaction and thus less •OH is formed. Since •OH is more toxic than the molecules that form it, a reduction in its formation could mean that this could possibly be one of the ways in which minocycline protects the brain after a stroke.

Finally, the effects of theanine and minocycline on brain GSH levels were investigated and neither of these agents caused a significant alteration.

8.2 CONCLUSIONS

Traumatic brain injury resulting from ischaemic insult leads to neuronal degeneration that involves free radical-induced damage, LP, the activation of neutrophils and the biosynthesis of endogenous toxins such as QA (Gariballa, 2000). The molecular pathways of ischaemic cell death involve a rise in cytosolic Na^+, a fall in ATP and a drastic increase in Ca^{2+} influx into the cells (Onténiente *et al.*, 2003). Free radicals however have a major role in the initiation of neuronal cell death after ischaemia therefore antioxidants are important role in limiting the oxidative stress that arises as a result of ischaemic injury. This is a popularly studied area in the search of therapeutic agents that may potentially reduce neuronal death and reducing the severity of stroke.

Minocycline and theanine were chosen in this study to investigate their potential neuroprotective properties in the events that lead to neuronal death and in a model of ischaemia. Theanine was shown to have the ability to inhibit LP *in vitro* after 5 and 10 days of treatment and to reduce the total enzymatic activity of TDO holoenzyme after 10 days of

treatment. It showed poor *in vitro* results for the scavenging of free radicals, iron binding, LP, changing the levels of $O_2^{\bullet-}$ and GSH and on reducing the swelling in the hippocampus induced by MCA occlusion. It was postulated that the effects of theanine as a radical scavenger and iron chelator were improved on treating the animals for 5 and 10 days.

Minocycline showed superior neuroprotective potential in that it attenuated LP *in vitro*, showed effective *in vitro* free radical scavenging properties and iron binding and reducing the post-ischaemic swelling in the hippocampus. These properties contributed to its neuroprotective properties. However it did exhibit some potential untoward effects such as the increasing of holoenzyme activity *in vitro* which may contribute to the biosynthesis of QA and increase the generation of $O_2^{\bullet-}$ when co-incubated with KCN in comparison to control brains. Treatment with both theanine and minocycline had no effect on GSH levels after the induction of ischaemia. Most drugs are studied at doses that are much higher than those used in a clinical setting but in this study the minocycline was used in concentrations that were lower than their usual clinical doses.

From the preceding evidence that these agents have potential neuroprotective properties and the mechanisms by which they act are outline. This study has shown various ways in which both drugs may interfere in the biochemical pathways which are important during a clinical relevant model of ischaemia.

CHAPTER 9

FUTURE RECOMMENDATIONS

Further analytical work to probe other neuroprotective properties and mechanisms of theanine and minocycline is necessary. These recommendations are outlined in this chapter.

Chapter 4 showed theanine as useful in inhibiting QA-induced LP after pre-treatment of 5 and 10 days which was completely different from the *in vitro* results. QA is an endogenous neurotoxin that is able to induce the Ca^{2+} overload intracellularly via interaction with the glutamate receptors and thus lead to the progression of excitotoxicity. Since both theanine (as an antagonist) and QA (as an agonist) are able to bind to NMDA receptors, it would be interesting to investigate the effects of QA alone and in combination with theanine on glutamic acid release in hippocampal synaptosomes. Such a study would prove to be useful in determining the interaction of theanine and QA at a receptor level. This study revealed that both theanine and minocycline prevented QA-induced LP. Further studies on whether these agents are able to reduce LP after ischaemia need to be conducted.

Ottino and Duncan (1996) hypothesised that any increase in COX activity could result in an increase in MDA production. The MDA produced can be determined by the TBA assay and linked to LP. Further studies of the effects of theanine and minocycline on COX activity would have to be conducted to determine other ways in which they may offer protection against LP.

TDO is an important enzyme in the KP and in the biosynthesis of QA. Levels of KP metabolites have an impact on the Ca^{2+} influxes and thus neuronal damage due to their interaction with glutamate receptors. Drugs that target KP or that change the balance between KYN (which is neuroprotective) and the toxic QA could prove to be potentially neuroprotective (Stone and Addae, 2002). Further studies on the drugs' effects on IDO and how these affect the levels of brain indoles would be beneficial for determining the overall effect that theanine and minocycline have on the KP.

Another area for further research would be a further investigation of the MCA occlusion stroke model. The areas in which the results of this experiment can be made more clinically relevant is to have a longer period of occlusion and reperfusion and using rats between the ages 2 – 3 years because this age range would mimic that of humans above the age of 50 years in which stroke usually occurs (Gupta and Briyal, 2004). The use of spontaneously hypertensive rats in stroke would be another clinically relevant study on the effects of these drugs on stroke.

Studies have shown that administration of the drug before and after ischaemia was induced, proved to be neuroprotective (Yrjänheikki *et al.,* 1999; Egashira, 2004; Cimino *et al.,* 2005). It would be worthwhile to investigate the potential neuroprotective effects of both drugs if administered before and after the stroke to determine the neuroprotective effects of theanine and minocycline. It would also be recommended that further studies would have to be carried out to investigate chronic administration of theanine and minocycline before a stroke in order to confirm its potential neuroprotective properties.

Theanine and minocycline have shown to be neuroprotective in stroke by various methods investigated in this study. It would be of great benefit if more studies were conducted on these drugs and their effects on stroke. Due to their wide use in the beverage industry (theanine in green tea) and as an antibiotic (minocycline), these drugs could prove to be a worthwhile investment into research towards neuroprotective agents in stroke.

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APPENDICES

APPENDIX I

Abstract for the 19th International Brain Research Organisation (IBRO) Schools Conference (24-30th August 2009)

Stroke (cerebral ischaemia) is the third leading cause for mortality and the leading cause of disability in the United States (Yasuhara *et al.*, 2008). Thus there is a dire need to develop or screen agents that have the ability to protect the brain against injury during stroke. A number of neurotransmitters are released during stroke. For example, the glutamic acid concentration rises in the brain when there is cerebral ischemia. This excess glutamate causes further damage due to the glutamic acid binding to receptors such as the NMDA receptors. This causes an influx of calcium into the cell resulting in neuronal death (Yashura *et al.,* 2008). There is also an increase in the generation of free radicals as a result of ischaemic injury which may lead to neuronal damage. In our search for agents which could protect the brain, an agent found in green tea, *viz* theanine has been shown to inhibit lipid peroxidation and that it is neuroprotective in cerebral ischaemia (Tsuge *et al.*, 2003). It has been shown that ischemia-induced neuronal death in hippocampal region is significantly prevented in a dose-dependent manner after the administration of theanine (Kakuda *et al*., 2002). These findings indicate that theanine may be clinically useful in preventing ischemic neuronal damage. The antioxidant activity of theanine was investigated *in vitro* and it was found to be a less effective radical scavenger of the 2, 2-diphenyl-1-picryl-hydrazyl ($DPPH^{\bullet}$) radical in comparison to vitamin C. These results show that theanine may be neuroprotective in free radical-mediated neuronal death after ischaemia and contributes to the antiradical properties of green tea

APPENDIX II

Abstract for the 43rd Annual Congress 2009 of the South African Society for Basic and Clinical Pharmacology (SASBCP) (23-26th September 2009)

ANTIRADICAL AND ANTIOXIDANT ACTIVITY OF THEANINE

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Introduction

Free radicals that are generated *in vivo* result in damage of DNA, small molecules and lipids (Halliwell *et al*, 1995). Lipid peroxidation results from free radicals attacking membrane lipids which leads to the destruction of the cell membrane, cell integrity and the proper functioning of membrane bound enzymes and receptors. Free radicals have also been shown to play a role in cardio vascular diseases (Dorman *et al.,* 2003). Importance of an antioxidant *in vivo* cannot be emphasized enough as these agents protect the human body against damage by ROS. Thus in the search for agents that have antioxidant activity, theanine which is a natural component of green tea was studied as it has been shown to prevent lipid peroxidation (Tsuge *et al.,* 2003).

The free radical scavenging activity of a potential antioxidant is evaluated *in vitro* by its ability to scavenge the stable 2, 2-diphenyl-1-picryl-hydrazyl ($DPPH^{\bullet}$) radical which acts as both an oxidizable substrate and as a reaction indicator molecule (Dorman *et al*, 2003).

Method

A modified method of free radical scavenging of Brand-Williams *et al.,* (1995) was used to measure the free radical scavenging ability of theanine and melatonin. Vitamin C was used as a positive control (Brand-Williams *et al.,* 1995; du Toit *et al.,* 2001).

Results

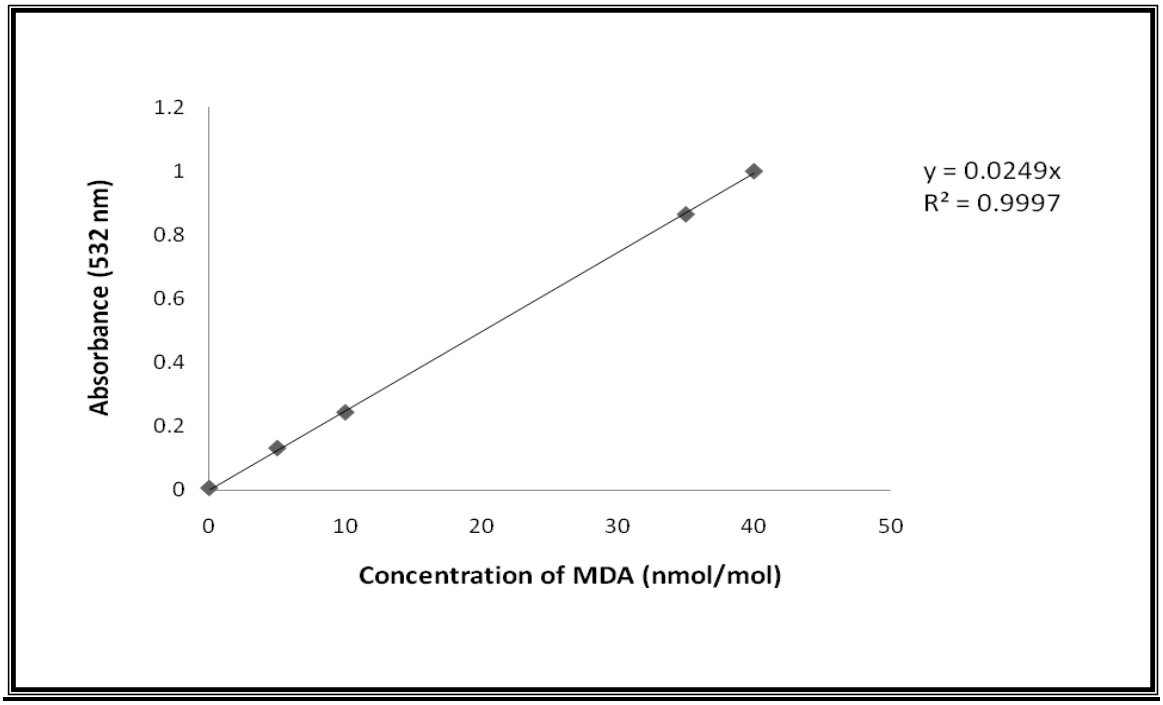
Theanine scavenges 32 % of the radicals on average over the entire concentration range tested which is significantly lower than the free radical scavenging ability of vitamin C which is 95 %. Flavonoids in tea account for most of the antioxidant activity in tea and thus the presence of Theanine in green tea also increases the antioxidant capacity of tea.

Conclusion

Theanine can be an antioxidant *in vitro* due to it antiradical activity.

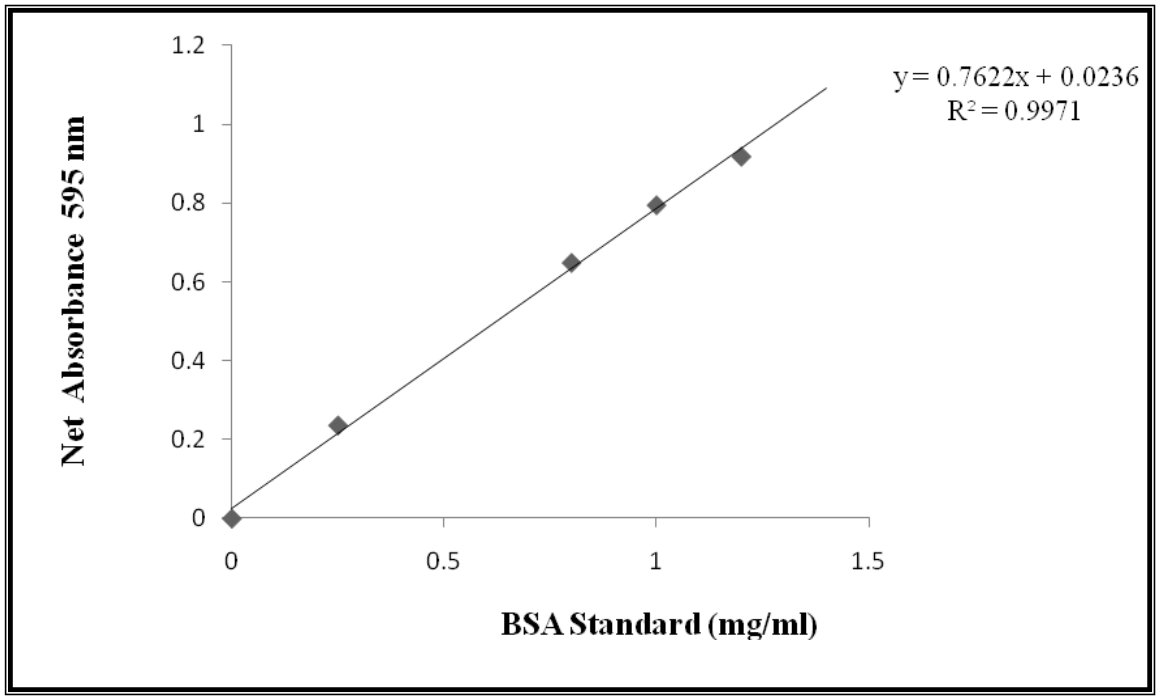
Keywords: Theanine, DPPH, antiradical, antioxidant, green tea

APPENDIX III



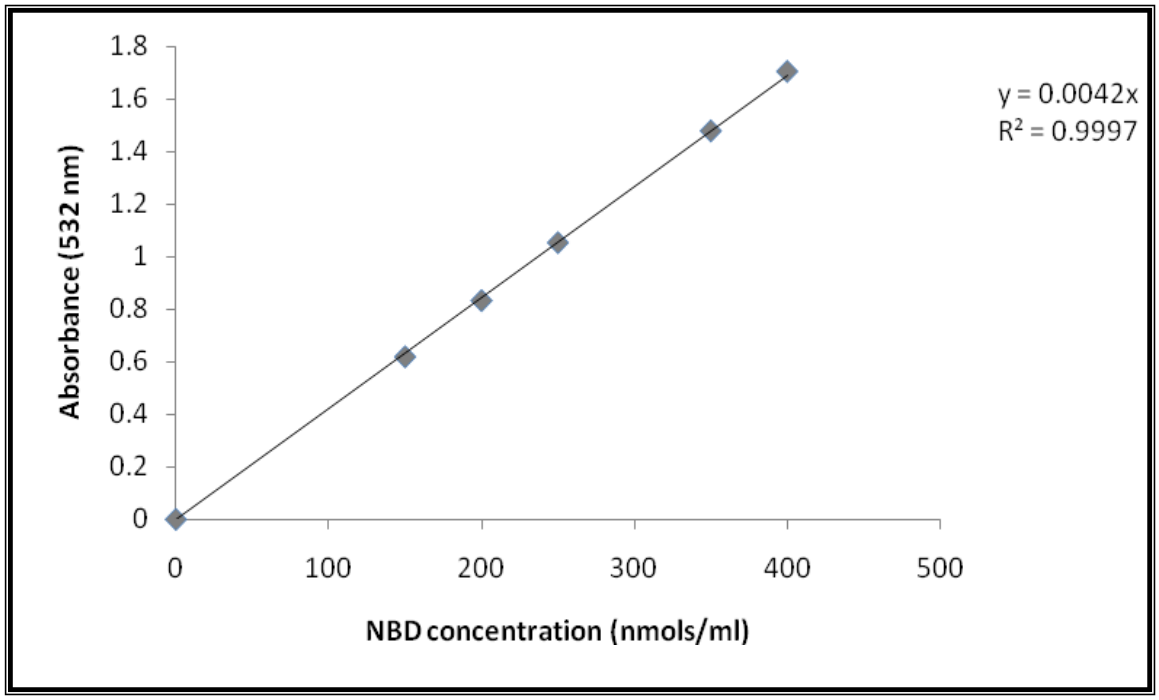
Appendix III: Malondialdehyde (MDA) standard curve.

APPENDIX IV



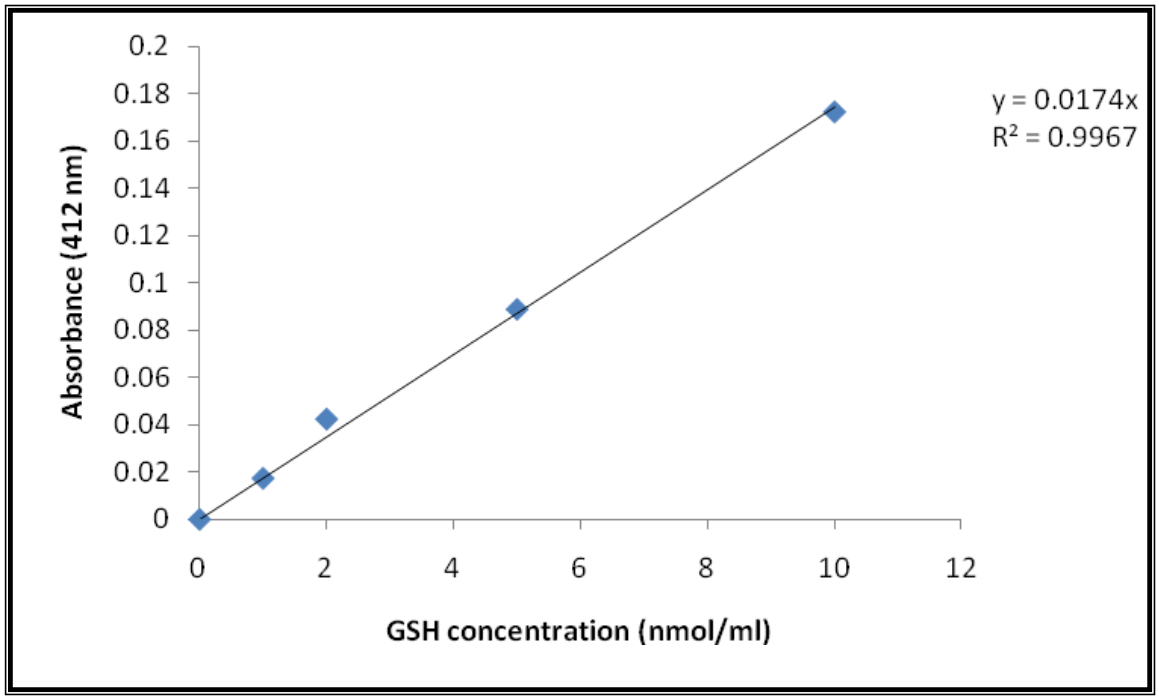
Appendix IV: Protein standard curve.

APPENDIX V



Appendix V: NBD standard curve.

APPENDIX VI



Appendix VI: Gluthathione standard curve.