CIMETIDINE AS A FREE RADICAL SCAVENGER

THESIS

Submitted in fulfilment of the requirements for the Degree

of

MASTER OF SCIENCE (PHARMACY)

In the Faculty of Pharmacy

Rhodes University

Grahamstown

by

ZAYNAB YUSUF LAMBAT

JUNE 2002

In loving memory of Mussa chacha and Hawa mahi, who both lost their lives to cancer.

Buried deep within each of us is a spark of greatness,

A spark that can be fanned into flames of passion and achievement….

That spark is not outside of you,
It is born deep within you

-James.A. Ray

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ACKNOWLEDGEMENTS

My parents and family for allowing me to do a Masters degree as well as their love and support, without which this work would not have been possible. **Thank- you** for being there!

My supervisor Professor Santy Daya for all his encouragement, guidance and support throughout this study.

The Pharmacy Department for all their assiatance and encouragement.

Professor Ben Potgeiter for always being approachable and willing to listen. Thank you for the guidance and encouragement.

Sally and Dave Morley for always ensuring that materials were available for the study.

Professor Iqbal Parker (UCT) and his research team for all their assistance regarding cell culture.

Vicky Longshaw from Biochemistry for all her help with cell culture.

The Dean of Research, Professor Duncan for his assistance regarding cancer.

Shai thank you for your help and encouragement and help with lipid peroxidation.

Dr. Janice Limson for her expertise with electrochemistry.

My fellow MSc colleagues and all my friends for their constant encouragement and support.

Nish for always being there for me. **Thank you!**

I would like to thank The Andrew Mellon Foundation Postgraduate Scholarship, NRF and Rhodes University for financial assistance towards this study.

Finally, to the Almighty without whom nothing is possible.

Abbreviations

A	Absorbance
A^o	Absorbance at time zero
AD	Alzheimer's disease
ANOVA	Analysis of variance
ASV	Anodic stripping voltammogram
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BHT	Butylated hydroxytoluene
^{o} C	Degrees Celsius
cAMP	5' cyclic adenosine monophosphate
CNS	Central nervous system
CSF	Cerebrospinal fluid
Cu^{2+}	Copper (II)
CV	Cyclic voltammetry
DMEM	Dulbecco's minimum essential media
DMH	1,2 dimethylhydrazine
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FCS	Foetal calf serum
Fe^{2+}	Iron (II)
Fe^{3+}	Iron (III)
g	Gram
GCE	Glassy carbon electrode

H_2	Histamine 2 receptor
H_2O	Water
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
k_{CIME}	Rate constant for cimetidine
k_{DR}	Rate constant for deoxyribose
KCN	Potassium cyanide
µl	microlitre
mg	milligram
ml	milliliter
µM	Micromolar
mM	Millimolar
MDA	Malondialdehyde
n	Sample size
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NBD	Nitroblue diformazan
NBT	Nitroblue tetrazolium
O_2	Oxygen
O_2^-	Superoxide anion radical
•OH	Hydroxyl radical
PBS	Phosphate buffered saline
PUFA	Polyunsaturated fatty acid
REM	Rapid eye movement
ROS	Reactive oxygen species
Sec	Seconds
SEM	Standard error of the mean

SHE	Standard hydrogen electrode
SOD	Superoxide dismutase
SPE	Solid phase extraction
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid-reacting substances
TCA	Trichloroacetic acid
TLC	Thin layer chromotography
UV	Ultraviolet
V	Volts
WHCO6	Wits Human Carcinoma of the Oesophagus

LIST OF FIGURES

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Publications

The articles published/ in press/ submitted thus far are listed below.

1) Lambat Z, Conrad N, Anoopkumar-Dukie S, Walker RB and Daya S. (2000). An investigation into the neuroprotective properties of ibuprofen. *Metab. Brain. Dis.* 15: 249-256.

2) Anoopkumar-Dukie S, Lambat Z, Lack B, McPhail K, Nyokong T, Maharaj D and Daya S. (2002) Indomethacin binds Fe^{2+}, reduces lipid peroxidation and scavenges Superoxide anions. *Metab. Brain. Dis*. **In Press.**

3) Lambat Z, Limson JL and Daya S. Cimetidine acts as a free radical scavenger by binding to transition metals. *Journal of Pharmacy and Pharmacology*. **In Press**.

4) Lambat Z and Daya S. The effect of cimetidine on free radical and lipid peroxidation levels in WHCO6 cancer cells. *British Journal of cancer*. **Submitted.**

ABSTRACT

The present study was undertaken to determine the effects and possible mechanism of action of cimetidine in cancer and Alzheimer's disease (AD). Throughout this study emphasis is placed on free radical levels since the magnitude of the relationship between diseases and the levels of free radicals vary from one disease to another. Studies were carried out to examine the effect of cimetidine on free radical levels using superoxide formation and lipid peroxidation as indicators of free radical levels.

The experiments revealed that addition of cimetidine, especially in high concentrations (0.5 and 1.0 $x10^{-6}$ M) significantly inhibited WHCO6 cancer cell growth rather than cancer cell growth, as no normal control was available. Free radical formation as well as hydroxyl radical formation were reduced in the deoxyribose assay. In addition, cimetidine exhibits properties of binding to metals such as copper and iron.

To maintain consistency in the experiments, a WHCO6 (Wits Human Carcinoma of the Oesophagus) cell line was used to investigate the effect of cimetidine in cancer. Neurodegeneration was induced in the rat brain using neurotoxins such as cyanide to investigate the relationship between cimetidine in AD.

A decrease in cancer cell growth was accompanied by a concomitant decrease in the levels of free radicals and lipid peroxidation, suggesting that

the growth-inhibitory effects of cimetidine on WHCO6 cancer cells *in vitro* may be due to free radical scavenging properties.

This proposal was further strengthened by determination of free radical levels in the rat brain. After treatment with neurotoxins to induce neurodegeneration, the levels of free radicals in the rat brain suggest that addition of cimetidine reduces free radical levels in the rat brain in a dose-dependent manner.

Further experiments were done in an attempt to uncover the underlying mechanism by which cimetidine exhibits free radical scavenging properties. Metal binding studies were done using electrochemical, HPLC and UV/Vis studies. The results show that cimetidine binds iron and copper. These metals have been implicated in free radical production via the Fenton reaction. By binding with cimetidine the metals become unavailable to produce free radicals and hence cimetidine indirectly reduces the formation of free radicals.

The final experiment was the determination of cimetidine as a hydroxyl radical scavenger in the deoxyribose assay. Cimetidine was shown to act as a potent hydroxyl radical scavenger, thereby confirming its activity as a free radical scavenger. In addition, cimetidine protects against damage to the deoxyribose sugar, a component of DNA.

Whilst there are many theories that explain the therapeutic role of cimetidine in degenerative disease, the actual mechanism of the role of cimetidine is emphasized as a free radical scavenger. Regardless of the mechanism of

action, cimetidine does inhibit tumour growth according to this study and also reduce free radical levels in neurodegeneration, which suggests a role for cimetidine as a possible additive in treatment of patients with such disease states. These findings have important clinical implications, and needs to be investigated further.

CHAPTER 1

Literature Review

1. INTRODUCTION

Cimetidine is a histamine- 2-receptor antagonist, which inhibits the secretion of basal and gastric hydrochloric acid and also reduces the output of pepsin. Cimetidine is currently used for the treatment of duodenal and gastric ulceration, reflux oesophagitis and for the reduction of gastric acid secretion for the management of Zollinger- Ellison syndrome. Cimetidine can be used in the form of a tablet, syrup, intravenously and as an injection fluid.

Cimetidine has a remarkably low rate of serious side effects and a great margin of safety. Cimetidine has anti-androgenic effects expressed as reversible gynaecomastia and less commonly impotence in a few patients. Cimetidine interacts with the cytochrome p-450 microsomal enzyme system and thus may delay the metabolism of drugs eliminated through this system.

Free radicals have been implicated as playing a role in the aetiology of many diseases. The evidence for a role in disease is of several types. For example, many human diseases present with increased production of free radicals or with increased level of free radical- generating substances as shown in Table 1 (Gieseg, 1999).

Table 1: Diseases possibly caused by free radical damage.

CANCER	Free radicals can react with DNA of the cell causing mutations. If the free radical damage is not repaired, the DNA sequence will change. This may result in the switching on of oncogenes or the switching off of tumour suppressor genes within the cell. Usually the cells require two or more genes to be altered before they become cancerous.
CATARACTS	The formation of cataracts involves the oxidation of the lens proteins. UV light and possibly iron generate free radicals which cause sugar molecules and other compounds to react with the lens proteins to form coloured compounds that block the passage of light through the lens.
ALZHEIMERS	Free radicals in the brain can lead to damage of proteins. These damaged proteins build up in specific areas causing the death of various neurons.

Recent research is based on finding methods to reduce damage caused by free radicals and a solution could be to use antioxidants or free radical scavengers as treatment.

In the following sections the relevant aspects of the vast literature pertaining to this study are presented.

Chapter 2 deals with studies of cimetidine addition on the growth of a malignant WHCO6 oesophageal cancer cell line.

Chapter 3 deals with the effect of cimetidine on the WHCO6 cell line as well as on rat brain homogenate to assess levels of superoxide anion, a potent free radical.

Chapter 4 examines the effect of cimetidine on the WHCO6 cell line and rat brain homogenate to assess lipid peroxidation levels.

Chapter 5 assesses the possibility of an interaction between cimetidine and transition metals such as Fe^{2+} and Cu^{2+}.

Chapter 6 deals with enzyme kinetics and the determination of rate constants for cimetidine as a scavenger of the hydroxyl radical in the deoxyribose assay.

Chapter 7 the results obtained are summarized and conclusions are drawn. Recommendations for future research are also suggested in the final chapter.

1.1 CIMETIDINE

1.1.1 HISTORY

Cimetidine originated from a research programme initiated by Dr.J.W.Black of Smith Kline and French laboratories where a specific antagonist of the histamine receptors was being sought. Gastric juice volume is proportionately reduced and intrinsic factor (also a product of parietal cells) is partly reduced though not to the extent where Vitamin B_{12} supplementation is required. Cimetidine reduces pepsin secretion following stimulation by histamine more effectively than stimulation by the vagus or gastrin.

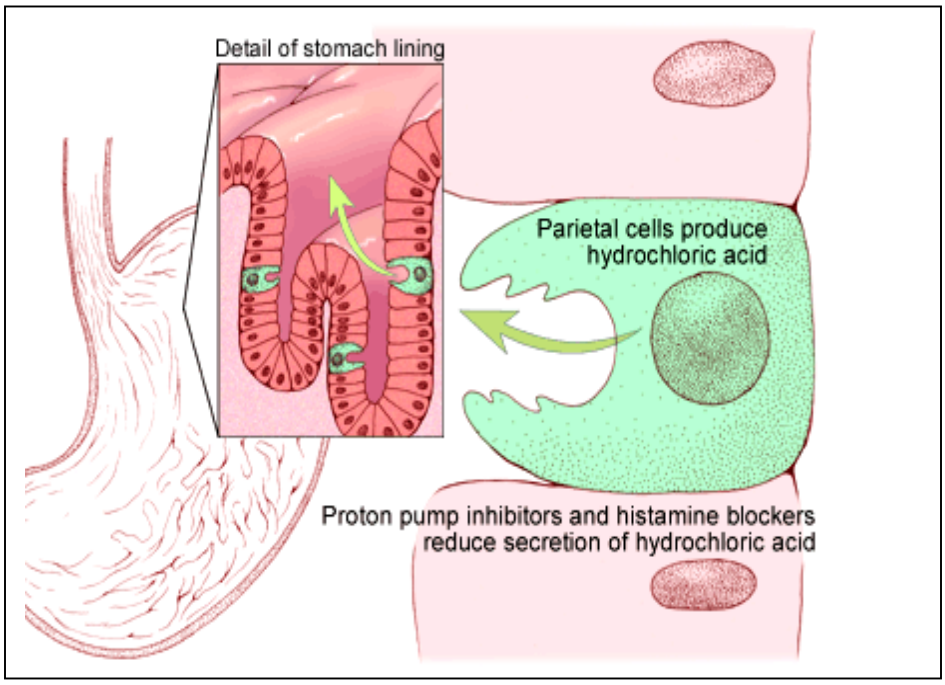


Figure 1 : The site at which cimetidine exerts its pharmacological effect

The chemical name for cimetidine is N”-cyano-N-methyl-N’- [2-{5-methyl-1H-imidazol-4-yl} methyl] thio] ethyl] guanidine.

Cimetidine can exist in two forms: as the free base or as the hydrochloride salt.

$C_{10}H_{16}N_6S$ or $C_{10}H_{16}N_6S \cdot HCl$

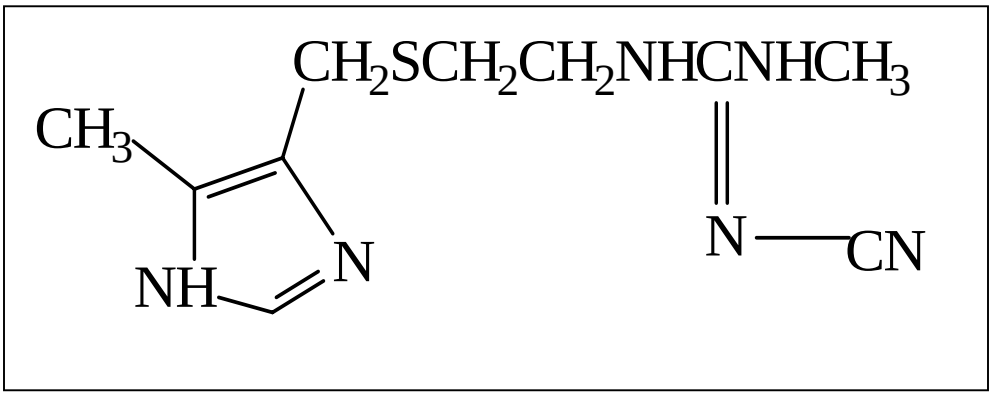


Figure 2 : Chemical structure of cimetidine

Since, cimetidine acts on histamine receptors and for a better understanding of the drug it is necessary to have a basic understanding of histamine itself.

1.1.2 HISTAMINE

Histamine (beta-imidazolylethylamine) is distributed widely throughout the body and is present in many different organs of the body. In 1927, it was found that histamine could be isolated from samples of liver (Best et al, 1927) in the body and confirmed by Best, Dudley, Dale and Thorpe that this amine was a natural constituent of the body.

Histamine is synthesized by the decarboxylation of the amino acid histidine under the influence of the enzyme histidine decarboxylase and therefore it is clear that the tissues do not rely on exogenous sources for their supplies of histamine. The activity of the endogenous enzyme is controlled by biochemical feedback mechanisms ensuring a more steady level of histamine.

Histidine decarboxylase is highly specific and utilizes pyridoxal phosphate as a coenzyme. Factors such as stimuli and catecholamines that provoke inflammation promote the formation of histidine decarboxylase and consequently histamine.

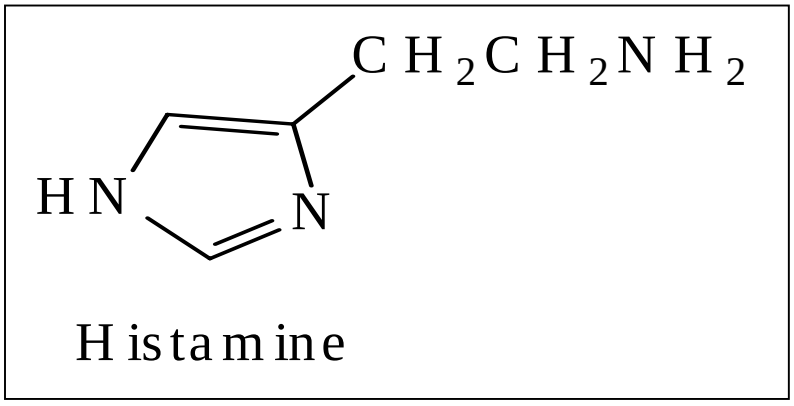


Figure 3 : Chemical structure of histamine

Lewis and co-workers discovered a substance called “Substance H”, which was being liberated from skin cells in response to injury caused by an antigen antibody reaction. This substance was found to be identical to histamine (Lewis, 1927).

Histamine is found in fairly high concentrations in the mammalian cells of up to 100µg/g, and is stored in mast cells. The skin, bronchial tree and intestine have a high concentration of mast cells. Histamine causes contraction of smooth muscle, dilation of capillaries and secretion of hydrochloric acid by the stomach. The relative intensity of the extent of these effects varies considerably in the animal species.

In 1920 Popielski was the first person to discover the effect of histamine on gastric acid secretion. The output of pepsin and hydrochloric acid was found to increase on administration of histamine.

Histamine has various effects on the cardiovascular system. These include:

- Dilation of Peripheral blood vessels.
- Increased capillary permeability causing leakage of various fluids leading to oedema.
- Increased contractility of the heart.
- Reduction in peripheral resistance due to dilation.

When histamine is injected intradermally a number of events occur:

- Localised red spot which extends around the site of injection.
- Development of a flare forming approximately 1cm from the site of injection.
- Formation of a “wheal” which is the last spot and occupies the same area as the original spot except that it is raised.

Histamine stimulates various nerve endings and hence is responsible for the development of pain and subsequent itching.

Metabolism of histamine is by two major pathways: Ring methylation and oxidative deamination. These two major pathways metabolise approximately 80% of histamine; the metabolites are excreted unchanged in the urine.

Histamine is released when exposed to a variety of agents. These include:

- Antigens which combine with antibodies.
- Mechanical trauma.
- Damage to the skin caused by insect bites and snake venom.
- Proteolytic enzymes.
- Monoamines.

Histamine receptors have not been extensively studied in terms of physical and chemical methods but have been classified pharmacologically in terms of antagonists. The first histamine receptor antagonists discovered were found to be specific competitive antagonists. Although these agents blocked the activity of histamine in terms of muscle contraction, it was found that these drugs did not inhibit all the pharmacological actions of histamine.

These agents partially blocked the vasodilator effects of large doses of histamine and completely failed to inhibit histamine stimulated acid production. Furthermore it was found that histamine functions like other biogenic amines and hormones. Some histamine receptors are located in the stomach, specifically on the parietal cells. The enzyme adenylate cyclase is activated causing an increase in cellular cyclic AMP (cAMP), which then causes an increase in gastric acid secretion (Grossman et al, 1978).

1.1.3 H_2-RECEPTOR ANTAGONISTS

Dr. J.W.Black and co-workers were searching for a molecule that would compete with histamine for its receptor site as well as antagonize histamine-induced gastric acid secretion (Brimblecombe et al, 1978). The antagonist needed was based according to the following criteria:

- Must be recognized by the receptor.
- Bind more strongly than histamine but not trigger the usual response.
- Close structural resemblance to histamine.

The breakthrough for such a compound came with the discovery of the guanidine analogue of histamine and consequently in 1964 the first true H2-receptor antagonists were discovered (Black et al, 1972). N-alpha-guanylhistamine appeared to act as a partial antagonist and at high doses antagonized histamine–induced acid secretion (Durant et al, 1975).

Studies of structure-activity relationships indicated that the imidazole ring in structure of guanidine was necessary for exerting the pharmacological effect. The activity of the compound was also dependent upon the length and extension of the side chain.

Furthermore, the length of the alkylene chain resulted in a marked increase in antagonist potency, which lead to the development of the drug, burimamide.

Although burimamide exerted the required pharmacological effect the potency of this compound was low and when administered orally had a reduced bioavailability. This lead to the development of the drug metiamide. Metiamide had an enhanced effect of reducing gastric inhibition when compared to burimamide (Schofield et al, 1975).

Unfortunately on continual use of metiamide it was found that although this compound promoted ulcer healing it was associated highly with granulocytopaenia and consequently had to be withdrawn.

The development of cimetidine occurred when the thiourea moiety of the metiamide side chain was replaced by a cyanoguanidine functional group.

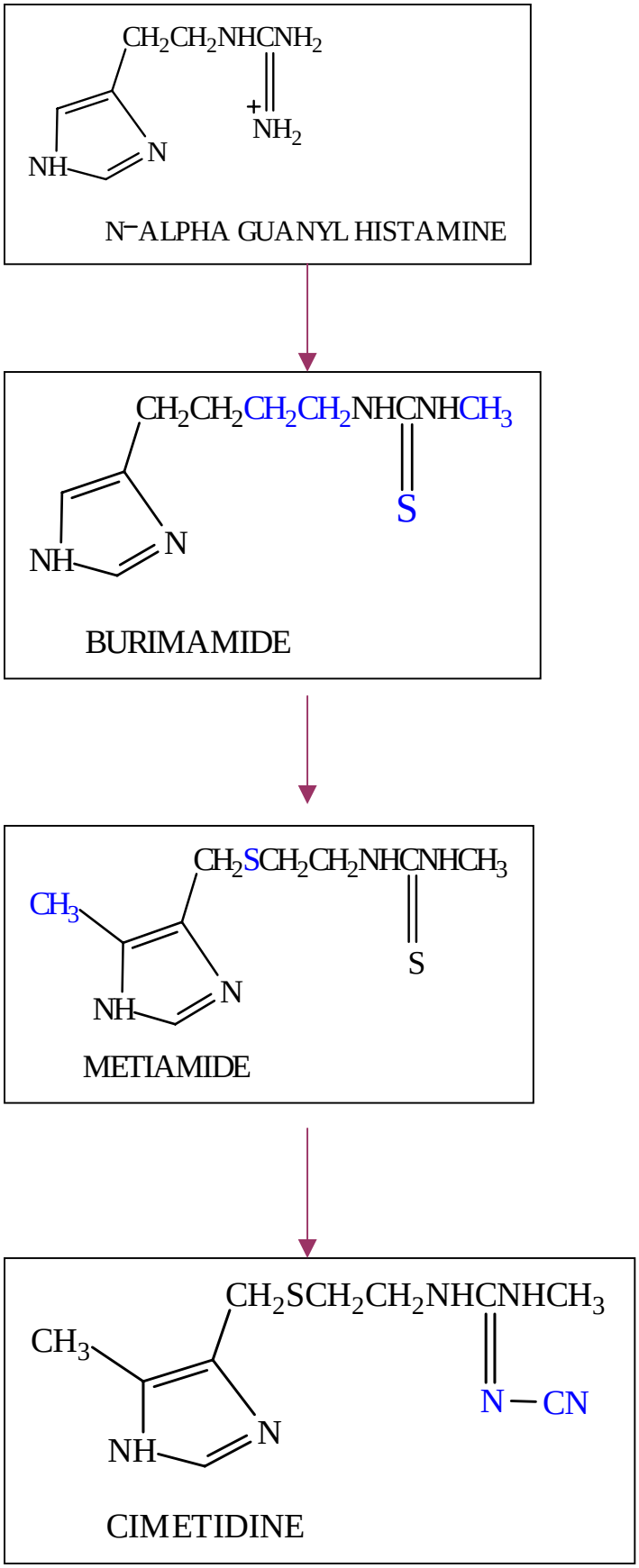


Figure 4: The development of cimetidine (Brimblecombe et al, 1978)

1.1.4 PHARMACOKINETICS OF CIMETIDINE

1.1.4.1 Absorption

Cimetidine is well absorbed in the gastrointestinal tract with oral bioavailability 37 to 90 %. Onset of action is seen 30 to 60 minutes after ingestion and effects peak at 1 to 2 hours. The plasma half-life of cimetidine is 123 ± 12 minutes. Administration of cimetidine with food delays the appearance of peak levels in blood, however the total absorption is not altered and hence it is not necessary to administer cimetidine in the fasting state (Bodemar et al, 1979).

1.1.4.2 Distribution

Cimetidine is widely distributed in the body and 22% is bound to plasma proteins in the serum. Initially it was reported that cimetidine was found rapidly distributed throughout the body of rats and dogs especially in glandular tissue, however it was found that there was no penetration of cimetidine into the brain or the eye (Cross, 1977). Later it was found that cimetidine was found to be present in cerebrospinal fluid in patients suffering from the cognition disorder of amnesia whilst receiving cimetidine therapy (Schentag et al, 1979).

1.1.4.3 Metabolism and excretion

Cimetidine is metabolised in humans by hydroxylation at the imidazole methyl group and by S-oxidation, the cytochrome enzymes catalyse both the reactions. The major part of cimetidine is excreted unchanged in the urine by

the kidneys (up to 66%), a conjugate with glucuronic acid (18% as cimetidine-N-glucuronide) and as the S-oxide (11% as cimetidine – sulphoxide).

1.1.5 DRUG INTERACTIONS

Cimetidine inhibits hepatic microsomal enzymes and hence the metabolism of many drugs may be reduced, leading to increased effects. In addition the inhibition of hepatic microsomal enzymes that activate drugs to their active forms will result in reduced effects. Examples of such drugs where interaction may occur are:
Oral anticoagulants (Warfarin)
Theophylline
Phenytoin
Barbiturates

1.1.6 CLINICAL EFFICACY OF CIMETIDINE

Cimetidine administration results in a significant reduction in gastric acid secretion *in vitro* and in intact animal preparations (Brimblecombe et al, 1975). In humans, administration of cimetidine lowers gastric acid secretion stimulated by food, histamine, insulin, pentagastric infusion and caffeine (Moberg et al, 1977).

Cimetidine is useful for the treatment of acute gastrointestinal haemorrhage in patients with fulminant hepatic failure and to treat gastrointestinal haemorrhage associated with gastritis and gastric or oesophageal erosions (MacDougall et al, 1977).

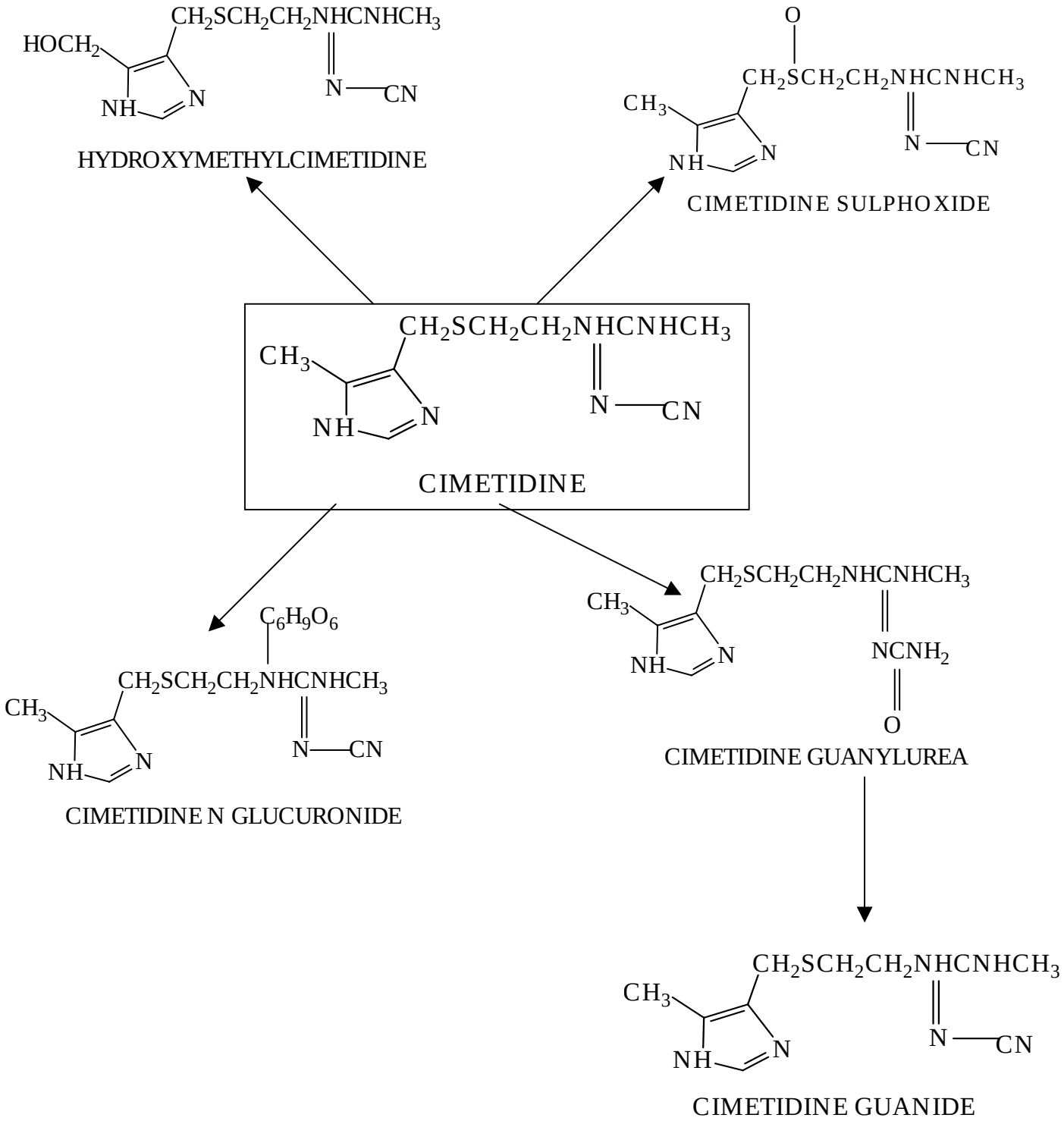


Figure 5: Metabolism of Cimetidine in humans (Walkenstein et al, 1978)

1.1.7 SIDE-EFFECTS OF CIMETIDINE

The general side effects of cimetidine will be considered:

- ENDOCRINE TOXICITY

  During treatment using cimetidine it was reported that hyperglycaemia and hyperosmolar nonketotic coma have occurred. However, these cases were in patients who were metabolically unstable (Jeffreys et al. 1978).

- HAEMOTOLOGIC TOXICITY

  Cimetidine has been implicated in the development of a number of cases of agranulocytosis, pancytopaenia and thrombocytopaenia (McDaniel et al, 1979).

- DERMATOLOGICAL REACTIONS

  It has been reported that cimetidine has been known to cause psoriasis, giant urticaria and Stevens-Johnson Syndrome (Hadfield, 1979).

- GASTROINTESTINAL TOXICITY

  Following abrupt discontinuation of cimetidine perforation of duodenal, oesophageal and gastric ulcers have been reported (Gill et al, 1977).

  Acute pancreatitis and paralytic ileus as well as a case of severe case of diarrhoea have been reported with cimetidine therapy (Gazala et al, 1978).

- MISCELLANEOUS EFFECTS

  On administration of cimetidine fever has been reported to occur and this is thought to be due to the blockade of H2-receptors in the thermoregulatory areas of the hypothalamus (Nistoco et al, 1978)

- CENTRAL EFFECTS

  An assortment of central effects have been reported with the use of cimetidine. Mental confusion, lethargy, restlessness, focal twitching, sweating, seizures, unresponsiveness and periods of apnoea are effects that have been reported (Mc Guigan, 1981).

1.2 FREE RADICALS

1.2.1 FREE RADICAL FORMATION

Atoms are most stable in their ground state. An atom is considered to be "ground" when every electron in its outermost shell has a complimentary electron that spins in the opposite direction. By definition a free radical is any atom with at least one unpaired electron in the outermost shell, and is capable of independent existence (Karlsson, 1997). A free radical is formed when a covalent bond between entities is broken and one electron remains with each newly formed atom.

An unpaired electron makes the molecule very reactive. The molecule takes an electron from other molecules in order to gain a full set of electrons. The structure of the molecule is subsequently changed and can often become a free radical itself.

1.2.1.1 Biological systems

In biological systems free radical species can be formed in one of three ways. These include haemolytic cleavage (Cheeseman et al, 1993); heterolytic fission (Cheeseman et al, 1993) and electron transfer reactions (McCay et al, 1980).

With the exception of unusual circumstances such as the influence of ionising radiation, free radicals are generally produced in cells by electron transfer reactions. These can be mediated by the action of enzymes or non-enzymatically, often through the redox chemistry of transition metals.

Animal cells in special circumstances generate free radicals intentionally because these agents can be useful entities if constrained or targeted. Some enzymes utilize a free radical at their active site in the process of catalysis; for example ribonucleotide reductase (Cheeseman et al, 1993).

Activated phagocytes also deliberately generate superoxide as part of their role in the immune system. Under normal circumstances the major source of free radicals in mammalian cells is through electron leakage from the electron transport chain located in the endoplasmic reticulum and mitochondria.

1.2.1.2 The electron transport chain

The electron transport chain is located in the mitochondria and acts as an integrated chain during biological oxidation (Veis at al, 1993).

The electron transport chain has two main functions:

1) *Formation of water*: The sequence of redox compounds acting as an integrated chain lead to the production of reduced equivalents which are then transferred to NAD, pass down the chain and eventually react with molecular oxygen to form water.
Water is probably formed from H^+ discharged at the CoQ-Cyt b step and OH^- arising from the reaction of molecular oxygen with cytochrome a_3.

2) *Oxidative Phosphorylation*: The transfer of hydrogen from a substrate (AH_2) to NAD generally involves a relatively small change in free energy. The oxidation of reduced coenzyme by molecular oxygen however releases a large amount of energy. In the electron transport chain, the energy is not only released in discrete steps but at certain points in the form of adenosine triphosphate (ATP) which is a form of energy that can be used by the cell.

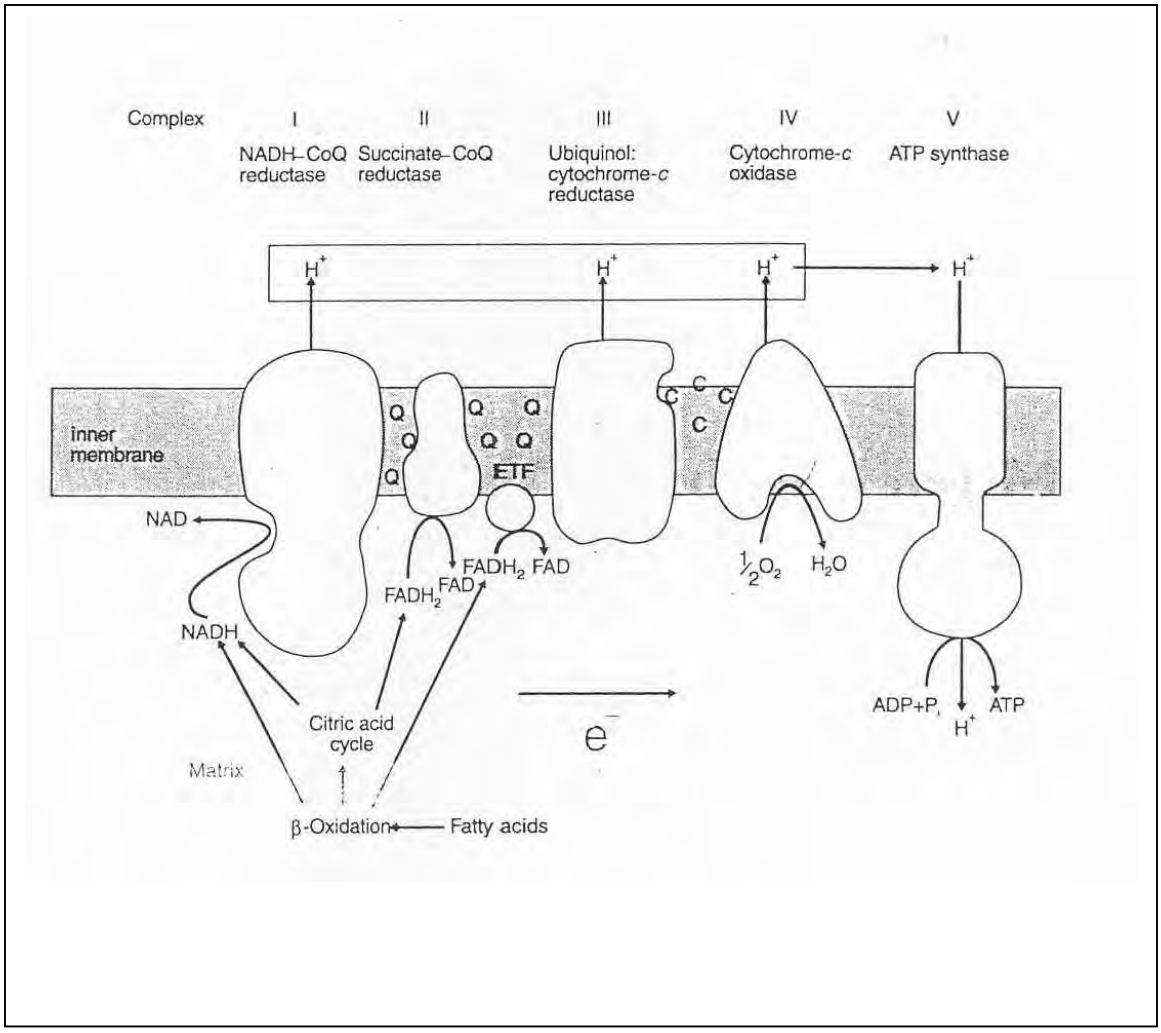
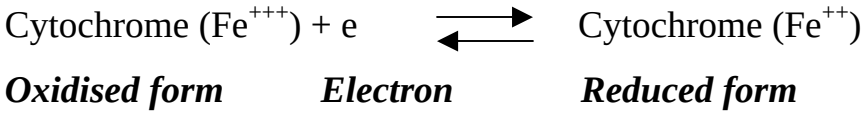


Figure 6: The mitochodrial respiratory chain (Katzung, 1998)

1.2.1.3 Cytochromes

Cytochromes were first discovered by McMunn in 1886, and then rediscovered by Keilin in 1925. The word cytochrome means cellular pigment, and the name is derived from the fact that all living cells show characteristic absorption bands in the visible region of the spectrum due to these iron porphyrin compounds.

The cytochromes are redox catalysts of key importance in cellular respiration. In the mitochondria, the cytochromes act together as a coordinated system for electron transfer. Electrons are transferred by the haem iron, which undergoes a change in valency in the process.



The cytochromes P450 are a group of related enzymes primarily involved in metabolism. Although the enzymes are somewhat related and share some general characteristics, each one is unique and has a distinct role.

Drug interactions involving the cytochrome P450 enzyme system generally result from one of two processes, enzyme inhibition or enzyme induction. Enzyme inhibition usually involves competition with another drug for the enzyme-binding site. This process usually begins with the first dose of the

inhibitor and onset and offset of inhibition correlate with the half-lives of the drugs involved (Dossing et al, 1983).
Enzyme induction occurs when a drug stimulates the synthesis of more enzyme protein, enhancing the enzyme's metabolising capacity.
Cimetidine is a known inhibitor of the cytochrome P450 enzymatic system (Rendiae et al, 1979). Cimetidine inhibits the following enzymes:

CYP2D6

CYP3A

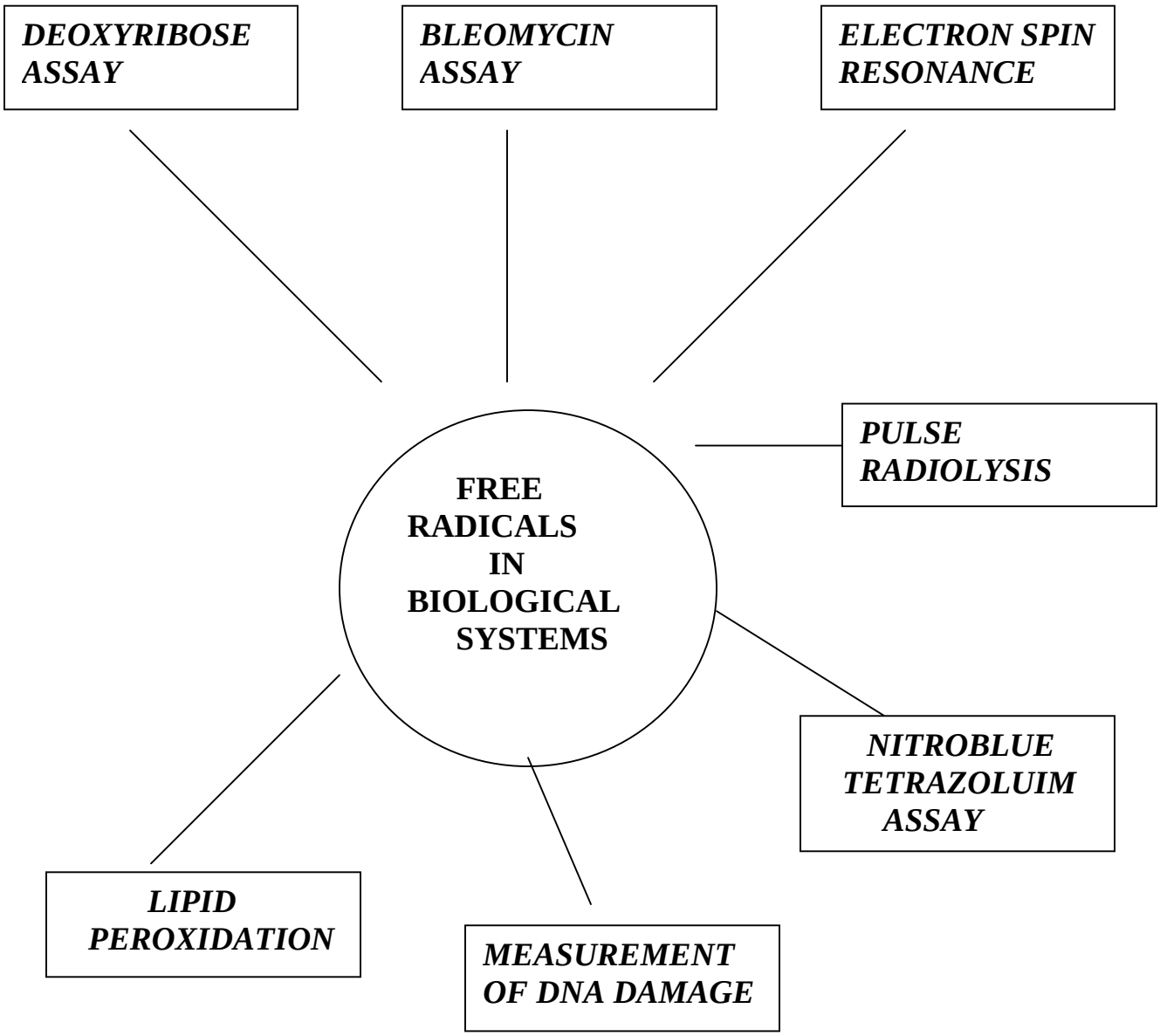
The cytochrome P450 enzymatic system is implicated in the production of propagating and initiating free radicals (Svingen et al, 1979). This production of free radicals is kept to a minimum by the high efficiency of enzyme-mediated electron transfer and by keeping metals ions tightly sequestered; these are the fundamentals of preventative antioxidant defence.

1.2.1.4 Measurement of free radicals

One of the principal problems associated with attempts at antioxidant intervention regimes is that reactive free radicals cannot easily be specifically targeted and scavenged in biological systems.

Lifetimes of free radicals are generally measured in microseconds and are extremely difficult to measure in a clinical situation. Free radical assessment is based on the measurement of products of free radical reactions and therefore it is essential that the role of free radicals in the causation of the disorders and their production as a consequence of disorders be clearly distinguished.

To do this it is necessary to understand the time course of the free radical production and the injury. Due to the problems associated with studying free radicals sensitive and specific methods should be applied to prove the presence of free radicals at the site of the injury (Cheeseman et al, 1993).



1.2.2 OXIDATIVE STRESS

Oxidative stress is due to the formation of reactive oxygen species (ROS). The reactive species derived from oxygen include the superoxide anion radical, hydrogen peroxide and the hydroxyl radical. Oxidative stress results when there is an imbalance between biochemical processes leading to the production of ROS and the cellular antioxidant cascade (Fahn et al, 1992)

Oxygen is vital for the survival of aerobic organisms; however, it is this very oxygen that can lead to their destruction. Aerobic organisms utilize approximately 98% of cellular oxygen at Cytochrome $_{a3}$, which is the terminal cytochrome on the electron transport chain in the mitochondria.

In mitochondria, 1-3 % of molecular oxygen may leak out and it is this small percentage of oxygen that gives rise to ROS. Although, a small amount of oxygen is responsible for producing ROS, this process is continuous and eventually a build up of ROS occurs which can then cause significant damage. This theory of oxidative stress has been used to scientifically explain the ageing process (Kirkwood et al, 1997)

Oxygen can be regarded as a radical as it possesses two unpaired electrons in different orbitals, maintaining a parallel spin. The nature of oxygen having unpaired electrons makes it a powerful oxidizing agent. Oxygen accepts electrons and maintains them in antiparallel spin, in reference to its two unpaired electrons in the given orbitals. Molecular oxygen can accept electrons maintaining them in a spin conversion resulting in O_2

monovalently being reduced to $O_2^{\cdot -}$. In the presence of superoxide dismutase $O_2^{\cdot -}$ can be converted to hydrogen peroxide (H_2O_2).

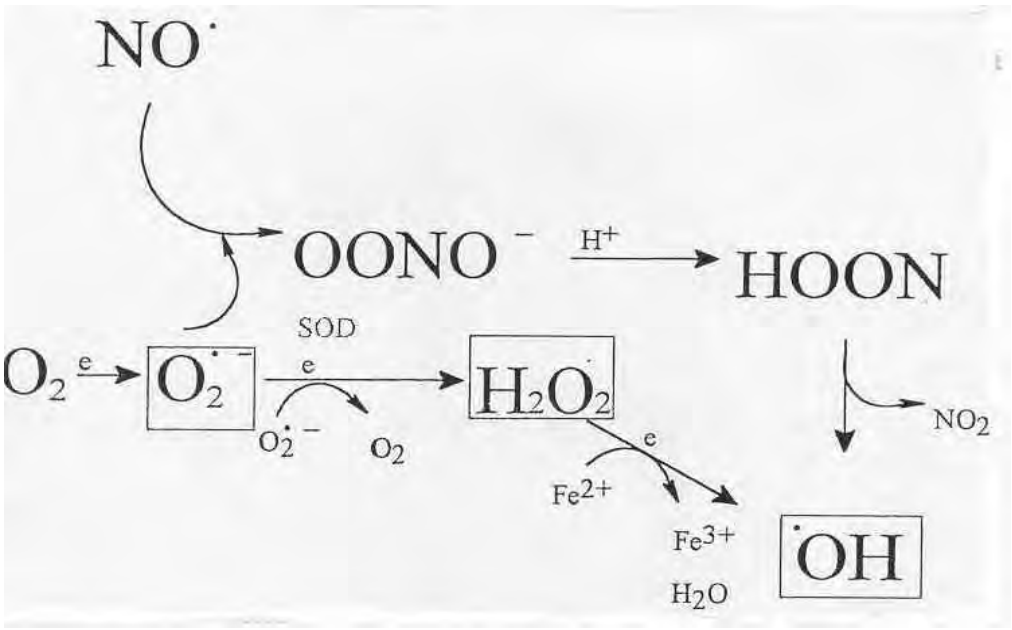


Figure 8: Generation of free radicals and reactive oxygen species from molecular oxygen (Reiter et al, 1994)

1.2.2.1 Superoxide anion

Although the superoxide anion is a free radical, it is not a particularly damaging species. It is mostly reductive in nature and its main significance is probably as a source of hydrogen peroxide and as a reductant of transition metal ions. At low pH values superoxide will protonate to form the

perhydroxyl radical, a more reactive oxidising species but at physiological pH less than 1% will be in the protonated form (Cheeseman et al, 1993).

Superoxide anions are generated enzymatically by a number of oxidases, other than cytochrome oxidase in the mitochondria. These include xanthine oxidase, which is found in the plasmalemma of phagocytic cells (Reiter et al, 1995).

Reduction of oxygen by the transfer of a single electron will produce the superoxide free radical anion:

$$O_2 + e^- \longrightarrow O_2^{-\bullet}$$

The superoxide anion free radicals produced from activated phagocytic cells are used to destroy engulfed bacteria (Curnutte et al, 1987) and tumour cells (Colton et al, 1987). Although, these effects are beneficial excessive activation of phagocytic cells can lead to oxidative stress.

1.2.2.2 Hydrogen peroxide

Hydrogen peroxide is often generated in biological systems via the production of superoxide: two superoxide molecules can react together to form hydrogen peroxide and oxygen.

$$2O_2^{-\bullet} + 2H^+ \longrightarrow H_2O_2 + O_2$$

Although hydrogen peroxide is not a free radical, at high concentrations it can be toxic to cells. Hydrogen peroxide is produced *in vivo* by many reactions. Hydrogen peroxide is unique in that it can be converted to the highly damaging hydroxyl radical or be catalysed and excreted harmlessly as water. Glutathione peroxidase is essential for the conversion of glutathione to oxidized glutathione, during which hydrogen peroxide is converted to water (Allessio el al, 1997). If hydrogen peroxide is not converted into water singlet oxygen is formed.

Singlet oxygen is not a free radical, but can be formed during radical reactions and also cause further reactions. Singlet oxygen violates Hund's rule of electron filling in that it has eight outer electrons existing in pairs leaving one orbital of the same energy level empty.

When oxygen is energetically excited one of the electrons can jump to an empty orbital creating unpaired electrons (Karlsson J, 1997). Singlet oxygen can then transfer the energy to a new molecule and act as a catalyst for free radical formation. The molecule can also interact with other molecules leading to the formation of a new free radical.

Hydrogen peroxide can be reduced further in the presence of transition metals such as iron and copper to the highly toxic hydroxyl radical (Reiter et al, 1995) by a process known as the Fenton Reaction.

1.2.2.3 The Hydroxyl radical

The most reactive free radical known is the hydroxyl radical, which can be formed by X-rays or gamma rays splitting water molecules. The hydroxyl

radical written •OH is so reactive that it takes one billionth of a second to react with neighbouring electrons at diffusion-controlled rates. It therefore will not diffuse a significant distance within a cell before reacting and has an extremely short half-life but is capable of causing great damage within a small radius of production (Buettner et al, 1978).

The hydroxyl radical can be formed from oxygen (O_2) and hydrogen peroxide ($H_2O_{2)}$ via the Harber-Weiss reaction.

$$O_2^{-\bullet} + H_2O_2 \longrightarrow O_2 + \bullet OH + OH^-$$

The interaction of copper or iron and H_2O_2 also produce the •OH as first observed by Fenton (Halliwell et al, 1985), which may be represented by the overall reaction:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + \bullet OH + OH^-$$

All transition metals, with the exception of copper, contain one electron in their outermost shell and can be considered free radicals. Copper has a full outer shell, but loses and gains electrons very easily making itself a free radical (Halliwell et al, 1985). In addition, iron has the ability to gain or lose electrons very easily ($Fe^{2+} \leftrightarrow Fe^{3+}$). This property makes iron and copper two common catalysts of oxidation reactions.

The hydroxyl radical reacts with almost every molecule in living cells, including DNA, which causes changes in the chemical composition of deoxyribose and of the purine and pyrimidine bases. The cytotoxic effect of

H_2O_2 on most mammalian cells seems to involve DNA damage, which can lead to genetic mutations and eventually carcinogenesis.

Another devastating effect of the hydroxyl radical is its effects on membrane lipids. Under normal conditions, the antioxidant defence system within the body can easily handle free radicals that are produced. During times of increased oxygen flux, free radical production may exceed that of removal and ultimately result in lipid peroxidation.

1.2.2.4 Lipid Peroxidation

The liquid-crystalline mosaic model of biological membranes, summarized by Singer and Nicholson (1972), considers the membrane as a “fluid” lipid bilayer into which proteins are inserted. The plasma membrane, being the barrier separating intra- and extracellular environments plays a significant role in the behaviour of cells. In particular, it is involved in regulating physiological processes and features like cell shape, growth rate, cellular recognition, communication, adhesiveness, migration, drug resistance, metastatic spread and immunological competence.

The cell membrane is composed of three chemically different classes of molecules: carbohydrates, lipids and proteins. Carbohydrates are attached covalently either to the lipid or protein molecules and are exposed to the aqueous environment. Lipids are organized into phospholipid bilayers or incorporated in the membrane as distinct structures. Proteins are attached to the lipid fraction with hydrophobic bonds formed between non-polar amino acid residues and acyl chains.

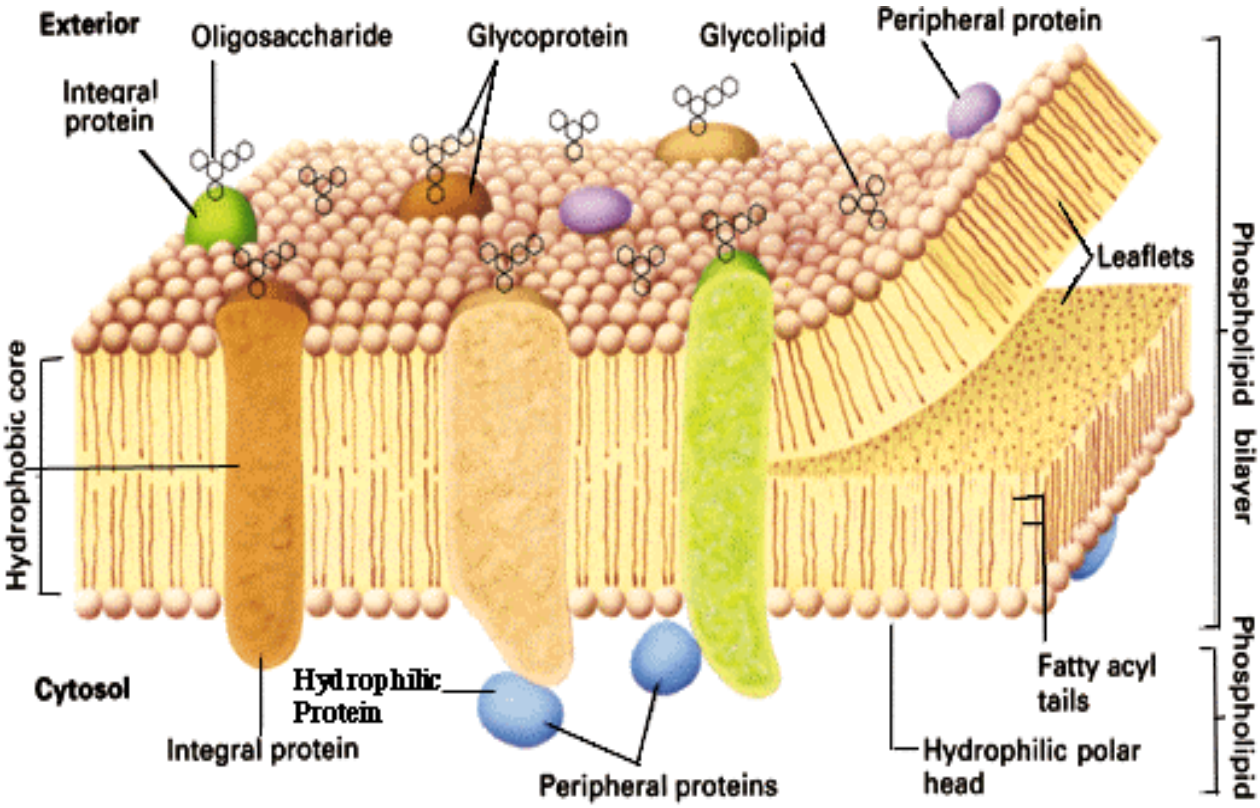


Figure 9: The Fluid Mosaic Model

Lipid peroxidation lowers the fluidity of the mitochondrial membrane with a consequent alteration of the ion flux around it. Studies on lipid peroxidation have suggested that peroxide clusters may be formed as a consequence and these may act as channels across the membrane making it permeable to ions (Bindoli, 1988).

Free radicals are able to cause alterations in membrane integrity ultimately resulting in lipid peroxidation, which is an irreversible reaction and the products of which can be toxic and cause further pathological changes (Barber et al, 1967).

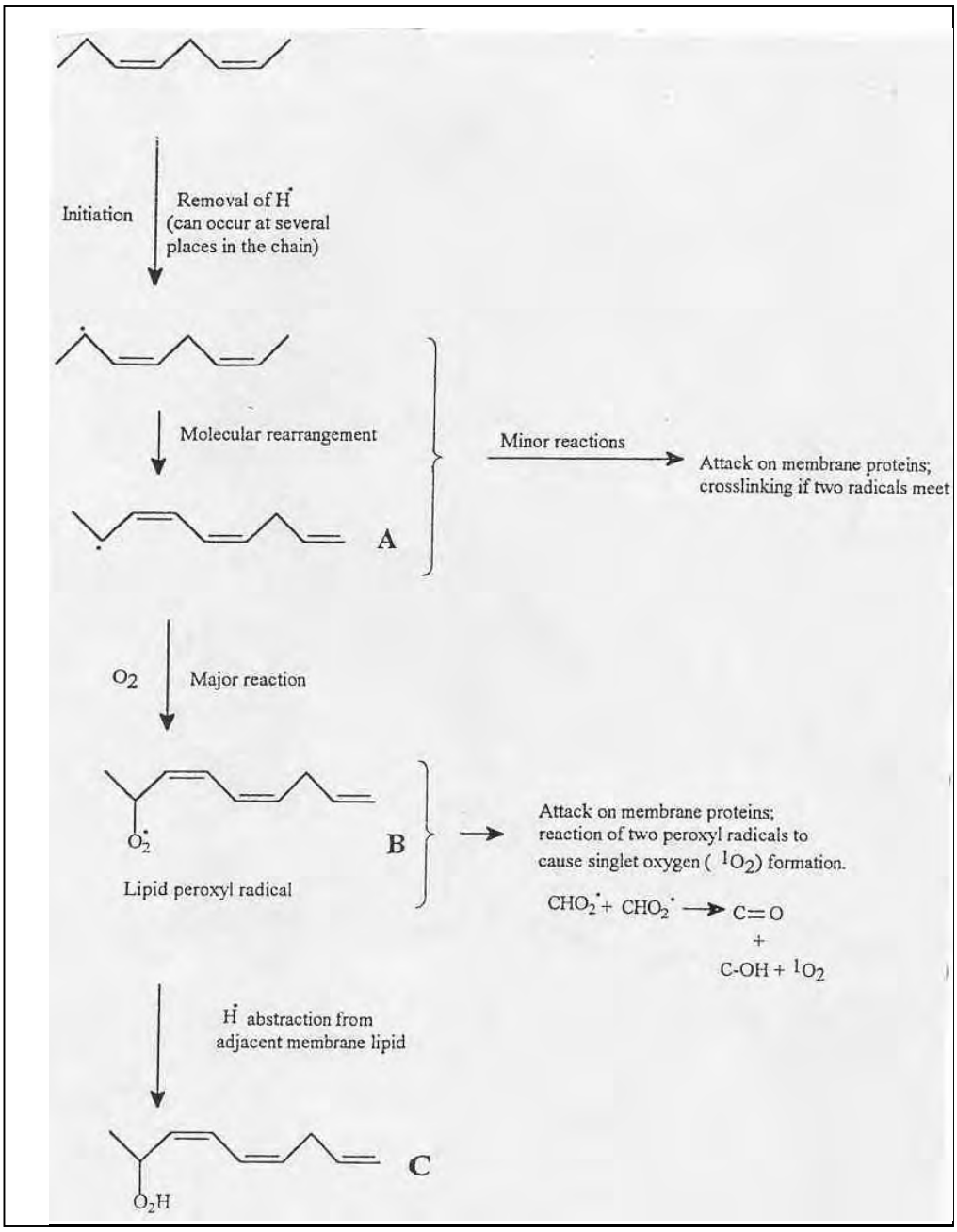


Figure 10: An outline of the mechanism of lipid peroxidation (Gutteridge and Halliwell, 1990)

1.3 FREE RADICALS AND DEGENERATIVE DISEASES

1.3.1 ALZHEIMERS DISEASE (AD)

Alzeihmer's disease is a neurodegenerative illness characterized by a loss of memory and cognitive functions- Alois Alzheimer, 1906.

AD is chronic in nature and has a high incidence in the elderly. In the USA, nearly half of demented patients aged 85 and older suffer from AD.

AD is a neurodegenerative disorder characterized by the presence of extracellular senile plaques and intracellular neurofibrillary tangles in the brain of affected individuals. The characteristics of AD include loss of memory, speech impairment, loss of cognition and memory as a result due to damage to brain regions and neuronal circuits. The neuronal death appears to be selective to cholinergic neurons of the parietal cortex, hippocampus and prefrontal cortex.

Recent research findings have shown that free radicals have been implicated in the cause of AD. Free radicals generated from oxidative mechanisms such as protein oxidation, DNA oxidation and lipid peroxidation play a major role in many diseases of the aged such as AD (Perry et al, 2000).

Oxidative stress leads to formation of oxygen radicals, which in turn attack vital structural components of neurons resulting in neurodegeneration. It has been postulated that the major constituent of senile plaques, β-amyloid

protein is theorized to possess a direct neurotoxic action, which is related to calcium overload and subsequent free radical generation (Daya, 1999).

The brain is particularly vulnerable to lipid peroxidation since membrane lipids in the brain are very rich in polyunsaturated fatty acids (PUFAs) providing the ideal medium for lipid peroxidation (Gutteridge, 1994). The brain also has a low concentration of enzymes which have free radical scavenging properties such as catalase, superoxide dismutase (SOD) and glutathione peroxidase.

1.3.1.1 The role of metals in AD

Metals such as iron, aluminium, copper and zinc are vital for physiological processes in the body and are also present in the brain for normal neuronal functioning. However, these metals can also be toxic to cells at low or elevated levels (Armstrong et al, 2001). At elevated levels the metals can influence cell behaviour by interacting with transduction molecules, causing oxidative stress and energy failure (De Moore et al, 2000). Furthermore, cell membranes and DNA can be damaged, enzyme specificity can be altered and cellular functions disrupted.

Aluminium ions are known to be neurotoxic. Aluminosilicates have been identified at the cores of senile plagues and aluminium has been found within neurons bearing neurofibrillary tangles (Good et al, 1992). The accumulation of aluminium in the brain could be due to a disrupted blood-brain barrier in AD (Halliwell, 1992). In certain circumstances aluminium has been shown to accelerate Fe^{2+} dependent peroxidation, as well as

interfere with cellular iron metabolism allowing for more iron to be available to catalyse free radical reactions (Good et al, 1992).

Sayre et al (1999) carried out a study to investigate the effect of accumulating redox active iron and copper in AD pathology and major producers of ROS. It was found that the metabolites are not only responsible for numerous oxidative stress markers that appear on neurofibrillary tangles and amyloid plaques, but the general oxidative stress observed in AD.
AD has been associated with imbalances between the rate of free radical production and free radical scavenging in the brain.

Recent studies have suggested a role for cimetidine as a free radical scavenger. Cimetidine has efficient antioxidant properties (Uchida et al, 1990) and may be used as possible treatment for AD.

However for cimetidine to be a source of treatment in AD it is necessary for it to penetrate the central nervous system to exert the desired therapeutic effect. To date there are many theories that support cimetidine penetrating the central nervous system.

1.3.1.4 Cimetidine's ability to cross the Blood-Brain Barrier

Borrow et al (1981) carried out a study to determine the effects of cimetidine on sleep, a relatively sensitive method for demonstrating the effects of drugs on the human brain. The mean duration of rapid eye movement (REM) sleep of placebo patients was 109.5 minutes and 95.8 minutes with those patients who were administered with cimetidine ($p<0.05$, two tailed $t=2.3009$). The total duration of sleep, interruption of sleep due to arousal and stages of sleep were unaffected by cimetidine administration and had no significance. An important action of drugs that affect the CNS is a reduction of REM sleep, which occurred with cimetidine (Oswald, 1973).

Nuotto and Matilla conducted a study to investigate whether oral administration of cimetidine would cause central effects in healthy subjects. Psychomotor measurements such as flicker fusion, body sway, choice reaction and tracking were assessed. The results showed that cimetidine improved flicker fusion and reduced body sway but was inactive otherwise on the psychomotor skills.

Mental confusion after administration of cimetidine occurs 24-48 hours after the first dose with signs and symptoms of restlessness, disorientation, agitation and confusion. Giving higher doses of cimetidine can result in seizures, twitching, unresponsiveness, hallucinations and apnoeic attacks (Schentag et al, 1979). The mental changes were related to elevated serum trough concentrations greater that 1.25 μg/ml as well as cerebrospinal fluid concentrations above 0.4 μg/ml.

Not all patients taking an overdose of cimetidine will suffer from central nervous system side effects. Studies conducted by Illingworth and Jarvie (1979) reported that there were no central effects in patients taking cimetidine.

A study conducted by Scott (1982) showed that cimetidine at threshold dosages penetrated the blood-brain barrier and was found to be present in the prefrontal areas of the cerebral cortex, which are those portions of the frontal lobes that lie anterior to the motor regions. Single doses of cimetidine up to 143.0 mg/kg did not induce mental confusion but cumulative doses of cimetidine induced mental confusion provided that the threshold dosage had been surpassed.

From the above studies it can be concluded that cimetidine although water-soluble has the ability to cross the blood brain barrier and can therefore exert its effect in the central nervous system. A report by Totte et al (1981) suggests that the sulphoxide metabolite of cimetidine that is more lipid soluble than cimetidine and thus more likely to penetrate the blood-brain barrier has been found in the CNS in abnormal levels. This could provide an explanation for cimetidine’s ability to penetrate the CNS.

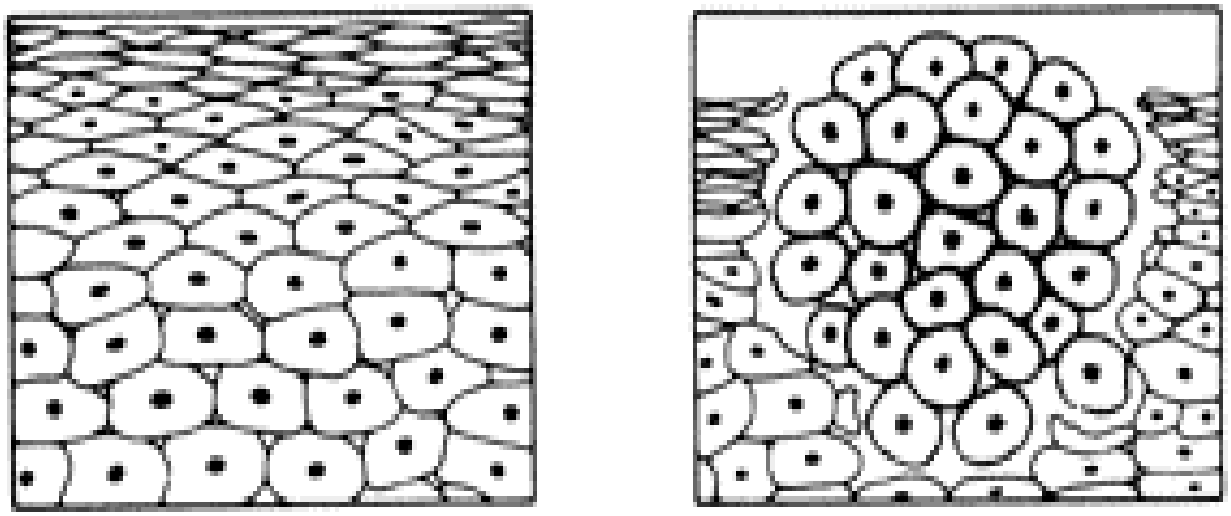
1.3.2 CANCER

Next to heart disease, cancer is the major cause of death in the USA, causing over 500 000 deaths annually (Harris et al, 1993). With present methods of treatment, one third of patients are cured with local measures (surgery or radiation therapy), which are quite effective when the tumour has not metastasised by the time of treatment. Early diagnosis might lead to increased cure of patients with such local treatment. At present, 50% of patients with cancer can be cured, with chemotherapy contributing to cure in about 17% of patients (Katzung, 1998).

Cancer is basically a disease of cells characterized by a shift in the control mechanisms that govern cell differentiation and proliferation. Cancer is a generic term used to refer to the uncontrolled growth of abnormal cells in a number of malignant tumours rather than a single disease.

The mechanism that causes transformation of normal cells to malignant cells is at present unclear. Many agents have been implicated in the transformation process. These can be physical or chemical in nature (Pitot et al, 1991). These include chemical carcinogens, radiation, oncogenic viruses, chronic mechanical trauma, genetic predisposition, chromosomal instability, ineffective DNA repair and the ageing process.

Abnormal proliferating cells may arise in any part of the body and not all tumours are malignant. Tumour cells that remain localized in certain tissues and cannot invade surrounding tissue are referred to as benign tumours and these are non-lethal. The cells that spread from their site of origin through the bloodstream and lymphatic system and destroy surrounding tissue are referred to as malignant tumours and these are fatal.



Normal Cells

Cells forming a tumour

Figure 11: Normal cells compared to malignant cells (Pitot et al, 1991)

The main target of malignancy is DNA, which can be affected in terms of structure or function. The theory of altered DNA causing cancer is central to most theories of carcinogenesis. With new advances in research technology in terms of molecular biology and tissue culture, specific genes responsible for pathogenesis of cancer can be studied and this has led to considerable progress in understanding the stages of tumour development (Friedberg, 1986).

1.3.2.1 Stages of cancer

There are three stages of cancer development: initiation, promotion and progression stages.

Table 2: Stages of cancer

INITIATION	Permanent and hereditable alteration of the cell phenotype occurs. The initiating agents bind to DNA and other macromolecules within the cells. The initiation phase requires a single application of a carcinogen and is dependent on a large extent on cell division and DNA synthesis (Pitot et al, 1991).
PROMOTION	The compounds, which generate free radicals, are promoters of tumours. The promoters stimulate the production of free radicals and decrease the effectiveness of the antioxidant defence system by inhibiting enzymes which have free radical scavenging properties such as catalase, superoxide dismutase (SOD) and glutathione peroxidase.
PROGRESSION	Benign tumour cells rapidly progress to malignant cells. Tumour cells detach from the primary tumour, infiltrate into the blood or lymphatic system and spread further to distant organs. On reaching the organ, tumour cells infiltrate the tissues and begin to proliferate. At this stage, tumour cells have the ability to metastasise and spread from the primary tumour (Roos et al, 1979).

1.3.2.2 Chemical Changes in malignant cells

Biological membranes are essential for cellular life and function to separate tissues from cells, and cells into organelles. This organisation of compartmentalising membranes is necessary for molecules to come together during metabolic reactions as well as balancing the concentration of ions and molecules within the compartments.

Lipid bilayers have numerous properties that are vital for cell survival. The most important is membrane fluidity, which is necessary for the functioning of proteins whose actions depend on mobility within the plane of the membrane (Ahmad et al, 1990).

Mobility of the molecules within the membrane is restrained by mutual interactions. There have been many reports indicating that the fluidity in the membrane increases due to tumour progression (Nicolau et al, 1974; Kier, 1990). Changes in membrane fluidity through physical and chemical perturbation are believed to alter numerous membrane functions such as receptor characteristics, transport and activity of membrane bound enzymes (Spector et al, 1985. Murphy, 1990).

Additional factors which influence the tumour cell membrane mobility is the plasma membrane composition, especially the phospholipid: cholesterol and polyunsaturated: monosaturated phospholipid ratios as well as the presence of lipid domains that alter molecular mobility in tumour cell membrane. An increased level of cholesterol and other lipid metabolites (choline, phosphoryl choline, ethanolamine and glycerophosphocholine) is indicative of fast lipid turnover associated with intense proliferation.

A lower proportion of polyunsaturated fatty acids usually characterizes tumour and activated cells compared to monounsaturated fatty acids (Schroeder et al, 1984; McDanagh et al., 1992). The decrease in fluidity of the tumour membrane causes changes in physical (membrane fluidity) and chemical properties. This effect is believed to be due to the formation of free radicals involving lipid peroxidation (Galeotti et al, 1986).

Numerous studies have suggested that oxidative modification of polyunsaturated fatty acids by lipid peroxidation cause a decrease in membrane fluidity and consequently enhance membrane rigidity (Kaplàn et al, 1995). The peroxidation effect on the membrane is believed to be due to a decrease in the unsaturated: saturated fatty acid ratio, change in the chain length percentage of fatty acids and covalent cross-linking between adjacent lipid radicals formed in membranes (Cutris et al, 1984).

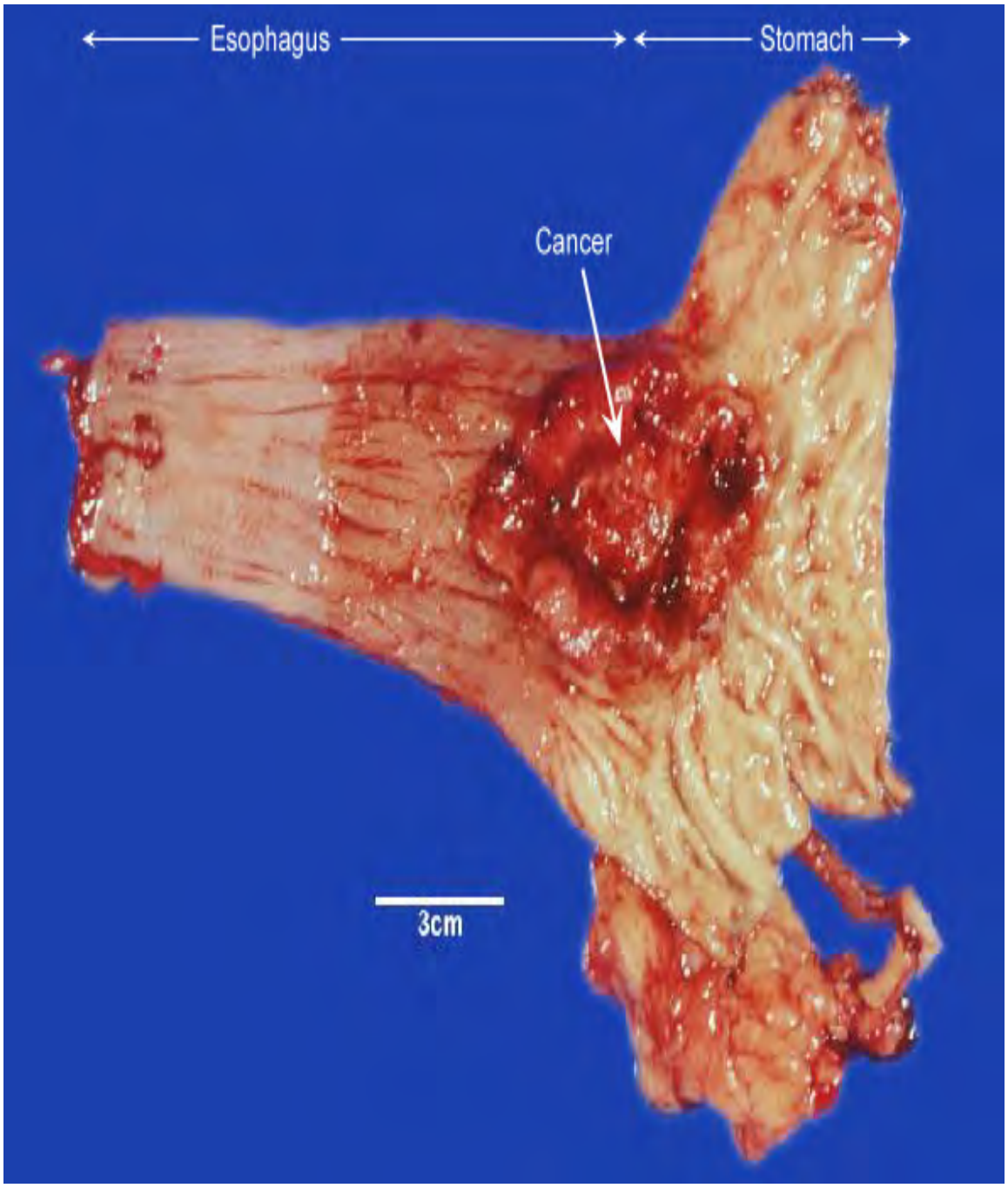


Figure 12:Cancer at the junction between the oesophagus and the stomach

1.3.3 STUDIES ON CANCER RESEARCH USING CIMETIDINE

1.3.3.1 Clinical trials

A study was conducted to investigate the effect of cimetidine on the extent of lymphocytic infiltration of primary colorectal adenocarcinoma. 151 patients were randomized to one of three treatment groups, given cimetidine or placebo control orally for five days prior to colorectal resection. There was a dose dependent trend for the extent of lymphocytic infiltration to increase with cimetidine treatment. The results showed that a short preoperative oral course of cimetidine results in trends towards enhanced lymphocytic infiltration and early survival in colorectal cancer patients (Morris et al, 1999)

A study was carried out in 181 randomised patients with gastric adenocarcinoma. Immediately after surgery the patients were randomized into two groups in a double-blind fashion: one to take cimetidine 400 mg twice daily for two years or until death, the other a placebo. Both groups were reviewed every 3 months. The average survival in the cimetidine group was 450 days compared to 316 days in the placebo group. At the end of each year from a total of five years of follow-up, the cimetidine group had roughly twice the survival rate of the placebo group (Tonenesen et al, 1988).

1.3.3.2 Animal studies

Colon cancer was induced in 40 Sprague Dawley rats using a 10 week course of 1,2 dimethylhydrazine (DMH). Twenty of the rats received cimetidine in their drinking water commencing 5 weeks after concluding the course of DMH. After five weeks of treatment the animals were sacrificed and the colon and rectum were assessed histologically for depth of tumour invasion and inflammatory cell response. The results showed that the control group had marked increase of tumour invasion as compared to the group treated with cimetidine, and therefore cimetidine inhibits colon cancer cellular proliferation as well as slows down tumour invasion (Adams et al, 1993).

Cancer progression in three horses treated with multifocal diseases is halted by cimetidine (Goetz et al, 1990).
In mice cimetidine reduced tumour formation (Gifford et al, 1981) as well as provided successful immunotherapy (Osband et al, 1981).

1.4 RESEARCH OBJECTIVES

The objectives of this study were to determine the effect of cimetidine in the following diseases: AD and Cancer. Since both these diseases have been postulated to be of free radical origin, the free radical scavenging ability of cimetidine was investigated.

To investigate the effect of cimetidine in AD, the rat brain was used as a model and production of free radicals had to be induced by neurotoxins such as cyanide and iron. To investigate the effect of cimetidine in Cancer, a cancer cell line was used as a model. In this study WHCO6 (Wits Human Carcinoma of the Oesophagus) was used.

1.4.1 Proposed experiments

a) Addition of cimetidine on the *in vitro* growth of WHCO6 cancer cells.

b) Determination of the effect of cimetidine on damage induced by superoxide anion free radicals on WHCO6 cancer cells and in cyanide treated rat brain homogenate.

c) Determination of the effect of cimetidine on lipid peroxidation levels on WHCO6 cancer cells. In addition lipid peroxidation levels were assessed in the rat brain homogenate using iron to generate free radicals to initiate lipid peroxidation.

d) Determination of interaction between cimetidine and trace metals using electrochemical, HPLC and UV/Visible studies.

e) Determination of the effect of cimetidine as a hydroxyl radical scavenger using deoxyribose as a substrate.

CHAPTER 2

Growth Studies of WHCO6 Cancer cells

2.1 INTRODUCTION

The number of people at high risk of developing cancer is rapidly increasing. Cancer is now the second major cause of death after cardiovascular diseases in the Western world. Despite significant progress in the area of cancer treatment there is a still a need for new and improved therapeutic strategies (Bertram et al, 1987). There is increased evidence of the importance of early detection and diagnosis of malignant disease in controlling the high incidence and low survival rates of patients with solid tumours (Bertram et al, 1987; Lippman et al, 1993).

Tissue culture has been widely used in studies concerning cancer cell growth. This technique has expanded significantly over the past few years and is now accepted in biological research. The development of continuous cell lines has attained great importance in research.

Since many properties of transformed cells grown in tissue culture resemble those of cancer cells, it is possible to study the factors that lead to uncontrolled growth or an inhibition of growth *in vivo* by the effects they have on tissue culture cells under *in vitro* conditions (Zubay, 1993).

Advantages of tissue culture include allowing one to grow the cells under defined conditions thus eliminating unwanted variables, which complicate matters *in vivo* (Liebler, 1993). In addition, growth of cell lines in simple

media is easy to maintain and has the ability to grow to high cell densities resulting in higher yields by the supplementation of various nutrients even though the cells are no longer organized into tissues (Freshney, 1986). The main concern with tissue culture is that the clonal origin of the cell line can undergo possible mutations resulting in a completely different cell line.

2.2 MATERIALS AND METHODS

MATERIALS

A highly metastatic human oesophageal cell line (WHCO6) was obtained from the Department of Medical Biochemistry, University of Cape Town. Dulbecco's Modified Eagles' Medium (DMEM) was obtained from the Department of Medical Biochemistry, University of Cape Town as well as Highveld Biological Association, South Africa. Cimetidine was purchased from Sigma Chemical Co., USA. Foetal Calf Serum (FCS) was purchased from Highveld Biological Association, South Africa. The haemocytometer was purchased from Neubauer, Germany.

METHODS

2.2.1 PREPARATION OF CULTURE REAGENTS

2.2.1.1 Preparation of cell culture media

Cell lines were grown at 37°C with 5% CO_2 in Dulbecco's Modified Eagles' Medium (DMEM). The medium composition is as follows:

Table 3:Composition of DMEM

COMPONENTS	**Concentration (mg/litre)**
AMINO ACIDS	
L-Arginine HCl	84.0
L-Cysteine	48.0
L-Glutamine	580.0
L-Histidine-HCl-H_20	42.0
L-Isoleucine	105.0
L-Leucine	105.0
L-lysine-HCl	146.0
L-Methionine	30.0
L-Phenylalanine	66.0
L-Threonine	95.0
L-Tryptophan	16.0
L-Tyrosine	72.0
L-Valine	94.0
Glycine	30.0
L-Serine	42.0

VITAMINS	
Choline chloride	4.0
Folic Acid	4.0
I-Inositol	7.2
Nicotinamide	4.0
D-Ca-Pantothenate	4.0
Pyridoxal-HCl	4.0
Riboflavin	0.4
Thiamine-HCl	4.0
Na Pyruvate	110.0
INORGANIC SALTS	
NaCl	6400.0
KCl	400.0
$CaCl_2$	200.0
$MgSO_4 \cdot 7H_20$	200.0
NaH_2PO_4	124.0
$NaHCO_3$	3700.0
$Fe(NO_3)_3 \cdot 9H_20$	0.1
OTHER COMPOUNDS	
D-Glucose	1000.0
Phenol Red	15.0

Preparation of the media was carried out as follows:

Media was filtered by positive filtration using a Falcon 7104 filter. During filtration the initial 20 ml of the medium was discarded while the remainder of the medium was collected into autoclaved Schott bottles. Aseptically dispensed media was then incubated at 37° with 5% CO_2 for one week to test for contamination.

Fig

2.2.1.2 Growth media preparation

Growth media was prepared by adding 10% FCS to the DMEM. This media was further incubated at 37° with 5% CO_2 for 48 hours to check for contamination.

2.2.1.3 Freezing media preparation

Initially, freezing media was prepared by filtering FCS and dimethylsulfoxide (DMSO) through a 0.45μM Millipore filter (Falcon7104) until the media contained 20% FCS and 10% DMSO. However, it was found that the cells did not store well and this could be due to the DMSO being toxic to the cells making it difficult to culture the cell line.

Although not directly toxic, DMSO is a very powerful solvent and is able to penetrate intact skin and where possible use of DMSO for freezing cells should be avoided. Glycerol is preferred in the freezing medium because it prevents or reduces at least the production of sharp edged water crystals that can pierce cell walls. The following freezing media was used:

20% FCS
15% sterile glycerol, which was autoclaved for 30 minutes.
65% DMEM

2.2.1.4 Trypsin preparation

The trypsin solution was prepared in 1litre of milli-Q water:

$Na_2HPO_4.2H_2O$	1.45g
NaCl	8g
KCl	0.2g
KH_2PO_4	0.2g
Trypsin	2.5g
EDTA	1.0g

The trypsin solution was set to a pH of 7.4 and filtered into autoclaved Schott bottles. The trypsin was stored at –20°C until required.

2.2.1.5 Preparation of Phosphate buffered Saline (PBS) solution

PBS was made up in double distilled water and set to a pH of 7.4.To 1litre of double-distilled water was added:

$Na_2HPO_4.2H_2O$	1.45g
NaCl	8g
KH_2PO_4	0.2g
KCl	0.2g

The solution of PBS was then autoclaved.

2.2.2 Routine Cell Culture Procedure

All cell culture procedures were carried out in a vertical laminar flow bench, which was previously sterilized by exposing to ultraviolet light and swabbing of 70% ethanol. Any equipment that was used was either bought sterile, autoclaved or swabbed with 70% ethanol. When not required for experimental purposes the WHCO6 cell line was incubated at 37° in 75 cm^3 culture flasks containing 25 ml of 10%(v/v) FCS supplemented media. During maintenance of the cell lines, growth media was changed on a regular basis (every two days).

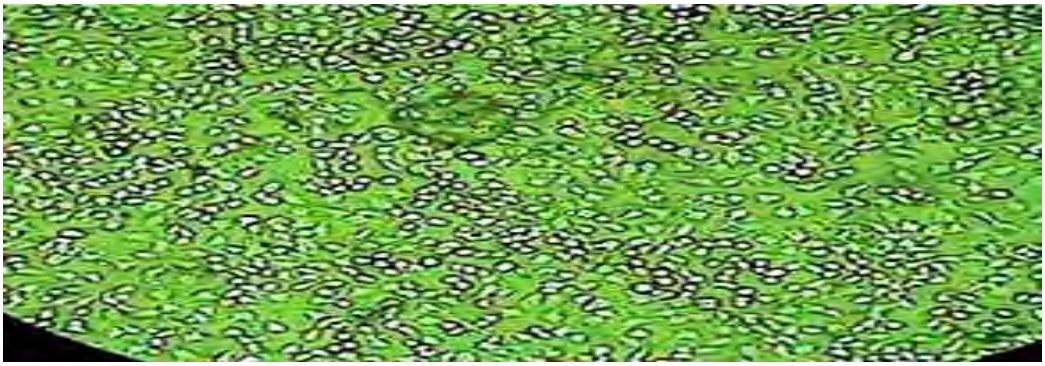
Once the cells were confluent it was vital to prepare stock solutions of the cell line or subculture the cells. The growth media was discarded and 10 ml of trypsin was added to each flask and incubated at 37°C with 5% CO_2 for 4 to 5 minutes. The cells detach from the flask during the incubation period after which the resulting suspension was centrifuged at 500 *g* for 5 minutes to pellet cells.

The supernatant containing the trypsin was discarded and the pellet was resuspended and cells redistributed in PBS to remove any traces of trypsin. The suspension was centrifuged at 500 *g* for 5 minutes, and the resulting pellet was finally suspended in growth medium. An aliquot of the cell suspension was removed (100 μl) and counted using a haemocytometer to give an indication of the number of cells present in the original flask.

The cells were then passaged into sterile flasks or stocks of the cells were made followed by the addition of media containing 10% FCS.

a)

b)



c)

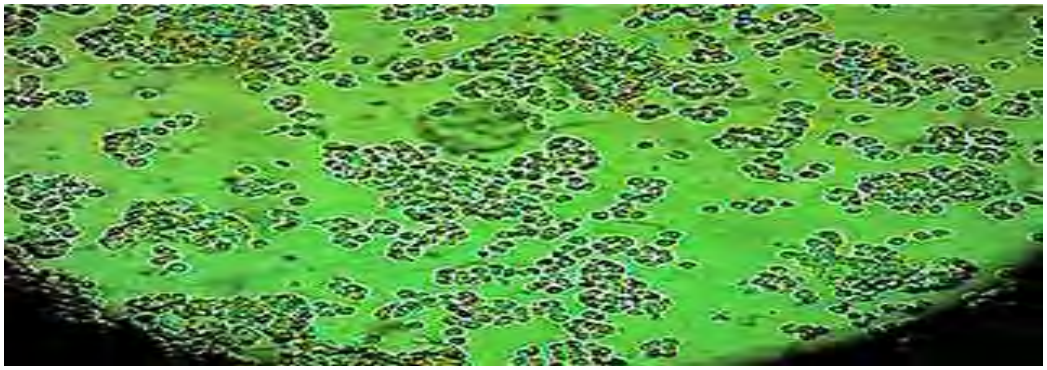


Figure 15:a) The initial stage of trypsinisation (high confluency)

b) Trypsinisation at time t= 2 minutes

c) Trypsinisation at time t= 5 minutes(detachment into single cells)

2.2.2.1 Haemocytometer Counting

Trypan blue was the dye used since it is one of the stains recommended for use in dye exclusion procedures for viable cell counting. This method is based on the principle that live (viable) cells do not take up certain dyes, whereas dead (non-viable) cells do. Staining facilitates the visualization of cell morphology.

Cells were stained 1:1 with trypan blue. 10 µl of the mixture was introduced under the cover slip of the haemocytometer and cells in each of the four chambers were counted. Cell counts were calculated by the following:

Cells per ml = the average count per square x dilution factor x 10^4
Total cells = cells per ml x original volume of fluid from which the sample was removed.

2.2.2.2 Freezing of cells

Once the cells are confluent the cell line may be subcultured or stored in a frozen state and regarded as stock. This is a protective measure in case the subcultured cells do not harvest, possibly due to contamination or in case of experimental error, there is a stock solution and hence the cell line can be cultured again. Cells were harvested and stock solutions were made and stored in cryogenic vials under liquid nitrogen (–70°C) until required.

2.3 THE EFFECT OF CIMETIDINE ON WHCO6 (Human cancer) CELL GROWTH

2.3.1 MATERIALS AND METHODS

The materials described in section 2.2 were used.

2.3.1.1 Cell culture procedure

Upon reaching confluency WHCO6 cells were trypsinised with 10ml of trypsin and centrifuged at 500 *g* for 5 minutes to pellet cells. The supernatant containing the trypsin was discarded, the pellet was resuspended and cells redistributed in PBS to remove any traces of trypsin. The suspension was centrifuged at 500 *g* for 5 minutes, and the resulting pellet was finally suspended in 5mls of growth medium.

Cells were counted using a haemocytometer and the volume of cell suspension required to seed 5 000 000 cells into 75 cm^2 flasks and 2000 000 into 25 cm^2 flasks was calculated.

2.3.1.2 Preparation of experimental medium

A stock solution of cimetidine (1mM) was made up in DMEM containing 10% FCS and dilutions were made to obtain final concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 $x10^{-6}$ M respectively. Controls were prepared which did not contain any cimetidine and the cells were grown in DMEM containing 10% FCS.

2.3.1.3 Growth of cell cultures

Six sets of 6 x 25 cm^2 flasks were seeded with a calculated volume (1ml) to seed 2000 000 WHCO6 cells.

Five sets of the flasks were supplemented with 10mls of growth media containing varying levels (0.2, 0.4,0.6,0.8 and 1.0 $x10^{-6}$M) of cimetidine.

The sixth set of flasks did not contain any cimetidine and the cells were grown in DMEM containing 10% FCS only and these were referred to as the control cultures. The flasks were incubated at 37°C with 5% CO_2 for 3 to 5 days with a media change prior to confluency. The flasks were incubated again and cells were harvested the following day.

2.3.1.5 Harvesting of cell cultures

Upon reaching confluency the cells were trypsinised with 10 ml of trypsin and centrifuged at 500 *g* for 5 minutes to pellet cells. The supernatant containing the trypsin was discarded and the pellet was resuspended and cells redistributed in PBS to remove any traces of trypsin. The cells were counted using a haemocytometer. The cell counts were indicative of cell growth and enabled the effects of cimetidine addition on growth of cells to be determined.

2.4 RESULTS

2.4.1 STATISTICAL ANALYSIS

Results obtained were analysed using a One-Way Analysis of variance (ANOVA) followed by a Student Newman-Keuls Multiple Range Test.

2.4.2 SUPPLEMENTATION OF WHCO6 CANCER CELLS WITH VARYING CONCENTRATIONS OF CIMETIDINE

The results show that supplementation of cimetidine (0.2 up to1.0 $x10^{-6}$ M) has an inhibitory effect on the growth of the WHCO6 cell line. WHCO6 cell growth was significantly ($p \leq 0.05$) inhibited by cimetidine especially at higher concentrations.

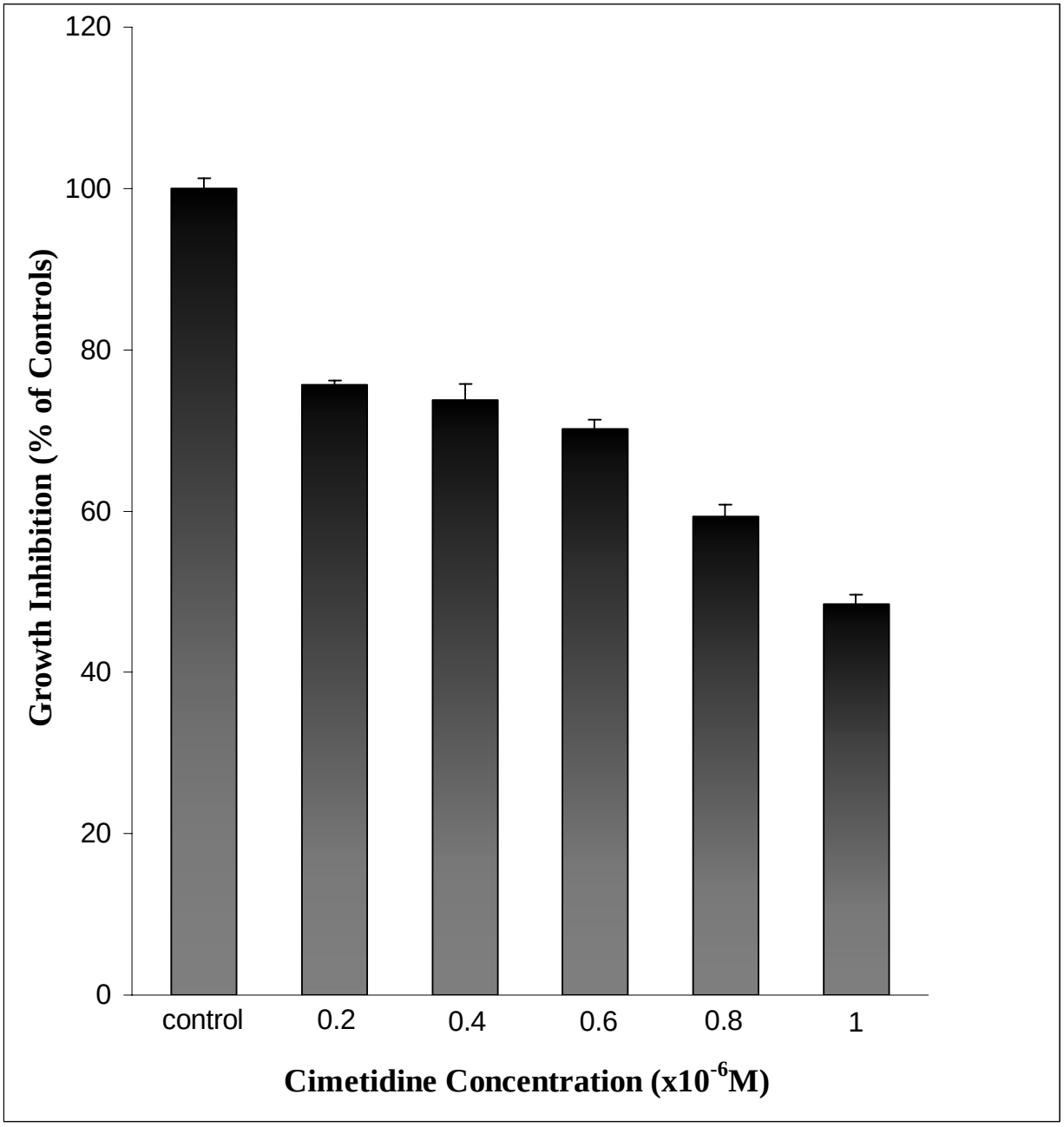


Figure 16: The effect of varying concentrations of cimetidine in WHCO6 cancer cells (Each bar represents the mean of 3 sets of 3 determinations ± SEM).

2.5 DISCUSSION

Cancer cells require free radicals for growth especially in the initiation and promotion stages (Pitot et al, 1991). From the results of the present study it can be concluded that cimetidine significantly inhibits the growth of the WHCO6 oesophageal cancer cells. Previous studies have shown that cimetidine suppresses the growth of cancer cells (Takahashi K et al, 2001.; Hansbrough J et al, 1986).

In this study addition of cimetidine (0.2 up to1.0 $x10^{-6}$ M) to WHCO6 cells with varying concentrations of resulted in a significant inhibition of cell growth, as compared to control cultures. Control experiments were carried out to confirm that the growth inhibition was due to cimetidine and not due to vehicle treatment of media and various supplements to the media.

Cimetidine has been analysed for use in cancer treatment by many researchers, since clinical trials with cimetidine demonstrated the potent effects on activation of tumour immunity and regression of tumour growth (Matsumoto, 1995). There are many theories, which attempt to explain the immunomodulatory effect of cimetidine.

Histamine has been shown to directly stimulate the *in vitro* growth of cancer cell lines (Adams et al, 1994). To a cancer cell, histamine is a source of nutrition and hence histamine promotes the growth of cancer cells. Cimetidine is a H_2-antagonist and antagonizes the stimulatory effects of exogenous histamine. However, whilst cimetidine is capable of antagonizing

the trophic effects of histamine on tumour cell growth *in vitro* (Morris et al, 1997), histamine also has important immune regulating effects.
A number of interrelated potential mechanisms have been proposed to explain the effect of cimetidine on survival in cancer patients.

Furthermore, cimetidine is known not only to be a H_2-antagonist but also a powerful free radical scavenger (Uchida et al, 1990). Numerous tumour initiators have been shown to produce oxygen radicals during their action. In recent years it has become clear that free radicals may be involved in the enzymatic activation of various chemical carcinogens. Substantial evidence has implicated free radicals, particularly those from oxygen, in both the initiation and promotion stages of carcinogenesis (Sun et al, 1990).

Whilst there are many theories that explain the therapeutic role of cimetidine in cancer, the actual mechanism of the role of cimetidine in treatment of cancer is unclear. Regardless of the mechanism of action cimetidine does inhibit tumour growth in this study and has been shown to enhance cell-mediated immunity in cancer patients (Kikuchi et al, 1986), which suggests a role for cimetidine as a possible additive agent in treatment of cancer patients.

CHAPTER 3

Superoxide anion Formation

3.1 INTRODUCTION

In the presence of oxygen, reactive species such as superoxide anion (O_2^-), hydroxyl radical (OH•), organic peroxide radicals, hydrogen peroxide and singlet oxygen are continuously generated intracellularly. If the free radicals continue to react and consequently cause more free radical formation, this can result in damage to macromolecular components like DNA, carbohydrates, proteins and lipids (Di Macsio et al, 1991).

Under normal circumstances, the major source of free radicals in mammalian cells is through electron leakage from the electron transport chains located in the mitochondria and endoplasmic reticulum. Faulty electron transfer at any point in the electron transport chain, results in an electron being accepted by atomic oxygen (O_2). The superoxide anion created (O_2^-) has a single unpaired electron, which seeks to react with an electron source to make a stable electron pair. Under physiological conditions, electrons leak from the electron transport chain, thereby converting about 1-3 % of oxygen molecules into the superoxide anion (Porter et al, 1995).

The current dominant view of the superoxide anion toxicity suggests that this radical damages living cells only indirectly by giving rise to more powerful oxidants such as the hydroxyl radical and hydrogen peroxide (Fridovich, 1986).

DNA damage by oxygen free radicals causes an accelerating degradation of mutation function over time. Accumulating mutations in the genes encoding electron transporters lead to decreased transfer efficiency, which in turn leads to a greater production of superoxide and hydroxyl free radicals.

Mutant mitochondria produce excess free radicals and DNA protein damage and leads to an impairment of the body's natural antioxidant defence systems (Kirkwood et al, 1997). Mutations in ribosomal and transfer RNA lead to inefficient translation of proteins encoded by the mutant genome. Mutations in genes have been implicated in many degenerative diseases like AD and cancer and this concept is termed the "mitochondrial theory of aging"(Dean et al, 1993).

3.2 MATERIALS AND METHODS

MATERIALS

All reagents were of the highest quality available. Nitro-blue tetrazolium (NBT) and Nitroblue diformazan (NBD) were purchased from Sigma (St.Louis, U.S.A).

METHODS

3.2.1 NITROBLUE TETRAZOLIUM ASSAY

This method is generally accepted as a simple and reliable method for assaying the superoxide free radical (Ottino et al, 1997). A modification of the assay used by Ottino and Duncan was used in this set of experiments. The principle of the assay is based on the ability of free radicals to reduce NBT to insoluble diformazan, which can be extracted with glacial acetic acid. The reaction mixture was incubated with 0.4 ml of 0.1% NBT in an oscillating water bath for 1 hour at 37^{o}C. Termination of the assay and extraction of reduced NBD was carried out by centrifugation of the samples at 2000 *g* and resuspension of the pellet in 2 ml glacial acetic acid. The absorbance of the glacial acetic acid containing the NBT extracts were measured at 560 nm using an ultraviolet-visible spectrophotometer and the resulting absorbance was converted to μmol diformazan using a standard curve generated from NBD (Appendix 1).

3.2.2 STATISTICAL ANALYSIS

The results obtained were analysed using a One Way Analysis of Variance (ANOVA), followed by a Student Newman-Keuls Multiple Range test.

3.3 THE ROLE OF CIMETIDINE 0N SUPEROXIDE FORMATION IN WHCO6 CANCER CELLS

3.3.1 INTRODUCTION

Reactive oxygen species have been suggested to play an important role in triggering the transformation of non-malignant to malignant cells (Balasubramaniyan et al, 1994). In recent studies, it has been suggested that reactive oxygen species play an important role in the stimulation of cell division (Oberley et al, 1995). In addition free radical products have been implicated in both the initiation and promotion stages of carcinogenesis (Thompson, 1991). As a result, one would expect tumour cells to have higher free radical levels than non-malignant cells (Balasubramaniyan et al, 1994).

Results from the growth studies (Chapter 2) have shown that addition of cimetidine significantly retards growth of the WHCO6 cell line. The actual mechanism by which growth is inhibited in unclear and whether the effect of cimetidine is due to an antioxidant mechanism. In this study attempts were made to determine the effect of addition of cimetidine on free radical levels in WHCO6 cancer cells.

3.3.2 MATERIALS AND METHODS

MATERIALS

All reagents were of the highest quality available. A highly metastatic human oesophageal cell line (WHCO6) was obtained from the Department of Biochemistry, University of Cape Town. Dulbecco's Modified Eagles' Medium (DMEM) was obtained from the Department of Biochemistry, University of Cape Town as well as Highveld Biological Association, South Africa. Foetal Calf Serum (FCS) was purchased from Highveld Biological Association, South Africa. The haemocytometer was purchased from Neubauer, Germany.

Cimetidine, nitro-blue tetrazolium (NBT), and nitroblue diformazan (NBD) were purchased from Sigma (St.Louis, U.S.A).

METHODS

3.3.2.1 Cell culture procedure

Cells were counted using a haemocytometer and the volume of cell suspension required to seed 5 000 000 cells into 75 cm^2 flasks and 2000 000 into 25 cm^2 flasks was calculated. Upon reaching confluency WHCO6 cells were trypsinised with 10ml of trypsin and centrifuged at 500 *g* for 5 minutes to pellet cells. The supernatant containing the trypsin was discarded and the pellet was resuspended in PBS to remove any traces of trypsin. The suspension was centrifuged at 500 *g* for 5 minutes, and the resulting pellet was finally suspended in 10mls of PBS (pH 7.4).

3.3.2.2 Superoxide anion formation assay

Cell suspensions (1.0ml) containing a standard volume (100 μl) of cimetidine with varying concentrations (0.2, 0.5, 0.8 and 1.0 $x10^{-6}$ M). The assay used was performed as outlined in Section 3.2.1. Final results are expressed as μmol of diformazan/10^5 cells.

3.3.3 RESULTS

Table 4: The effect of cimetidine on superoxide anion formation in WHCO6 cancer cells

Cimetidine Conc. ($x10^{-6}$M)	**Superoxide formation (μmol diformazan / 10^5 cells)**
CONTROL	7.480 ± 0.01864
0.2	6.855 ± 0.02103
0.5	4.110 ± 0.0151
0.8	3.60 ± 0.0105
1.0	2.425 ± 0.01411

The results indicate that there is a significant decrease ($p \leq 0.005$) in superoxide radical formation on addition of cimetidine when compared to control cultures. This occurs especially at higher concentrations of Cimetidine (0.8 and 1.0 $x10^{-6}$M).

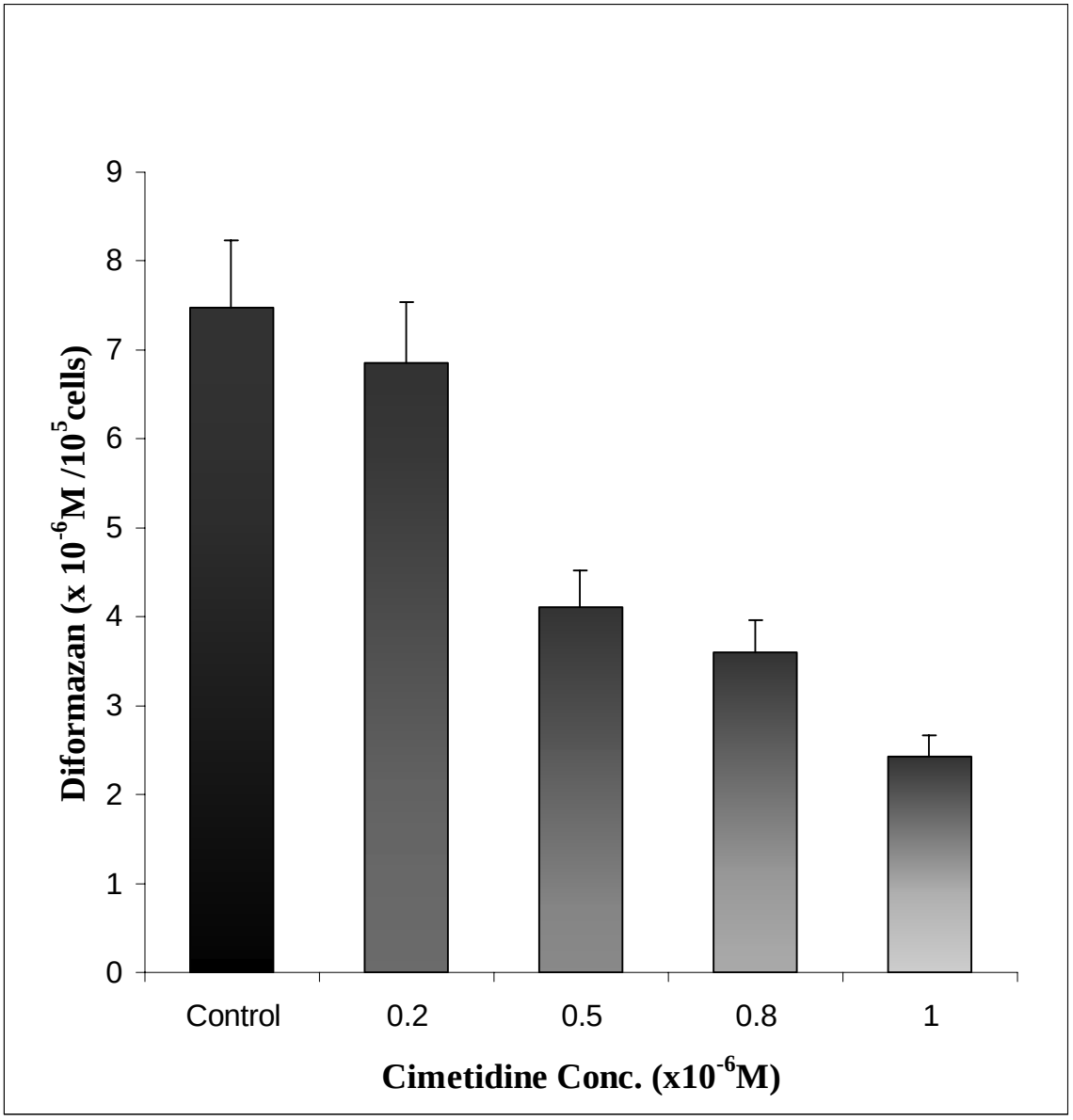


Figure 17: The effect of varying concentrations of cimetidine on superoxide formation inWHCO6 cancer cells (Each bar represents the mean of 6 determinations ± SEM).

3.4 THE ROLE OF CIMETIDINE ON CYANIDE-INDUCED SUPEROXIDE ANION PRODUCTION IN RAT BRAIN HOMOGENATE

3.4.1 INTRODUCTION

The brain is a primary target in cyanide toxicity (Gunasekar et al, 1996). It is believed that cyanide-induced neurotoxicity could be as a result of oxidative stress and inhibition of antioxidant enzymes (Ardelt et al, 1989). The neurotoxicity in the brain induced by cyanide has been attributed to the production of cellular anoxia. Cyanide produces dopaminergic toxicity, characterized by loss of dopaminergic neurons in the basal ganglia, this results in tonic and clonic convulsions and in some individuals a Parkinson-like condition may develop as a result of post toxicity (Ludolph, 1995).

Cyanide treatment on the brain results in increased intracellular calcium, which generates reactive oxygen species leading to cellular damage. Nitric oxide, which is a promoter of hydroxyl radical formation is a mediator of convulsions associated with cyanide toxicity (Yamamoto, 1992).

It has been suggested that cimetidine possesses free radical scavenging properties (Uchida et al, 1990). In this study, attempts were made to determine the effect of cimetidine on cyanide-induced free radical production. A decrease in free radical production would suggest that cyanide-induced neurotoxicity is partly due to free radical generation and hence, supporting the theory of cimetidine as a potential free radical scavenger.

3.4.2 MATERIALS AND METHODS

MATERIALS

Cimetidine, potassium cyanide (KCN), nitro-blue tetrazolium (NBT), and nitroblue diformazan (NBD) were purchased from Sigma (St.Louis, U.S.A).

METHODS

3.4.2.1 Animals and housing conditions

The Rhodes University Ethics Standards Committee for Animal Research approved all the experiments that involved the use of animals. The rats were housed in individual cages, and were maintained as described in Appendix 3.

3.4.2.2 Tissue preparation

The rats were sacrificed at designated times by cervical dislocation and decapitation. Using a pair of scissors, an incision was made through the bone from the foramen magnum to near the orbit of each eye. The top of the skull was lifted using a pair of forceps, exposing the brain. The brain was removed for either immediate use or stored at -70ºC until required.

Prior to homogenisation, tissues that were stored at -70ºC were thawed and weighed at room temperature. The tissue samples were homogenised in a Teflon® coated glass homogeniser for 60 seconds on ice. A 10% w/v homogenate was prepared with 0.1M PBS of pH 7.4.

3.4.2.3 Superoxide anion formation assay

Homogenate (1 ml) containing varying concentrations of KCN (0, 0.5, 1.0mM) alone or in combination with cimetidine (0.5, 1.0 and 2mM) was incubated with 0.4 ml of 0.1% NBT. The assay used was performed as outlined in Section 3.2.1. Final results are expressed as μmol of diformazan/mg tissue.

3.4.3 RESULTS

Table 5: The effect of cimetidine on KCN-induced superoxide anion formation in rat brain homogenate.

Concentrations of KCN and cimetidine used	Superoxide formation (μmol diformazan / mg tissue)
CONTROL	19.631 ± 0.8136
0.5 mM KCN	30.503 ± 0.3207
1.0 mM KCN	34.3855 ± 0.3416
1mM KCN + 0.5mM Cimetidine	29.006 ±0.2842
1mM KCN + 1.0mM Cimetidine	26.197 ± 0.2813
1mM KCN + 2.0mM Cimetidine	23.52 ± 0.0522

Exposure of whole rat brain homogenate to varying concentrations of KCN (0.5 and 1 mM) increased superoxide formation compared to the control. In the presence of cimetidine (0.5 and1 mM), the superoxide formation significantly ($p \leq 0.05$) decreased as shown in Figure 18.

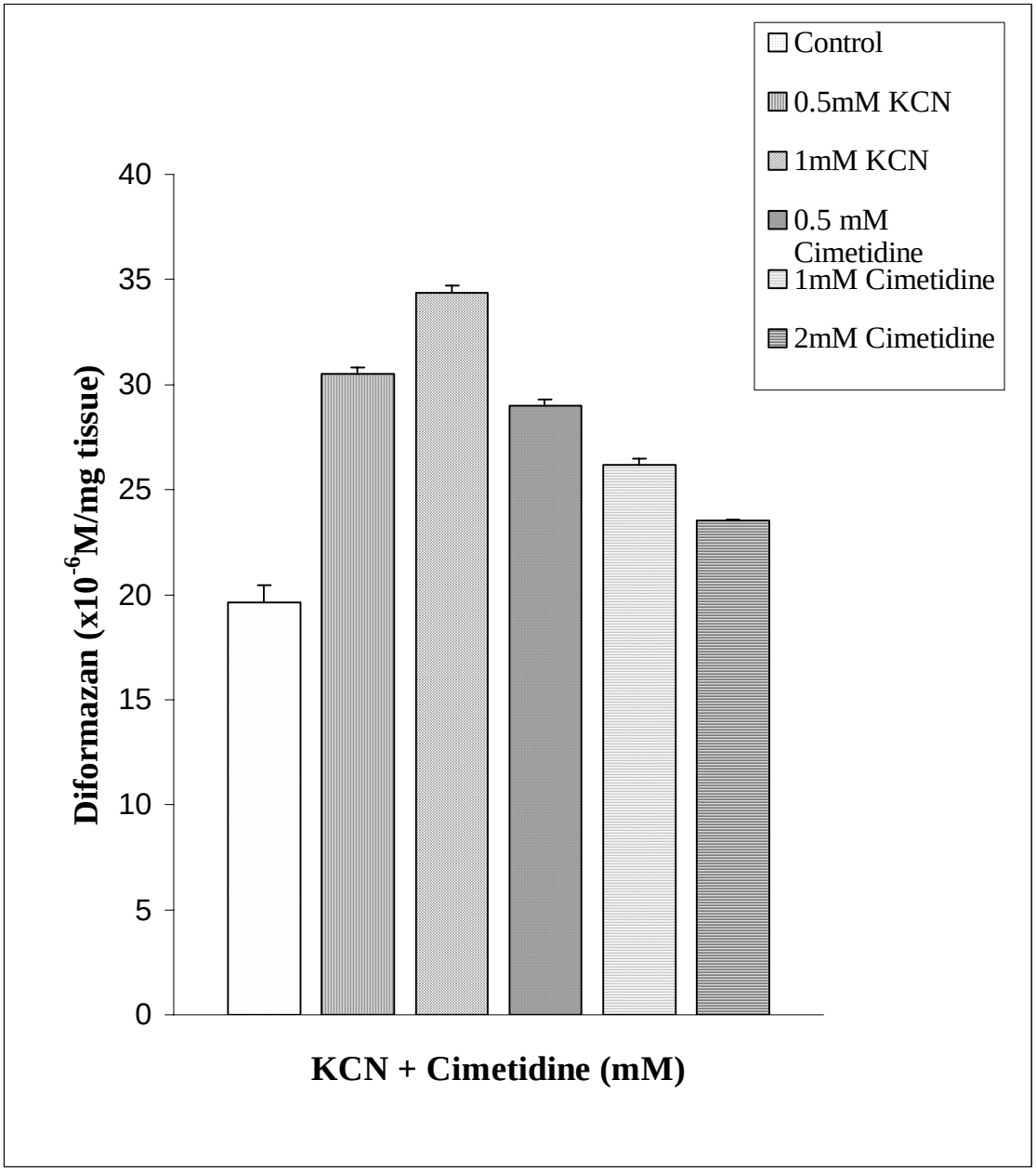


Figure 18: The effect of cimetidine on KCN- induced superoxide anion formation in rat brain homogenate (Each bar represents the mean of 6 determinations ± SEM).

3.5 DISCUSSION

The results of the present study indicate that cimetidine markedly reduces superoxide formation in malignant cells as well as in cyanide treated rat brain homogenate. Free radical reactions are part of normal human metabolism. When produced in excess, radicals can cause tissue injury. However, tissue injury can itself cause more radical reactions, resulting in an increase of the damage initially caused (Halliwell, 1992).

In tumour cells there were already high levels of superoxide formation that were subsequently reduced on addition of cimetidine, especially at higher concentrations. In rat brain homogenate the production of free radical formation had to be induced by cyanide, hence suprapharmacological concentrations of cimetidine had to be used. Potassium cyanide causes neuronal damage in the brain and therefore was used to generate the superoxide free radicals.

Results from the previous study on the growth of cell cultures of cancer cells indicate that cimetidine retards growth of such cells. Although the exact mechanism of action of cimetidine on such cells is unknown, the theory of cimetidine acting as a free radical scavenger is much more emphasized. Since, free radicals are implicated in degenerative diseases, the use of cimetidine in such cases would be of beneficial use. From the results, it can be concluded that cimetidine exhibits both neuroprotective and chemoprotective effects, thus having possible beneficial therapeutic role in disease of free radical origin.

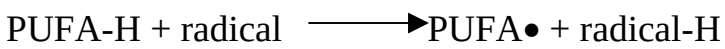
CHAPTER 4

Lipid Peroxidation

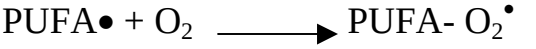
4.1 INTRODUCTION

Kohn and Liversedge first introduced the thiobarbituric acid test in 1944. The test was used as a measure of lipid rancidity in the food industry. Kohn and Liversedge described the colorimetric reaction of TBA with an unknown substance formed during aerobic incubation of tissue homogenates. It was later found that the unknown substance was the secondary product of lipid peroxidation and identified to be malondialdehyde (MDA) by Patton and Kurtz. This method has been widely used in biological systems to assess lipid peroxidation (Halliwell et al, 1985).

It has been considered that lipid peroxidation has been involved in many aging and pathological disorders due to oxidative damage (Okhawa et al, 1979). The lipid peroxidation process can be represented as follows: the reactive species that are generated during the Fenton reaction initiate lipid peroxidation by abstracting a hydrogen atom from a polyunsaturated fatty acid (PUFA) chain in a membrane lipid leaving behind a carbon-centered radical in the membrane (PUFA•)



The carbon-centered radical react with O_2 to form peroxyl radicals:



Peroxyl radicals can attack membrane proteins and can abstract hydrogen atoms from additional fatty acid chains:

PUFA- O_2 + PUFA-H $\longrightarrow$ PUFA- O_2H + PUFA•

Therefore, abstraction of a single hydrogen can set off a free radical chain reaction, that leads to the conversion of many membrane lipids into lipid hydroperoxides (lipid- O_2H), which are destructive to the membrane in terms of function (Halliwell, 1992).

The most widely used index of lipid peroxidation is MDA formation, which is assayed by the thiobarbituric acid (TBA) assay. The lipid material is heated at low pH with trichloroacetic acid (TCA). One molecule of MDA reacts with two molecules of TBA resulting in the formation of a TBA-MDA complex as well as protein-bound MDA. The complex is a pink chromatogen, and has an absorption maximum of 532nm (Mead et al, 1986)

Figure 19: The reaction of MDA with TBA (Mead et al, 1986)

4.2 MATERIALS AND METHODS

MATERIALS

All reagents were of the highest quality available. 1, 1, 3, 3-tetramethoxypropane (98%), butylated hydroxytoluene (BHT), and 2-thiobarbituric acid (98%) (TBA) were purchased from Sigma (St.Louis, U.S.A). Trichloroacetic acid (TCA) was purchased from Saarchem (Krugersdorp, South Africa). IsoluteJ C_{18} solid phase extraction (SPE) columns were obtained from International Sorbent Technology, Mid Glamorgan, UK.

METHODS

4.2.1 LIPID PEROXIDATION ASSAY

The method used in this experiment was a modification of the method used by Anoopkumar-Dukie *et al*., 2001. The reaction mixture was incubated in an oscillating water bath for 1 hour at 37°C. At the end of the incubation period, 0.5ml BHT (0.5mg/ml in methanol) and 1ml TCA (15% in water) were added to the mixture. The tubes were then placed in a hot water bath maintained at 80°C for up to 20 minutes to release protein-bound MDA. To avoid adsorption of MDA onto insoluble protein, the samples were cooled and centrifuged at 2000g for 15 minutes.

Following centrifugation, 1 ml of the protein free supernatant was removed from each tube and a 1 ml aliquot of TBA (0.33% in water) was added to this fraction. The tubes were sealed and incubated in a boiling water bath at acidic pH for 30 minutes.

After cooling, TBA-MDA was separated from other possible interfering thiobarbituric acid-reactive substances (TBA-RS) using a IsoluteJ C_{18} solid phase extraction (SPE) column that was pre-washed with 2 ml of methanol followed by 2 ml distilled water. The sample (1ml) was loaded onto the column that was subsequently washed with 2 ml distilled water. The TBA-MDA complex was eluted with 1 ml methanol.

The methanol was then evaporated using an N-EVAP analytical evaporator at 65^{o}C under a gentle stream of nitrogen. The residue was dissolved in distilled water (0.5 ml) containing 0.1 mg/ml resorcinol. These samples were analysed by HPLC as described above. The MDA levels were obtained from a calibration curve generated using 1, 1, 3, 3- tetramethoxypropane in the same way described above (appendix 2).

The ratio of the peak height of TBA-MDA to the peak height of resorcinol (external standard) was plotted against the concentration of MDA in the complex injected.

4.2.2 STATISTICAL ANALYSIS

The results obtained were analysed using a One Way Analysis of Variance (ANOVA), followed by a Student Newman-Keuls Multiple Range test.

4.3 THE ROLE OF CIMETIDINE IN FREE RADICAL FORMATION AND LIPID PEROXIDATION IN WHCO6 CANCER CELLS

4.3.1 INTRODUCTION

At present the basic assumption appears to be that free radicals mediate disturbances that may play an important role in triggering the transformation of non-malignant to malignant cells (Balasubramaniyan et al, 1994). Substantial evidence has implicated free radicals; particularly those derived from oxygen, in both the initiation and promotion stages of carcinogenesis (Slater et al, 1991). Although evidence at present suggests a link between the generation of free radical species and tumour initiation and promotion, the molecular mechanisms involved in alteration of a normal cell to a malignant cell are unclear.

One potential mechanism by which free radicals may cause malignancy is by targeting DNA and subsequently modifying phenotypic expression. Damage to DNA by free radicals causes mutations that may be carcinogenic (Witz, 1991; Troll et al, 1985).

Reactive oxygen species are continuously generated intracellularly in aerobic organisms (Oberley et al, 1995), and if allowed to react uncontrollably are capable of causing extensive damage to macromolecular components within the cells such as DNA, carbohydrates, proteins and lipids (Diplock, 1991).

In normal cells, highly effective antioxidant mechanisms have been developed to protect against attack by free radicals and lipid peroxidation (Cheeseman et al, 1993). These protective mechanisms or antioxidant defences can be classified into two main categories: Enzymatic and non-enzymatic cellular antioxidants. Enzymatic defence mechanisms include the superoxide dismutase, catalase and glutathione peroxidase enzymes, and the non-enzymatic cellular antioxidants include the minerals selenium, copper, zinc and magnesium, and the vitamins A, E and C (Packer, 1991. Di Macsio et al, 1991).

These defence mechanisms under normal circumstances provide adequate protection from free radical attack and lipid peroxidation. However, the possibility exists that the effectiveness of the defence mechanisms may be decreased or completely overwhelmed when the cells become malignant (Horton et al, 1987). Tumour cells are believed to have defective enzymatic antioxidant defences (Oberley et al, 1995). As a result, one would expect tumour cells to have high free radical and lipid peroxidation levels. Results from the growth studies (Chapter 2) show that cimetidine significantly decreases the growth of the WHCO6 oesophageal cancer cells.

Since it is unclear whether the growth inhibitory effects of cimetidine was due to the histamine antagonizing effect or due free radical scavenging properties (Uchida et al, 1990), attempts were made to determine the effect of cimetidine on lipid peroxidation levels in WHCO6 cancer cells.

4.3.2 MATERIALS AND METHODS

MATERIALS

All reagents were of the highest quality available. Highly metastatic human oesophageal cell line (WHCO6) was obtained from the Department of Biochemistry, University of Cape Town. Dulbecco's Modified Eagles' Medium (DMEM) was obtained from the Department of Biochemistry, University of Cape Town as well as Highveld Biological Association, South Africa. Foetal Calf Serum (FCS) was purchased from Highveld Biological Association, South Africa. The haemocytometer was purchased from Neubauer, Germany.

Cimetidine, resorcinol, 1, 1, 3, 3- tetramethoxypropane (98%), butylated hydroxytoluene (BHT), and 2-thiobarbituric acid (98%) (TBA) were purchased from Sigma (St.Louis, U.S.A). Trichloroacetic acid (TCA) was purchased from Saarchem (Krugersdorp, South Africa). IsoluteJ C_{18} solid phase extraction (SPE) columns were obtained from International Sorbent Technology, Mid Glamorgan, UK.

METHODS

4.3.2.1 Cell culture procedure

Cells were counted using a haemocytometer and the volume of cell suspension required to seed 5 000 000 cells into 75 cm^2 flasks and 2000 000 into 25 cm^2flasks was calculated. Upon reaching confluency WHCO6 cells were trypsinised with 10ml of trypsin and centrifuged at 500 *g* for 5 minutes to pellet cells. The supernatant containing the trypsin was discarded and the pellet was resuspended in PBS to remove any traces of trypsin. The suspension was centrifuged at 500 *g* for 5 minutes, and the resulting pellet was finally suspended in 10mls of PBS (pH 7.4).

4.3.2.2 Lipid peroxidation assay

Cell suspensions (1.0 ml) containing a standard volume (100 μl) of cimetidine with varying concentrations (0.2, 0.5, 0.8 and 1.0 $x10^{-6}$M). The assay used was performed as outlined in Section 4.2.1. Final results are expressed as nmoles of MDA/10^5 cells.

4.3.3 RESULTS

Table 6: The effect of cimetidine on lipid peroxidation in WHCO6 cancer cells

Cimetidine Conc. ($x10^{-6}$M)	**LIPID PEROXIDATION (nmol MDA / 10^5 cells)**
CONTROL	4.591 ± 0.2147
0.2	3.776 ± 0.1721
0.5	3.5984 ± 0.116
0.8	3.3264 ± 0.1791
1.0	3.2614 ± 0.1561

The results indicate that there was a significant decrease ($p \leq 0.05$) in lipid peroxidation levels on addition of cimetidine when compared to control cultures.

This was seen especially at higher concentrations of cimetidine (0.8 $x10^{-6}$ M and 1.0 $x10^{-6}$ M).

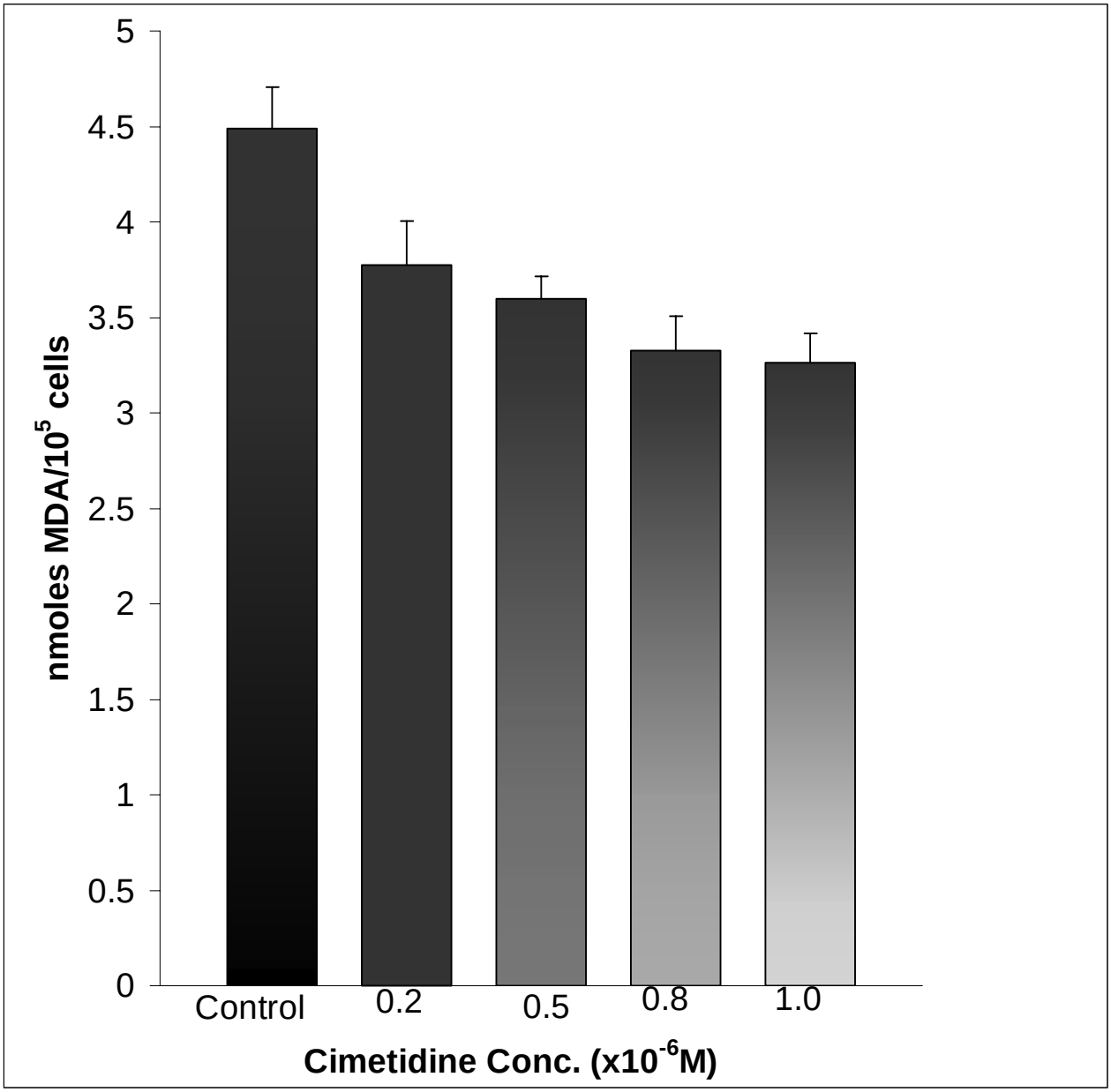


Figure 20: The effect of varying concentrations of cimetidine on lipid peroxidation in WHCO6 cancer cells (Each bar represents the mean of 6 determinations ± SEM).

4.4 THE ROLE OF CIMETIDINE IN IRON-INDUCED LIPID PEROXIDATION IN RAT BRAIN HOMEGENATE

4.4.1 INTRODUCTION

Transition metals promote lipid peroxidation in two ways:

1) Catalyse the formation of oxygen free radical species capable of initiating lipid peroxidation.
2) Catalysing the decomposition of preformed lipid peroxides to propagate lipid peroxidation. (Rikans et al, 1997).

In AD, most studies have shown that iron and ferritin levels are significantly elevated in the cortical grey matter regions, producing free radical species for lipid peroxidation via the Fenton reaction (Pitchumoni et al, 1998.), thus supporting a possible causative role for iron in AD.

In this study, iron was used to generate free radicals since the role of iron on free radical and lipid peroxidation reactions has been studied extensively and its role in this regard is widely accepted (Fahn et al, 1992). Iron is responsible for the decomposition of lipid peroxides to peroxy radicals, which can cause further lipid peroxidation.

4.4.2 MATERIALS AND METHODS

MATERIALS

All reagents were of the highest quality available. Cimetidine, resorcinol, 1, 1, 3, 3- tetramethoxypropane (98%), butylated hydroxytoluene (BHT), and 2-thiobarbituric acid (98%) (TBA) were purchased from Sigma (St.Louis, U.S.A). Iron sulphate was purchased from BDH Laboratory Supplies, Poole, England. Trichloroacetic acid (TCA), Ascorbic acid, Fe_2SO_4, FeCl3, $CuSO_4$ and EDTA were purchased from Saarchem (Krugersdorp, South Africa). IsoluteJ C_{18} solid phase extraction (SPE) columns were obtained from International Sorbent Technology, Mid Glamorgan, UK.

4.4.2.1 Animals and housing conditions

The Rhodes University Ethics Standards Committee for Animal Research approved all the experiments that involved the use of animals. The rats were housed in individual cages, and were maintained as described in Appendix 3.

4.4.2.2 Tissue preparation

The rats were sacrificed at designated times by cervical dislocation and decapitation. Using a pair of scissors, an incision was made through the bone from the foramen magnum to near the orbit of each eye. The top of the skull was lifted using a pair of forceps, exposing the brain. The brain was removed for either immediate use or stored at -70ºC until required.

Prior to homogenisation, tissue that was stored at -70ºC was thawed and weighed at room temperature. The tissue samples were homogenised in a

Teflon® coated glass homogeniser for 60 seconds on ice. A 10% w/v homogenate was prepared with 0.1M PBS of pH 7.4.

4.4.2.3 Lipid peroxidation assay

A modification of the lipid peroxidation method was used. In a final volume of 1ml containing the following reagents iron sulphate (5 mM), EDTA (100 μM), H_2O_2 (2.8 mM), cimetidine (0.0, 0.5 and 1 mM), and ascorbate (100 μM), which were added in the sequence listed.

The reaction mixture was incubated in an oscillating water bath for 1 hour at 37°C. The experiment was continued as outlined in Section 4.2.1. Final results are expressed as nmoles/mg tissue.

4.4.3 RESULTS

Table 7: The effect of cimetidine on iron-induced lipid peroxidation in rat brain homogenate.

Concentrations used of iron and cimetidine	LIPID PEROXIDATION (nmol MDA / mg tissue)
CONTROL	2.933 ± 0.4423
5mM IRON	5.685 ± 0.541
5mM IRON + 0.5mM Cimetidine	2.55 ± 0.1506
5mM IRON + 1.0mM Cimetidine	2.213 ± 0.2501

Exposure of whole rat brain homogenate to iron sulphate (5mM) increased lipid peroxidation compared to the control.

In the presence of cimetidine (0.5 and1mM), the iron induced lipid peroxidation significantly decreased ($p \leq 0.05$) as shown in Figure 21.

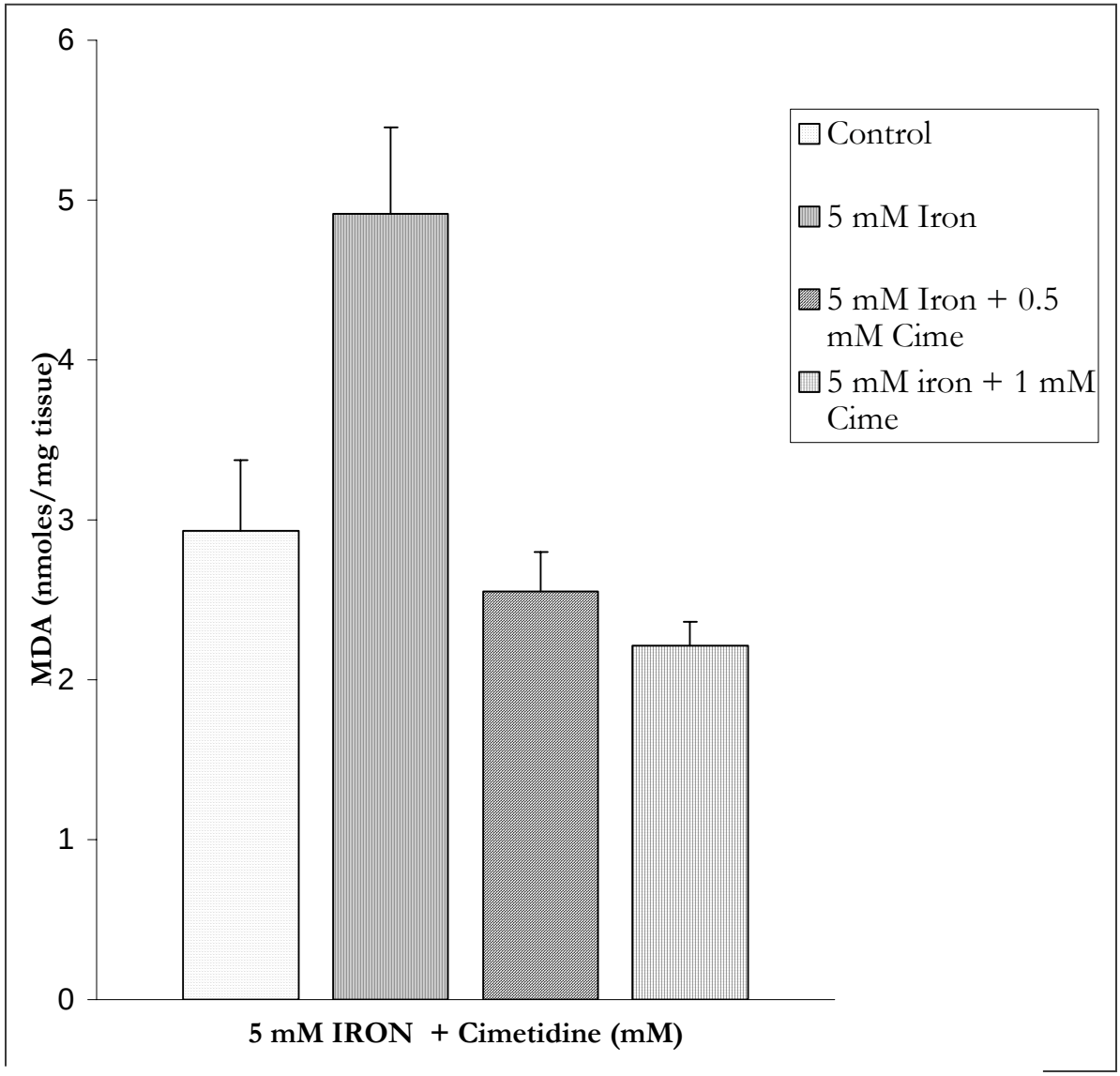


Figure 21:The effect of iron-induced lipid peroxidation in rat brain homogenate

(Each bar represents the mean of 4 determinations ± SEM)

4.5 DISCUSSION

Elevated levels of lipid peroxidation are present in diseases such as Alzheimer's disease and cancer due to oxidative modification of the membrane (PUFA). Steric hindrance occurs and as a result the bilayer membrane becomes very rigid due to restriction of movement of molecules (Choi et al, 1995).

From the results obtained it can be concluded that cimetidine reduces lipid peroxidation in both the cancer cells and the rat brain homogenate. The actual mechanism by which cimetidine quenches free radicals and thereby prevents lipid peroxidation is unclear, although it has been suggested that cimetidine acts as a hydroxyl radical scavenger (Uchida et al, 1990).

A number of abnormalities in lipid metabolism have been observed in both tumour cells and cultured cells following transformation by oncogenic substances (Bailey et al, 1977).

Cimetidine is known to be a potent cytochrome P-450 inhibitor. The cytochrome P-450 electron transport chain is an enzyme system that is concerned with metabolism in the body. This enzymatic system has been implicated in the propagation and initiation of free radicals and consequently lipid peroxidation (Svingen et al, 1979).

Cancer is a neoplastic multistage process and malignant cells are rapidly dividing and hence the cytochrome P-450 enzyme system would be more active in tumour cells than in a normal cell. This results in enhanced lipid peroxidation that was observed with the WHCO6 cancer cells in comparison to the control culture cells in the current study. A possible explanation for the results obtained could be due to cimetidine inhibiting cytochrome P-450 and hence reducing free radical damage and therefore lipid peroxidation as seen in this study.

The use of iron to generate free radicals, in particular the hydroxyl radical caused extensive damage to cell membranes and caused an increase in lipid peroxidation as compared to the control set of experiments. On addition of cimetidine the extent of lipid peroxidation was significantly reduced and this could be due to possibly a mechanism by which cimetidine is binding the iron making it unavailable to cause further damage due to free radical formation.

It had been suggested that cimetidine binds to transition metals and forms complexes. Copper and cimetidine form a complex that possesses a superoxide dismutase (SOD) like activity. SOD possesses antioxidant effects since it is an enzyme that removes superoxide radicals. Hence, the copper-cimetidine complex exhibiting a strong SOD-like activity (Kimura et al) can be beneficial for future therapeutic treatment.

This concept of cimetidine binding metals to form complexes was investigated further in the next chapter.

CHAPTER 5

Interaction of cimetidine and transition metals

5.1 INTRODUCTION

Biological oxidation occurs in the electron transport chain located in the mitochondria (Veis at al, 1993).

Addition of Oxygen:

$CH_3CHO + ½\ O_2 \longrightarrow CH_3COOH$

Acetaldehyde **Acetic acid**

Removal of Hydrogen:

CH (OH) COOH **CO· COOH**

$+2H^+$

CH_2COOH CH_2COOH

Malic acid Oxaloacetic acid

Removal of Electrons:

Cytochrome (Fe^{2+}) ⟶ Cytochrome (Fe^{3+}) + Electron

Figure 22: Methods of biological oxidation (Plummer, 1971)

Metallochemical reactions can be the underlying cause of free radical formation leading to disease. Highly reactive and biologically reactive hydroxyl radicals can be formed in biological tissue when suitable transition metals are available. The role of iron in the generation of free radicals has been well documented (Halliwell, 1992). Both copper, iron and redox-active metal ions are able to promote the formation of ROS within humans (Bush, 2000).

Metals such as iron, copper, zinc and manganese in brain tissue are present in biological tissue in concentrations that are sufficient to damage or deregulate many proteins and metabolic systems by promoting free radical formation.

In the previous chapter cimetidine reduced lipid peroxidation induced by iron. The mechanism by which cimetidine exerts its action was unclear. Due to the fact that iron is involved in free radical production as well as in the electron transport chain, an attempt was made to investigate the interaction of cimetidine with transition metals. In addition it was determined whether the mechanism of a possible antioxidant effect of cimetidine is due to a metal interaction forming complexes between transition metals and cimetidine, hence reducing the amount of free radicals being generated.

5.2 MATERIALS AND METHODS

Studies were conducted using electrochemistry, UV/VIS spectrophotometry and High performance Liquid Chromatography (HPLC).

5.2.1 ELECTROCHEMISTRY

In electrochemistry, a three-electrode system is used, which comprises of the working electrode, a reference electrode and an auxillary electrode.

5.2.1.1 The working electrode

The working electrode is constructed of a material which has a large potential range, low electrical resistance, easily reproducible surface and non-destructible under a range of conditions. In this study, glassy carbon electrodes were used. These have excellent mechanical and electrical properties, a wide potential window, chemically inert and exhibit reproducible performance. However, the transfer compared to other electrodes (Platinum, gold and mercury) is much more reduced with the glassy carbon electrodes (Phips, 1974)

5.2.1.2 The reference electrode

The standard hydrogen electrode (SHE) is universal for determining the half-cell potentials for many oxidation- reduction reactions. In this case the silver/silver chloride electrode was used. This consists of a silver wire anodised with silver chloride in a glass tube.

5.2.1.3 The auxillary electrode

Usually constructed of an inert metal such as platinum in the form of a wire, loop or foil. Electrochemistry involves passing a current between working electrode and the auxillary electrode.

5.2.1.4 Cyclic Voltammetry

This method is used to characterize species in solution. Electroactive analysis is carried out to produce characteristic redox patterns when a potential window is scanned. A change in potential is indicative of new species in solution.

Cyclic and stripping voltammograms were obtained with the Bio Analytical Systems (BAS) CV-50W voltammetric analyser using a BAS C2 cell stand to maintain constant atmosphere. A 3mm diameter glassy carbon electrode (GCE) was employed as a working electrode for voltammetric experiments.

A silver/silver chloride ([KCl = 3 M]) and a platinum wire were employed as reference and auxiliary electrodes, respectively, in all electrochemical work. Prior to use, the glassy carbon electrode was cleaned by polishing with alumina on a Buehler pad, followed by washing in nitric acid and rinsing in water and the buffer solution. Between scans, the GCE was cleaned by immersion in a dilute acid solution and rinsed with water.

For cyclic voltammetric experiments, appropriate concentrations of the metal and cimetidine in buffer were introduced into a glass cell and degassed for 5 min with nitrogen before scanning a potential window.

5.2.1.5 Adsorptive voltammetry

This method is used to examine metal-ligand complex formation at an electrode. The technique is dependent upon the tendency of analytes to preconcentrate at the surface of a working electrode. The introduction of a ligand into a metal solution causes an increase of the preconcentration of the metal at the electrode if it forms a metal-ligand complex. The formation of this complex causes an increase in the current response due to metal reduction. A shift in the reduction potential indicates that a new species is being reduced.

For adsorptive stripping experiments appropriate concentrations of the metal (Fe^{2+} or Fe^{3+}) and of the ligand (cimetidine) were introduced into an electrochemical cell. The electrolyte used was pH 7.3 Tris-HCl buffer for Fe^{3+} and pH 3.5 citric acid buffer for Fe^{2+} The solution was then deaerated with nitrogen for 5 min, after which a flow of nitrogen was maintained over the solution throughout the measurement.

Optimum deposition potential of 0.10 V or 0.15 V vs Ag/AgCl was applied for 60 s to effect the formation and adsorption of the metal and ligand species onto the glassy carbon electrode. The voltammograms were then scanned in the negative direction from the deposition potential to –0.6 V vs Ag/AgCl at the scan rate of 0.1 V s^{-1} to strip the adsorbed metal-ligand species from the electrode. During the stripping step, current response due to the reduction of the metal –ligand species were measured as a function of potential. All potential values quoted are referenced against the silver/silver chloride reference electrode.

5.2.2 UV/VIS STUDIES

The UV/Visible spectra were monitored with a Cary 500 UV/VIS/NIR spectrophotometer. Concentrations of each metal were kept constant (1mg/ml) and spectra measured on subsequent additions of cimetidine (1mg/ml) at different concentrations. Spectra were scanned immediately after addition of cimetidine solution to the metal solutions from a wavelength of 190-800 nm.

5.2.3 HPLC ANALYSIS

Samples were analysed on a modular, isocratic high performance liquid chromatographic (HPLC) system. The chromatographic system used consisted of a Spectraphysics Iso Chrom LC Pump, a Linear UVIS 200 Detector, and a Rikadenki Recorder. Samples were introduced into the system using a Rheodyne fixed loop injector, fitted with a 20μl loop. A mobile phase of 40: 60, acetonitrile: water was used. The retention time (5minutes) and the peak height of cimetidine (1mM) was measured alone as well as with subsequent additions with the metals Fe_2SO_4, $FeCl_3$, $CuSO_4$ (1mM).

5.2.4 STATISTICAL ANALYSIS

The results were analysed using a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test. The level of significance was accepted at $p<0.05$ (ZAR, 1974). A $p< 0,05$ between groups was accepted as being statistically significant.

5.3 RESULTS

5.3.1 ELECTROCHEMISTRY

Cimetidine is electrochemically inactive at a bare glassy carbon electrode, meaning that no redox waves are observed for this compound. However, adsorptive stripping voltammetry (ASV) and cyclic voltammetry (CV) produce reproducible oxidation and reduction waves for copper and iron species in solution. By monitoring the changes of the redox patterns for the metal species in the presence of cimetidine, the interaction between these metals and the ligand could be examined.

Figure 23: a) Anodic stripping voltammogram (ASV) for Fe^{3+} alone in solution ($1x10^{-5}$ M)

b) ASV for Fe^{3+} in the presence of $1x10^{-5}$ M cimetidine

c) ASV for Fe^{3+} in the presence of 2 $x10^{-5}$ M cimetidine

Figure 24: a) Cyclic Voltammogram (CV) of Cu^{2+} (5×10^{-5}M) in solution b)c)d) CV OF Cu^{2+} in the presence of 2×10^{-5}M, 6×10^{-5}M and 8×10^{-5}M cimetidine, respectively.

Figure 25: a) CV of Cu^{2+} alone(5×10^{-5}M)

b) CV of Cu^{2+} and cimetidine (7×10^{-5}M) after third scan

Figure 23a shows the ASV for Fe^{3+} alone at a potential of –0.47 V vs Ag/AgCl. In the presence of increasing concentrations of cimetidine, figure 23b and c show a strong increase in the current response for Fe^{3+}. At the higher concentration of 2.0×10^{-5} M cimetidine, a potential shift to –0.49 V is observed. Theoretically, an increase in the observed current and a potential shift are strong indicators that the ligand (in this case cimetidine) forms a bond with the metal, facilitating its transport to the electrode and hence bringing about an increase in current response. A potential shift is a strong indication that a new species is being reduced, in this instance, a cimetidine – Fe^{3+} complex as opposed to just the Fe^{3+} alone.

For studies with Fe^{2+}, it was necessary to work at low pH to avoid the oxidation of Fe^{2+} to Fe^{3+}. A very similar response was observed for Fe^{2+} in the presence of cimetidine as compared to Fe^{3+}, suggesting a bond between $Fe^{2+}$and cimetidine. However, in the presence of cimetidine, the Fe^{2+}could be immediately oxidized to Fe^{3+}, as the colour change to yellow suggests. It is thus likely that in these studies with Fe^{2+} that cimetidine binds Fe^{3+} present in solution.

Reproducible adsorptive stripping voltammograms for Cu^{2+} were not obtained, and therefore cyclic voltammetry was used to gauge the interaction between Cu^{2+} and cimetidine. Figure 24a shows the CV for Cu^{2+} alone in solution, at a potential of –0.11V vs Ag/AgCl. In the presence of increasing concentrations of cimetidine, the Cu^{2+} peak decreases and shifts to more positive potentials. Upon multiple scans of a solution of Cu^{2+} and cimetidine, it becomes apparent that a new complex is being formed. For comparison, figure 25a shows the CV of Cu^{2+} alone. Figure 25b shows the

CV after the third scan, clearly showing a peak shift for Cu^{2+} from –0.11 V to –0.076V, while a new oxidation wave at 0.10V is clearly visible. It is suggested that the copper peak decreases as it is being used to produce the Cu^{2+} -cimetidine complex visible at 0.10V in figure 25b.

5.3.2 UV/VIS. STUDIES

Table 4:The major bands observed for Cu^{2+} and cimetidine

Peak	**Wavelength (nm)**
Cu^{2+}	200; 212.9
Cu^{2+} + Cimetidine (1:2)	224; 332; 372
Cu^{2+} + Cimetidine (1:3)	224; 337; 378
Cu^{2+} + Cimetidine (1:5)	337; 378

The major band observed for Cu^{2+} was at 200nm. This band shifted to 224nm in the presence of cimetidine. Two new bands are observed on addition of cimetidine at 338 and 378nm. All bands increased in absorbance with an increase in cimetidine concentration. These shifts and the new bands strongly suggest an interaction between Cu^{2+} + Cimetidine. A distinct colour change was observed from light blue to jade for the copper solution in the presence cimetidine.

Table 5:The major bands observed for Fe^{3+} and cimetidine

Peak	**Wavelength (nm)**
Fe^{3+}	218, 234, 251, 266, 290, 330.
Fe^{3+} + Cimetidine (1:2)	365
Fe^{3+} + Cimetidine (1:3)	294, 311, 367
Fe^{3+} + Cimetidine (1:5)	294, 338,370

Fe^{3+} showed distinct bands at a range of wavelengths: 218; 234; 251; 290; 330; 366. All of these bands shift in the presence of cimetidine. At a ratio of 1:3, Fe^{3+}: cimetidine, a new band at 311 was observed. For Fe^{3+}: cimetidine in a ratio of 1:5, bands at 338nm and 370nm were observed, similar to those observed for Cu^{2+} and cimetidine.

Table 6:The major bands observed for Fe^{2+} and cimetidine

Peak	**Wavelength (nm)**
Fe^{2+}	289.7
Fe^{2+} + Cimetidine (1:2)	201; 235; 370.8
Fe^{2+} + Cimetidine (1:3)	206; 236; 378.5
Fe^{2+} + Cimetidine (1:5)	201; 216; 239; 378

The spectra obtained for the interaction between ferrous sulphate and cimetidine showed a peak for Fe^{2+} alone at 289.7 nm. In the presence of cimetidine, new bands at 206, 216 and 235nm, similar to those observed for Fe^{3+} alone. On addition of cimetidine a colour change for Fe^{2+} was observed

from pale blue/green to yellow, which darkened with time and increasing concentrations of cimetidine. This colour change in addition to the similarity between the bands with Fe^{3+} suggests that the cimetidine oxidizes the Fe^{2+} to Fe^{3+}.

5.3.3 HPLC ANALYSIS

The wavelength for analysis was 228nm for cimetidine. Cimetidine was released at a retention time of 5 minutes. The peak height was 164mm.
In the presence of Fe^{2+}, the peak height for cimetidine decreased to 82mm. With Fe^{3+}, a decrease in the peak height of the cimetidine peak to 91mm was observed along with a new peak with a retention time of 2 minutes and a peak height of 56mm.

Similarly for Cu^{2+}, a decrease in the peak height of the cimetidine peak to 78mm was observed, also with the appearance of a new peak with a retention time of 2 minutes and a peak height of 15mm. The peak with retention time of 2 minutes observed for both Fe^{3+} and Cu^{2+} strongly supports the theory of a cimetidine-metal complex with these metals.

5.4 DISCUSSION

Studies gauging the interaction of cimetidine with the metals Fe^{2+}, Cu^{2+} and Fe^{3+}, strongly confirm that cimetidine binds certain metals. Electrochemical studies show that both Cu^{2+} and Fe^{3+} form bonds with cimetidine. HPLC confirms this with the presence of a new band for cimetidine in presence of both Cu^{2+} and Fe^{3+}. UV/VIS studies corroborate these findings for Cu^{2+} and Fe^{3+}. Along with a colour change for Fe^{2+} in the presence of cimetidine and the similarity of the bands observed for Fe^{2+} and cimetidine with Fe^{3+}, it is suggested that cimetidine oxidizes Fe^{2+} to Fe^{3+}. It is postulated by the presence of similar bands observed at 338nm and ~ 370nm for Fe^{3+} and Cu^{2+}, that once cimetidine oxidises Fe^{2+}, it binds both Fe^{3+} and Cu^{2+}.

In addition it is commonly known that cimetidine inhibits certain Cytochrome P450 enzymes. Since the cytochrome p450 enzyme system has been implicated in producing free radicals (Svingen et al, 1979), the inhibition of such a system will consequently cause a decrease in free radical production.

These findings raise interesting questions concerning the manner in which cimetidine acts to reduce free radical damage and lipid peroxidation. It is suggested that cimetidine acts by binding Fe^{3+} and Cu^{2+} in a non-toxic form, thereby preventing these metals from generating free radicals. By oxidizing Fe^{2+} it is suggested that cimetidine mops up toxic free Fe^{2+} and binds it in a non-toxic form as Fe^{3+}- cimetidine complex.

In conclusion, since some free radical production is inevitable in neuronal cells, and lipid peroxidation is the major consequence of free radical action, antioxidant defence mechanisms have evolved to protect such cells from extensive damage (Uchida et al, 1990). Whether the mechanism of action of cimetidine is due to cytochrome p450 enzyme inhibition or metal binding, this study shows that cimetidine can be used to enhance the antioxidant defence mechanism. Cimetidine can be acting as a free radical scavenger or acting by preventing formation of free radicals.

CHAPTER 6
The effect of Cimetidine on Free radical formation using Deoxyribose as a substrate

6.1 INTRODUCTION

The deoxyribose sugar of the DNA backbone has 5 carbons and 3 oxygens. The hydroxyl groups link with the phosphate groups to form the DNA backbone. Deoxyribose lacks a hydroxyl group when compared to ribose, the sugar component of RNA.

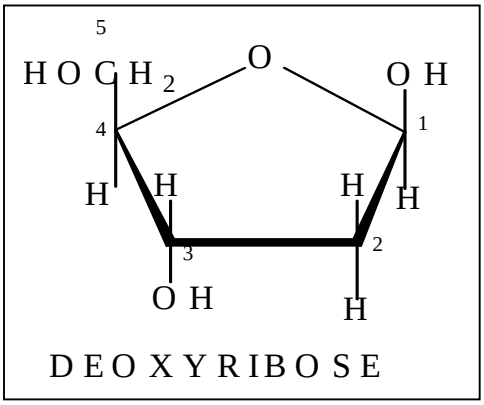


Figure 26: The structure of Deoxyribose

Oxidative damage to DNA is a result of interaction of DNA with free radical species, especially the hydroxyl radical. Superoxide anion and hydrogen peroxide are not normally reactive towards DNA. However, in the presence of metals such as iron and copper by means of the Fenton reaction these can be converted to the highly damaging hydroxyl radical as discussed in Chapter 5.

The hydroxyl radical can produce a variety of modifications in DNA. Oxidative attack by the hydroxyl radical on the deoxyribose moiety will lead to the release of free bases from DNA generating strand breaks with various sugar modifications. The hydroxyl radical reacts with all types of biologically important molecules and can cause changes in DNA repair, replication and transcription that can lead to mutations.

Figure 27: The areas where free radicals attack on DNA (Aust et al, 1985)

Free radicals attack all positions of the deoxyribose sugar, leading to single and double strand breaks in DNA. The free radicals can cause deamination which lead to point mutations. Deamination produces a mispair and replication produces two products:

G-C —*deamination*→ G-U

G-U —*DNA replication*→ G-C AND A-U

A-U —*DNA replication*→ A-T

The high mutation rate can occur especially in the mitochondrial genome as it is located in close proximity to the respiratory chain in the mitochondria where there is an increased amount of potent mutagens namely free radicals due to faulty electron transfer (Kirkwood, 1997).

The deoxyribose assay can be used to calculate the rate constant for the reaction of a scavenger molecule with the hydroxyl radical. The previous reliable method to measure rate constants was determined by pulse radiolysis (Gutteridge et al, 1981). In a situation where the use of pulse radiolysis is unavailable, it is still possible to determine rate constants between hydroxyl radicals and a test compound, as the results are similar when using the deoxyribose assay.

The deoxyribose assay may be considered an alternative to using pulse radiolysis, as it is a simple, cheap and reliable method to determine rate constants using deoxyribose as a substrate.

The hydroxyl radical formed in the reaction between iron (III)-EDTA and H_2O_2 in the presence of ascorbic acid attacks 2-deoxyribose (DR) to form products that on heating with thiobarbituric acid (TBA), at low pH to yield a pink chromogen, which can be measured using ultra-violet spectrometry.

$OH^{\bullet}$ + Deoxyribose $\rightarrow$ fragment $\rightarrow$ MDA

2TBA + MDA $\rightarrow$ chromogen

The hydroxyl radical that escapes scavenging will become available to attack the 2-deoxyribose molecule, and the cimetidine present in the reaction mixture will compete for the hydroxyl radical and thereby reduce the extent of damage caused to the deoxyribose hence providing a protective effect towards DNA.

The deoxyribose assay can be used to calculate the rate constant for the reaction of a scavenger molecule, in this case cimetidine with the $OH^{\bullet}$.

6.2 MATERIALS AND METHODS

MATERIALS

All reagents were of the highest quality available. Cimetidine, 2-deoxyribose and 2-thiobarbituric acid (TBA) were purchased from Sigma (St.Louis, U.S.A). Trichloroacetic acid (TCA), Ascorbic acid, $FeCl_3$ and EDTA were purchased from Saarchem (Krugersdorp, South Africa).

METHODS

6.2.1 DEOXYRIBOSE ASSAY

Arouma et al describe the method used in this study. Two different concentrations of 2-deoxyribose were used (2.8mM and 1.4mM). In a final volume of 1 ml containing the following reagents $FeCl_3$ (25 mM), EDTA (100 μM), H_2O_2 (2.8 mM), Cimetidine (0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM and 1.0 mM), KH_2PO_4/KOH buffer at pH 7.4 (10 mM) and ascorbate (100 μM), were added in sequence.

The reaction mixture was incubated in an oscillating water bath for 1 hour at 37°C. At the end of the incubation period, 1ml of 1% (w/v) TBA in 50mM NaOH and 1ml 2.8% TCA were added to the mixture which was then heated to develop colour due to the malondialdehyde (MDA)-like product of deoxyribose damage. The tubes were then placed in a hot water bath maintained at 80°C for up to 20 minutes. The absorbance was then measured at 532nm to give an indication of deoxyribose damage.

The initial attack of $OH^{\bullet}$ on DR is the rate-determining step in the formation of products like MDA. In the reaction mixture containing cimetidine:

Rate of reaction of $OH^{\bullet}$ with DR $= k_{DR} [OH^{\bullet}] [DR]$

Rate of reaction of Cimetidine with $OH^{\bullet} = k_{cime} [OH^{\bullet}] [Cime]$

Where k_{DR} and k_{cime} are second-order rate constants for deoxyribose and cimetidine reacting with hydroxyl radical.

The absorbance A, obtained is a measure of the rate of reaction. The absorbance, A°in the presence of Cimetidine is given by:

$$A° = k_{DR} [OH^{\bullet}] [DR] + k_{cime}[OH^{\bullet}] [Cime]$$

6.2.2 STATISTICAL ANALYSIS

The results obtained were analysed using a One Way Analysis of variance (ANOVA), followed by a Student Newman-Keuls Multiple Range test.

6.3 RESULTS

The literature value of k_{DR} obtained from pulse radiolysis is 3.1×10^9 M^{-1} sec^{-1}. In a situation where the use of pulse radiolysis is unavailable, it is still possible to determine rate constants between hydroxyl radicals and a test compound, as the results are similar when using the deoxyribose assay.

The equations can be combined to give:

$$1/A = 1/A^\circ (1 + k_{cime} [Cime]/k_{DR}[DR])$$

Hence, a plot of 1/A vs [Cime] should give a straight line of slope $k_{cime} /k_{DR}[DR] A^\circ$ with an intercept on the y-axis of $1/A^\circ$ and hence, the rate constant for the reaction of Cimetidine with $OH^\bullet$ can be obtained from the slope of the line.

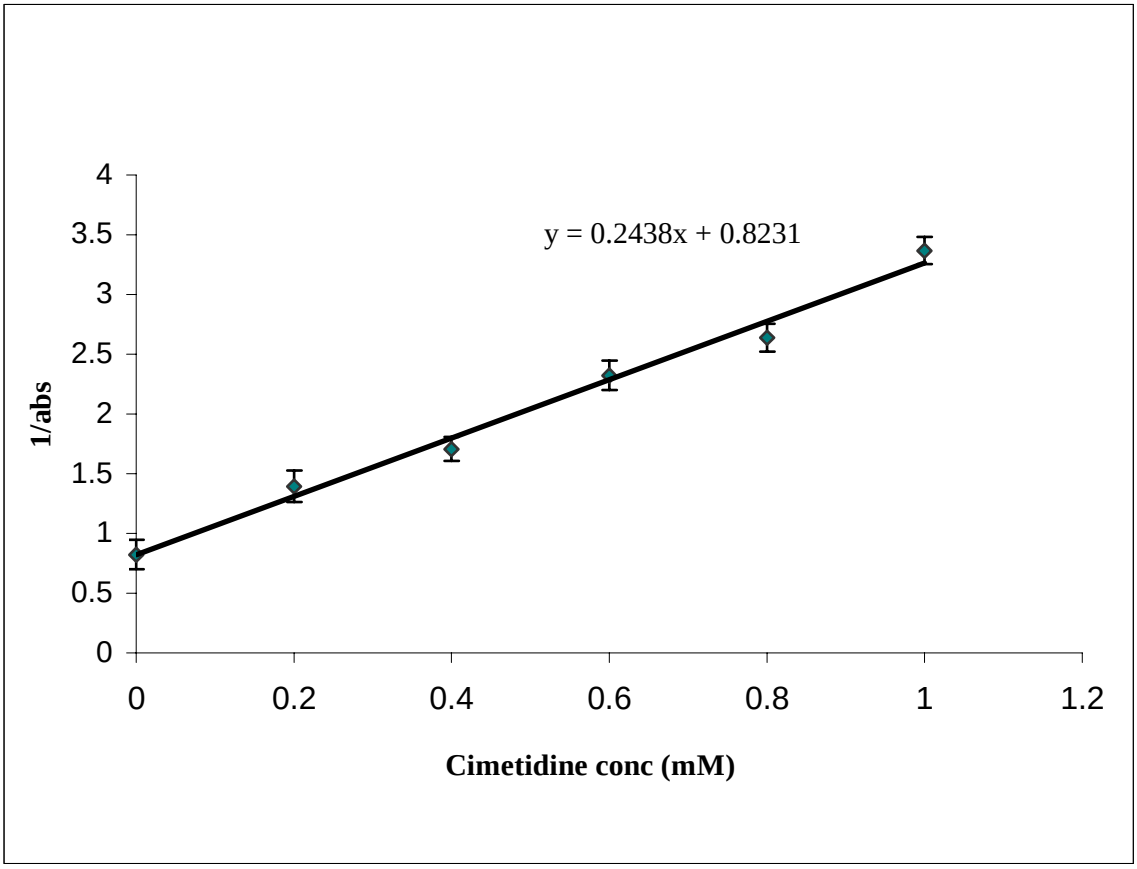


Figure 28: Hydroxyl radical scavenging by cimetidine at 2.8mM Deoxyribose. **(Each point represents the mean of triplicate determinations ± SEM).**

$$A^\circ = 1/A$$

$$= 1/ 0.8231$$

$$= 1.2149$$

$$\text{Slope} = k_{cime}/ 3.1 \times 10^9 \text{ (DR) x } A^\circ$$

$$0.2438 = k_{cime}/3.1 \times 10^9 \text{ (1.4) x } 1.2149$$

$$\mathbf{k_{cime} = 1.2854 \times 10^9 \ M^{-1} \ sec^{-1}}$$

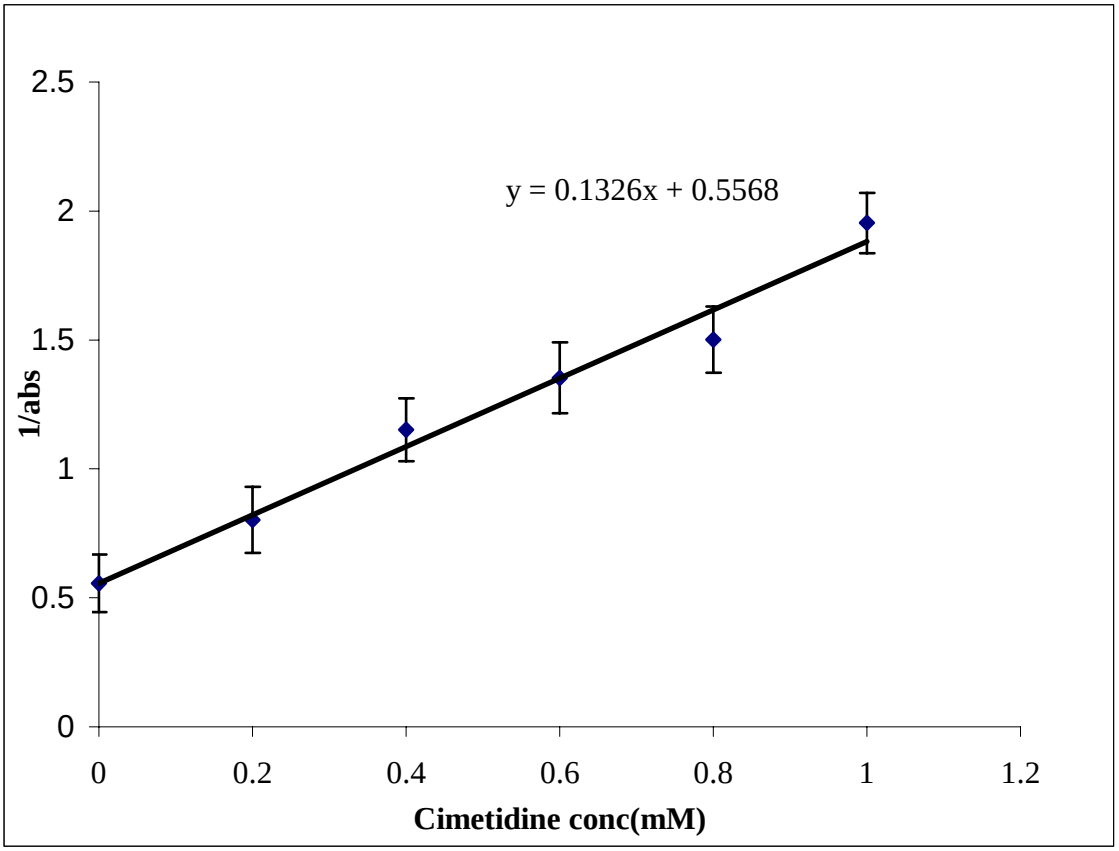


Figure 29: Hydroxyl radical scavenging by cimetidine at 1.4 mM Deoxyribose. **(Each point represents the mean of triplicate determinations ± SEM).**

$$A^\circ = 1/A$$

$$= 1/ /0.5568$$

$$= 1.7960$$

$$\text{Slope} = k_{cime}/ 3.1 \times 10^9 \ (DR) \times A^\circ$$

$$0.1326 = k_{cime}/3.1 \times 10^9 (2.8) \times 1.7960$$

$$\mathbf{k_{cime} = 1.5359 \times 10^9\ M^{-1}\ sec^{-1}}$$

6.4 DISCUSSION

Although pulse radiolysis will remain the choice for accurate determinations of rate constants with hydroxyl radicals, the deoxyribose assay for detecting hydroxyl radicals is a simple, cheap and accurate method.

The chemistry of the reaction involved is complex, and the generation of hydroxyl radicals can be obtained during the latter part of the experiment. However, the fact that linear competition plots are obtained suggests that the assumptions made under the kinetic analysis are valid and can provide useful data on the potency of a particular drug to exhibit antioxidant activity towards the hydroxyl radical. An association has been demonstrated between the generation of $OH^{\bullet}$ and subsequent damage to deoxyribose (Arouma et al, 1992).

DNA damage by the hydroxyl radical suggests an accelerating degradation of cellular function. Accumulating mutation in the genes can lead to decreased transfer efficiency, which consequently leads to a production of more free radicals. Mutations have been implicated in the aetiology of degenerative diseases such as AD disease and cancer. In tumour cells mutations are so highly amplified that they can often be detected in body fluids.

The results of the present study show that cimetidine reacts directly with the hydroxyl radical to inhibit deoxyribose degradation. In addition, our findings confirmed that this association involving both Fenton reactants induced deoxyribose degradation by $OH^{\bullet}$. Cimetidine exhibits greater antioxidative

activity than many known hydroxyl scavengers like mannitol and dimethyl sulfoxide (Uchida et al,1990).

In conclusion, the results of this study confirm that the deoxyribose assay is a simple, easy and reliable method for detecting hydroxyl radicals (Halliwell el al,1987). In addition, cimetidine can be considered as a potential free radical scavenger due to its ability to reduce deoxyribose damage by binding the hydroxyl radical at various concentrations. Hence, cimetidine could be used as an excellent antioxidant for biological systems, which could reduce the destructive damage caused by free radical species, especially the hydroxyl radical, the most damaging of the free radicals.

CHAPTER SEVEN

Conclusions and Recommendations for Future Work

7.1 SUMMARY OF RESULTS

7.1.1 Chapter 2: Growth studies of WHCO6 cancer cells

This study examined the effect of cimetidine on the growth of WHCO6 cancer cells. The results show that cimetidine is able to inhibit growth of WHCO6 cancer cells under cell culture conditions.

7.1.2 Chapter 3: Superoxide anion Formation

Cimetidine reduced the formation of superoxide anion in both WHCO6 cancer cells and in cyanide-induced rat brain homogenate. The purpose of this study was to assess the general effect of cimetidine on formation of free radicals.

7.1.3 Chapter 4: Lipid peroxidation

This study examined the effect of cimetidine on WHCO6 cancer cells and iron treated rat brain homogenate in terms of lipid peroxidation levels. The study was conducted to examine the effect of a particular free reactive oxygen species, the hydroxyl radical. The results from the study showed that cimetidine significantly reduced lipid peroxidation in WHCO6 cancer cells and rat brain homogenate and hence could be acting as a potential hydroxyl radical scavenger.

7.1.4 Chapter 5: Interaction of cimetidine and transition metals

This study was performed in order to substantiate the results from Chapters Three and Four. The actual mechanism by which cimetidine was acting to reduce levels of free radicals was determined. The results show that cimetidine binds transition metals and in doing so reduces free radical formation especially the hydroxyl radical that depends on metals such as iron and copper for formation via the Fenton Reaction. In addition, cimetidine is a known inhibitor of the cytochrome-p450 enzymatic system, which is implicated in free radical formation. Thus, if the Cytochrome P-450 enzymatic system is inhibited; consequently free radical production would be reduced.

7.1.5 Chapter 6: The effect of cimetidine in free radical formation using deoxyribose as a substrate

The purpose of this study was to link the disease states of concern pertaining to the subject of this thesis in terms of free radical formation. The hydroxyl activity was assessed using deoxyribose. The hydroxyl radical has been implicated in AD and its effect on deoxyribose is linked with cancer, which originates due to mutations of DNA. The results of this study show that cimetidine acts as a hydroxyl scavenger and in doing so prevents damage to DNA.

7.2 CONCLUSIONS

Section 1.5 outlines the main research objectives of this study. The objectives of this study was to determine the effect of cimetidine in AD and cancer, whether cimetidine exhibits free radical scavenging properties and finally the possible mechanism by which cimetidine acts in order to exhibit antioxidant properties.

Cimetidine has significant effects on free radical levels, especially the hydroxyl radical which is the most damaging of all free radical species. From studies on superoxide anion formation and lipid peroxidation levels, the results tentatively suggest that cimetidine is effective in reducing levels of free radicals in rat brain homogenate and WHCO6 cancer cells. In particular, using iron to generate the radicals assessed hydroxyl radical scavenging properties. The results from lipid peroxidation assay as well as the deoxyribose assay confirm that cimetidine exhibits properties of a hydroxyl radical scavenger.

The metal binding studies also reveal that cimetidine has an ability to bind to transition metals. The mechanism by which the binding occurs could be by forming complexes with the metals. Furthermore, this theory can be supported by the known fact that cimetidine is a cytochrome P-450 enzyme inhibitor and hence, free radical formation propagated from the electron transport chain in the mitochondria is reduced as cimetidine can either bind to iron present in the cytochromes or inhibit enzymes to reduce free radical formation. However, this would need to be investigated further by carrying

out more studies on the cytochrome p-450 enzyme system as well as further studies to understand the complexity of the metal binding interactions.

The deoxyribose assay confirmed that cimetidine acts as potent hydroxyl radical scavenger. In addition, the importance of the hydroxyl radical to cause damage in degenerative diseases in emphasized. Cimetidine protects the deoxyribose, an important component of DNA by scavenging free radicals and hence prevents damage caused to the DNA, which could result in mutations leading to disease, in particular cancer.

The results from this study have important clinical applications and would possibly be of beneficent use in the treatment of AD and cancer.
AD is targeted in the elderly. Cimetidine would be beneficial not only in the treatment of AD but in treating the gastric acid secretion problems that are associated with the elderly.

The elderly are a known group of patients with multiple disease states. Consider an elderly patient with gastric heartburn and AD. An antacid would be suitable, however, some antacids contain aluminium and since this metal has been implicated in AD it would be better to avoid any medication containing aluminium. An alternative therapy would be to use cimetidine as treatment is such a case, since the heartburn as well as AD would be treated. Hence, cimetidine would be beneficial in this target group especially by exerting a dual effect.

In cancer, from section 1.4.2 it can be seen that cimetidine has beneficial immunological implications in cancer. Although, cimetidine cannot be used

as sole treatment for cancer due to the severity of the disease, it can be used as an adjunct along with chemotherapy in cancer treatment.

7.2.1 LIMITATIONS AND FUTURE RECOMMENDATIONS

In this study experiments have been carried out *in vitro* and the rat brain has been used as a model to mimic AD. The human brain is more evolved compared to the rat brain and the rat brain has less antioxidants compared to the human brain, so free radical levels would be expected to be higher than normal as seen in this study. For cimetidine to be effective in humans, clinical trials would have to be carried out to support the theory of cimetidine as treatment for AD.

Furthermore, cimetidine needs to be investigated further in cancer by doing additional work with DNA. Cimetidine is being used as a treatment in colorectal cancer, since this is the area where cimetidine exerts its pharmacological response. Further studies should be done on parietal cell cancers which would support the theory that cimetidine exerts beneficial effects by acting as a free radical scavenger. In addition, histamine has been implicated in cancer. Levels of histamine can be assessed in cancer cells and thereby prove that cimetidine could exert a beneficial immunological response in cancer simply by acting as a histamine antagonist.

The results from this study imply that cimetidine has important clinical implications. One problem that would be experienced with cimetidine would be interaction with other drugs and hence before initiating therapy with cimetidine a medication history of the patient taking current medication would have to be assessed. However, there is sufficient evidence of the

beneficial uses of cimetidine and these should be examined in further detail, since findings from this study have generated several possible areas of research.

APPENDIX 1

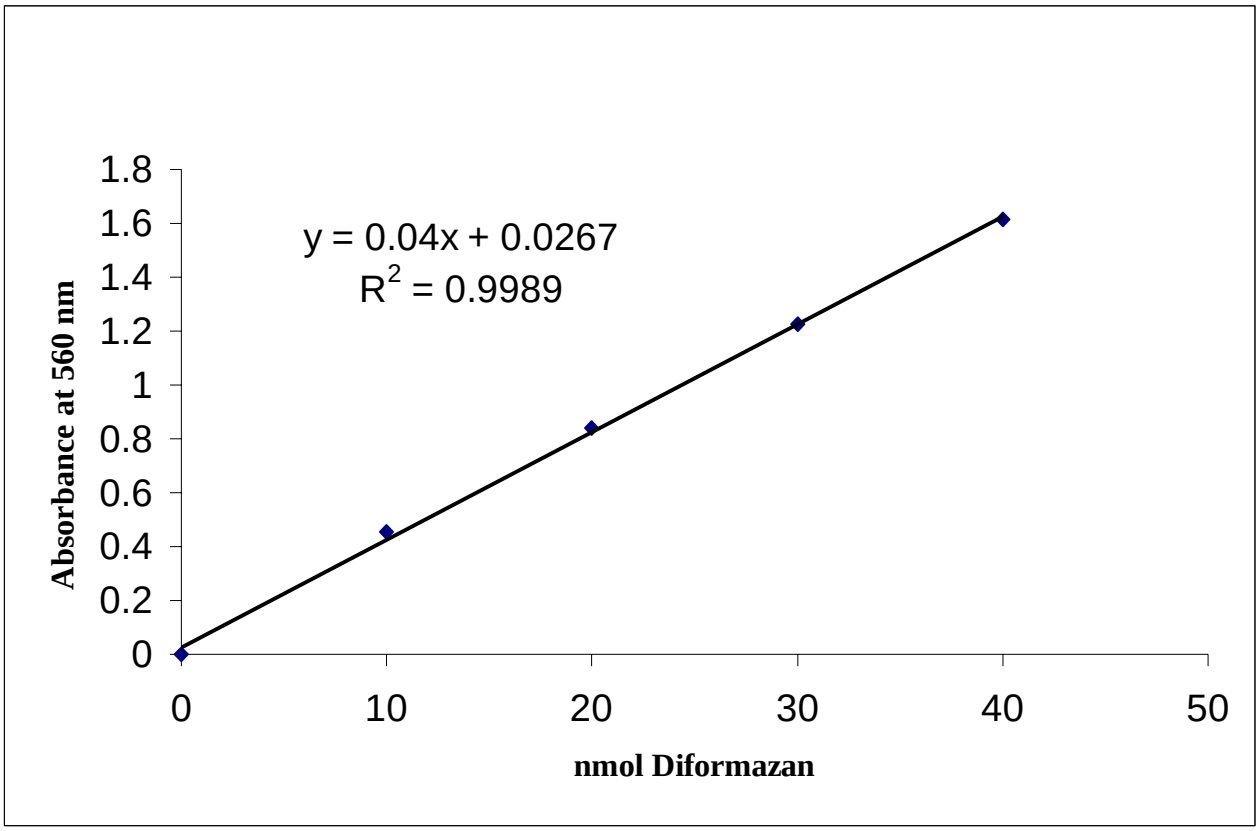


Figure 30: Diformazan Standard Curve

APPENDIX 2

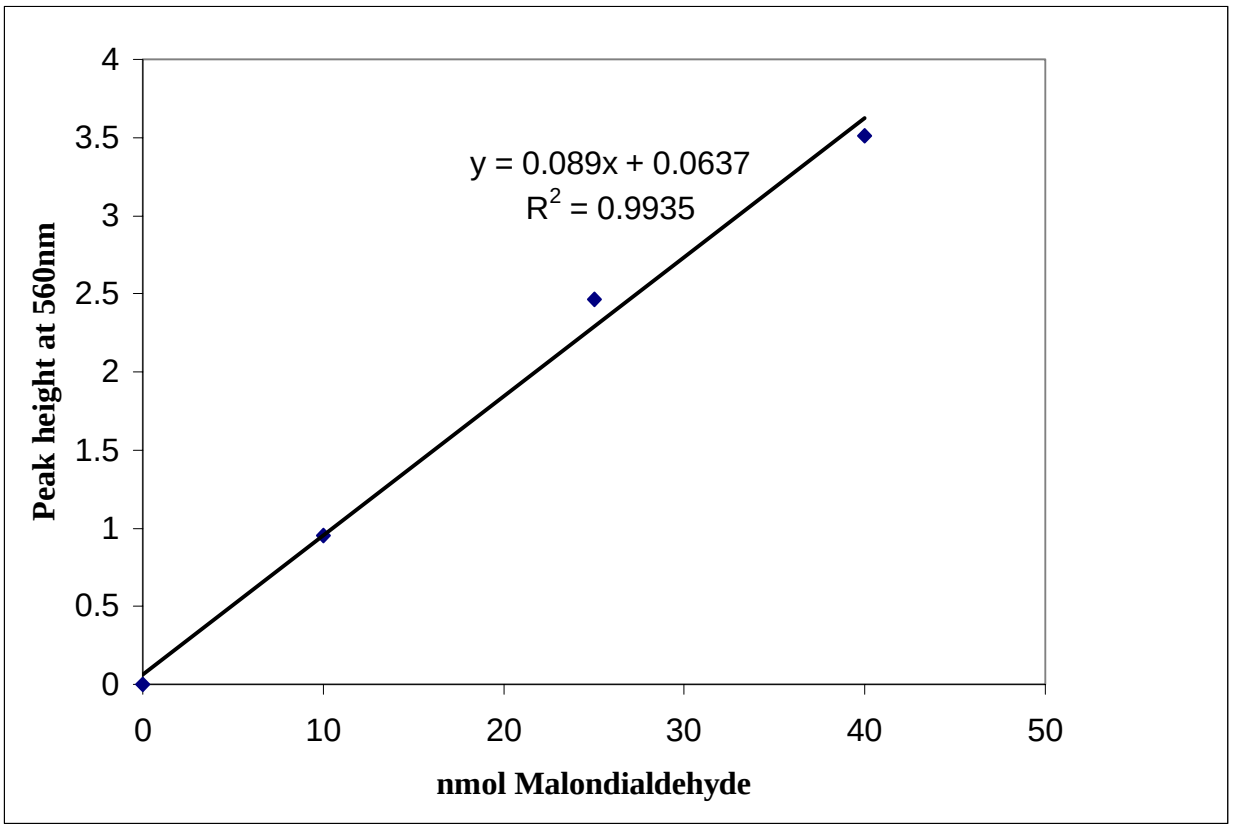


Figure 31: Malondialdehyde Standard Curve

APPENDIX 3
ANIMALS AND HOUSING CONDITIONS

The Rhodes University Ethics Standards Committee for Animal Research approved all the experiments that involved the use of animals. All experiments were performed using adult male albino rats of the Wistar strain obtained as an outbred colony from a single breeder, the South African Institute for Medical Research (SAIMR, Johannesburg). Unless otherwise stated, all the animals were approximately 3 months old weighing 250-300g.

The animals were housed in a windowless, well-ventilated constant environment room (CER) under a diurnal lighting cycle; 12 hours light: 12 hours darkness (lights on at 0600, Central Africa n Time [CAT]). Artificial lighting was provided by standard cool-white fluorescent bulbs (75W) with an intensity of illumination of approximately 300µ Watts/ cm^2 . Ambient temperature of the animal room was maintained between 22ºC and 25ºC. Animals were housed 5 to 6 per cage to minimize stress (Gambardella et al, 1994).

Cage cleaning and feeding were performed daily and only in the photophase, to avoid induction of secondary exogenous rhythms. Every effort was made to minimize stress to the rats, including experimental handling time.

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