EFFECTS OF CROSS-FOSTERING AND MELATONIN SUPPLEMENTATION ON THE DEVELOPMENT OF HYPERTENSION AND RENAL ANTIOXIDANT/OXIDANT SYSTEM IN SPONTANEOUSLY HYPERTENSIVE RATS

by

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PREFACE

The exact cause of primary hypertension remains unknown but genetic, lifestyle and other environmental factors have been implicated in the pathogenesis of primary hypertension. There is also a belief that a number of lifestyle diseases might have their origins in the foetus and that early intervention during fetal life or immediate postnatal life might modify the subsequent development of these diseases. This thesis documents a study examining the consequences of both intrauterine and extrauterine environmental modifications on the development of hypertension in spontaneously hypertensive rats (SHR), where the hypertension is genetic. The study was conducted in four stages or phases and the layout of the thesis is as follows.

Chapter 1 provides a review of the literature on the subject of hypertension and oxidative stress, leading to the hypotheses and the objectives of this research. Chapter 2 describes the general materials and methods used in the four phases of the study. Chapter 3 reports the first stage of the study examining the differences in blood pressure profile and the antioxidant system between SHR and their controls, the WKY rats. Chapter 4 documents the second stage of the study where the effect of cross-fostering of offspring between SHR and WKY rats on blood pressure and renal antioxidant system was examined. Chapter 5 reports the third stage of the study, which examined the effect of antenatal and postnatal melatonin supplementation on blood pressure and renal antioxidant system. Chapter 6 documents the fourth stage of the study where the effect of both antenatal and postnatal melatonin supplementation together with cross-fostering on the development of hypertension and renal antioxidant systems is examined. Chapter 7 discusses the overall discoveries and summarises the important findings.

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LIST OF ABBREVIATIONS, SYMBOLS, ACRONYMS USED

Abbreviation	Definition
4-HNE	4-hydroxy-2-nonenal
8-OHdG	8-hydroxy-2-deoxyguanosine
AA-NAT	arylalkylamine N-acetyltransferase
AFMK	N-acetyl-N-formyl-5-methoxykynuramine
AMK	N-acetyl-5-methoxykynuramine
Ang	angiotensin
ANOVA	analysis of variance
AOE	antioxidant enzyme
ATP	adenosine triphosphate
b	base
bp	base pair
CAT	catalase
cDNA	complementary deoxyribonucleic acid
C_T	threshold cycle
Cu/Zn-SOD	copper/zinc superoxide dismutase
ddH_2O	distilled-deionized water
DMSO	dimethyl sulfoxide
dNTP	deoxynucleoside triphosphate
DNA	deoxyribonucleic acid
DOCA	deoxycorticosterone acetate
dR	fluorescence value
dsDNA	double stranded deoxyribonucleic acid
EC-SOD	extracellular superoxide dismutase
eNOS	endothelial nitric oxide synthase
GCS	glutamycysteine synthetase
GMP	guanosine monophosphate
GPx	glutathione peroxidase

GPx-1	cytosolic glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione/glutathione
GST	glutathione S-transferase
GST-M1	glutathione S-transferase mu 1
GSSG	oxidized glutathione/disulfide glutathione
HDL	high density lipoprotein
HRP	horseradish peroxidase
H_2O_2	hydrogen peroxide
iNOS	inducible nitric oxide synthase
kDa	kilo Dalton
K_m	Michaelis constant
LDL	low density lipoprotein
LOO•	lipid peroxyl radical
MDA	malondialdehyde
Mn-SOD	manganese superoxide dismutase
mRNA	messenger ribonucleic acid
n	sample size
NAC	N-acetylcysteine
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
$O_2•^-$	superoxide anion
$^1O_2$	singlet oxygen
•OH	hydroxyl radical
$ONOO^-$	peroxynitrite
NO	nitric oxide
NOS	nitric oxide synthase
PCO	protein carbonyl
RAS	renin-angiotensin system

RNA	ribonucleic acid
RNS	reactive nitrogen species
ROOH	lipid hydroperoxide
ROS	reactive oxygen species
RT-PCR	real-time polymerase chain reaction
QPCR	quantitative polymerase chain reaction
SBP	systolic blood pressure
S.E.M.	standard error of the mean
SGLT-1	sodium dependent glucose co-transporters-1
SHR	spontaneously hypertensive rats
SOD	superoxide dismutase
SP-SHR	stroke-prone spontaneously hypertensive rats
SCN	suprachiasmatic nucleus
TAS	total antioxidant status
TBA	thiobarbiturate acid
TBARS	thiobarbituric acid reactive substances
WKY	Wistar Kyoto

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KESAN PELIHARAAN SILANG DAN PEMBERIAN MELATONIN KE ATAS PERKEMBANGAN HIPERTENSI DAN SISTEM ANTIOKSIDAN/OKSIDAN GINJAL DALAM TIKUS HIPERTENSI SPONTAN

ABSTRAK

Pelbagai intervensi di awal hayat SHR telah dilakukan dalam percubaan untuk memprogram semula profil peningkatan tekanan darah dalam SHR tetapi manfaatnya adalah terhad dan sementara sahaja. Kajian ini memeriksa kesan peliharaan silang, pemberian melatonin semasa antenatal dan postnatal atau kombinasi kedua-duanya ke atas perkembangan hipertensi dan sistem antioksidan/oksidan ginjal dalam tikus SHR dan WKY. Pada peringkat pertama kajian, tekanan darah dan profil antioksidan diukur dalam tikus SHR dan WKY. Seterusnya, kesan peliharaan silang diperiksa apabila anak tikus SHR dan WKY berusia satu hari dipelihara silang oleh ibu tikus yang berlawanan strain dan anak tikus jantan dipelihara sehingga berusia 16 minggu. Pada peringkat seterusnya, pemberian melatonin (10mg/kg berat badan/hari) melalui air minuman kepada ibu SHR dan WKY mulai hari pertama mengandung sehingga hari ke-21 postpartum, dan kemudiannya pemberian melatonin dilanjutkan kepada sebahagian daripada anak tikus jantan sehingga berusia 16 minggu. Pada peringkat seterusnya, anak daripada ibu WKY dan SHR yang diberi melatonin dipelihara silang dari usia satu hari sehingga bercerai susu dan pemberian melatonin diteruskan kepada sebahagian daripada anak tikus jantan yang telah bercerai susu sehingga berusia 16 minggu. Tekanan darah sistolik (SBP) anak tikus jantan dicatat pada usia 4, 6, 8, 12 dan 16 minggu serta tisu ginjalnya dipungut untuk menganggar status antioksidan/oksidan pada setiap penghujung kajian menurut kategori usia.

Min SBP dalam SHR adalah lebih tinggi secara signifikan mulai usia 6 minggu manakala aktiviti glutation peroksidase (GPx) dan paras protein GPx-1 dalam SHR adalah lebih rendah secara signifikan antara usia 8 hingga 16 minggu, berbanding dengan WKY kawalan. Sebaliknya, aktiviti katalase (CAT) dan paras proteinnya adalah lebih tinggi secara signifikan dalam SHR mulai usia 6 minggu, manakala mRNA CAT adalah lebih tinggi secara signifikan mulai usia 4 minggu. Selain daripada itu, status antioksidan keseluruhan (TAS) adalah lebih tinggi secara signifikan dan penanda pemperoksidaan lipid (TBARS) adalah lebih rendah secara signifikan dalam SHR pada usia 16 minggu. Tahap hidrogen peroksida (H_2O_2) adalah lebih rendah secara signifikan dalam SHR pada usia 8 hingga 16 minggu. SBP mencatatkan penurunan secara signifikan dalam SHR yang dipelihara oleh ibu WKY berbanding kawalan sehingga usia 12 minggu. TAS didapati lebih rendah secara signifikan dalam WKY yang diperlihara oleh ibu SHR pada usia 4 minggu, manakala TBARS didapati lebih rendah secara signifikan dalam SHR yang dipelihara oleh ibu WKY pada usia 4 minggu berbanding dengan kawalan berpadankan usia masing-masing.

Anak SHR yang menerima melatonin melalui ibu sehingga hari ke-21 postnatal hanya mencatat penurunan SBP secara signifikan sehingga usia 8 minggu berbanding SHR kawalan berpadankan usia yang tidak dirawat. Walau bagaimanapun, jika rawatan melatonin (melalui air minuman) diteruskan selepas bercerai susu, SBP dalam anak SHR menunjukkan penurunan signifikan sehingga usia 16 minggu. Aktiviti GPx dan glutation s-transferase (GST) dalam SHR yang menerima melatonin sehingga usia 16 minggu adalah lebih tinggi secara signifikan pada usia 16 minggu, dan tahap glutation keseluruhan adalah lebih tinggi secara

signifikan pada usia 4, 12 dan 16 minggu berbanding dengan SHR yang tidak dirawat.

SBP adalah lebih rendah secara signifikan hanya pada usia 12 dan 16 minggu dalam SHR yang diperlihara oleh ibu WKY dan diberi melatonin sepanjang tempoh eksperimen berbanding dengan SHR yang dipelihara oleh ibu WKY dan tidak dirawat, tetapi SBP adalah lebih rendah secara signifikan sepanjang tempoh eksperimen dalam SHR yang tidak dirawat sama ada dipelihara oleh ibu SHR atau WKY. Aktiviti GPx dan GST mencatatkan peningkatan signifikan dalam SHR yang dipelihara oleh WKY dan diberi melatonin pada usia 16 minggu jika dibandingkan dengan SHR yang tidak dirawat sama ada dipelihara oleh ibu SHR atau WKY.

Sebagai kesimpulan, kajian ini mencadangkan keabnormalan major dalam sistem pertahanan antioksidan ginjal dalam SHR, khususnya aktiviti CAT dan GPx. Pemberian melatonin sama ada semasa tempoh antenatal atau postnatal, dan peliharaan silang dapat menurunkan kadar kenaikan SBP tetapi kesannya bersifat sementara dan memerlukan pemberian melatonin secara berterusan. Pemberian melatonin juga telah memperbaiki sistem glutation dalam SHR. Walau bagaimanapun, kombinasi pemberian melatonin dan peliharaan silang tidak menunjukkan sebarang kesan tambahan terhadap penurunan tekanan darah jika dibandingkan dengan pemberian melatonin atau peliharaan silang secara berasingan. Walaupun keabnormalan dalam katalase berlaku sebelum kenaikan sebenar SBP dalam SHR, ia tidak mungkin dikaitkan secara langsung dengan patogenesis tekanan darah tinggi dalam strain ini pada peringkat ini.

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ABSTRACT

Various interventions in early life in SHR have been attempted to re-programme the raised blood pressure profile in SHR but with limited and only transient benefits. This study examined the effects of either cross-fostering or antenatal and postnatal melatonin supplementation alone or in combination on the development of hypertension and renal antioxidant/oxidant system in SHR and WKY rats. In the first stage of the study, blood pressure and antioxidant profiles in SHR and WKY rats were measured. After this the effect of cross-fostering was examined where one-day-old SHR and WKY pups were cross-fostered to the dam of the opposite strain and the male offspring were then raised up to 16 weeks of age. In the next stage, melatonin (10mg/kg body weight/day) was supplemented via drinking water to pregnant SHR/WKY from pregnancy day-1 and up to postpartum day-21, and then continued to be given to some of the weaned male offspring up to 16 weeks of age. In the following stage, one-day-old offspring of melatonin supplemented SHR and WKY dams were cross-fostered and after weaning some of these cross-fostered male offspring continued to receive melatonin via drinking water up to 16 weeks of age. Systolic blood pressure (SBP) of all the male offspring was recorded at the age of 4, 6, 8, 12 and 16 weeks, and the kidneys were collected for the estimation of antioxidant/oxidant status at the end of each age category.

Compared to WKY controls, mean SBP in non-supplemented SHR was significantly higher from the age of 6 weeks onwards; glutathione peroxidase (GPx) activity and GPx-1 protein level was significantly lower in SHR aged 8 to 16 weeks.

In contrast, significantly higher catalase (CAT) activity and its protein level was found in SHR aged 6 weeks and onwards, whereas elevated CAT mRNA was found from as early as 4 weeks of age in SHR. In addition, significantly higher total antioxidant status (TAS) and lower lipid peroxidation marker (TBARS) was found in SHR aged 16 weeks. The level of hydrogen peroxide (H_2O_2) was significantly lower in SHR aged 8 to 16 weeks. In cross-fostered SHR, SBP was significantly lower than that in SHR controls till the age of 12 weeks. TAS was lower in cross-fostered WKY rats aged 4 weeks whereas TBARS was lower in cross-fostered SHR at 4 weeks of age when compared to their respective age-matched controls.

When compared to age-matched untreated SHR, SBP in SHR offspring that received maternal melatonin up to postnatal day-21, was significantly lower up to 8 weeks of age only. However, SBP in SHR offspring that continued receiving melatonin (via drinking water) after weaning remained significantly lower till the age of 16 weeks. GPx and GST activities were significantly higher at the age of 16 weeks, and total glutathione level was significantly higher at 4, 12 and 16 weeks in SHR receiving melatonin till the age of 16 weeks when compared to untreated SHR.

In melatonin supplemented cross-fostered SHR, SBP was significantly lower at the age of 12 and 16 weeks when compared to untreated cross-fostered SHR, but was significantly lower throughout the experimental period when compared to untreated or in-fostered SHR. Activities of GPx and GST were higher in melatonin-treated cross-fostered SHR aged 16 weeks, when compared to age-matched in-fostered SHR and non-melatonin supplemented cross-fostered SHR.

In conclusion, this study suggests the presence of a major abnormality in the renal antioxidant defence system in SHR, particularly in CAT and GPx activities, Melatonin supplementation whether antenatal or postnatal and cross-fostering reduce

the rate of rise in SBP but the effect is transient and requires a continuous administration of melatonin. Melatonin supplementation also improves the glutathione system in SHR. A combination of melatonin supplementation and cross-fostering however did not confer any additional impact on the blood pressure lowering effect of either melatonin or cross-fostering alone. The precise mechanism by which these two maneuvers delayed the rise in blood pressure remains unclear. Although the abnormality in catalase was present before the actual rise in SBP in the SHR, it is however not possible to directly link this abnormality to the pathogenesis of high blood pressure in this strain at this stage.

CHAPTER 1
GENERAL INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Hypertension is a major risk factor contributing to cardiovascular, cerebrovascular and renal diseases, which together contribute to the high mortality rate worldwide. It is estimated that there are 4.8 million individuals with hypertension in Malaysia (Ministry of Health Malaysia, 2008). The third National Health and Morbidity Survey document indicates a prevalence of hypertension among adults aged 30 years and above as 42%. From the survey it is alarming to note that close to two thirds of individuals with hypertension in Malaysia were unaware that they were hypertensive and therefore remained undiagnosed (Ministry of Health Malaysia, 2008). Hypertension is often referred to as a silent killer as it remains symptomless and is often only detected co-incidentally or during a routine medical examination. It is therefore important that hypertension is diagnosed early and well managed as its early and proper management has been shown to significantly reduce complications like strokes, myocardial infarction, congestive heart failure and end-stage renal disease (Collins and MacMahon, 1994; Arguedas *et al.*, 2009; de Lusignan *et al.*, 2009; Stenvinkel, 2010).

The public impact and economic burden of hypertension extends far beyond that related to the treatment of high blood pressure. For example, in Malaysia, according to the Ministry of Health records, about RM145 million was spent on antihypertensive medicines alone in the year 2004 (Ministry of Health Malaysia, 2008). This estimation, however, does not include the additional costs that arise from the management of complications associated with hypertension like heart

failure, myocardial infarction and renal failure and even the loss of working hours etc (Sarojini and Lim, 2004; Ministry of Health Malaysia, 2008).

Hypertension is generally classified as either primary or secondary hypertension. The etiology and pathogenesis of primary or essential hypertension remain unidentified and under intense study. Essential, primary, or idiopathic hypertension is defined as high blood pressure in which secondary causes such as renovascular disease, renal failure, pheochromocytoma, aldosteronism, or other causes of secondary hypertension are not present (Carretero and Oparil, 2000). On this basis essential hypertension accounts for 95% of all cases of hypertension (Carretero and Oparil, 2000). It is considered a heterogeneous disorder, with different patients having different causal factors that lead to high blood pressure. Although the causes of essential hypertension remain largely unknown, some information is available indicating the role of some genetic variations and intermediary phenotypes that might cause or be responsible for the high blood pressure. Existing evidence suggests that the genetic contribution to blood pressure variation is about 30% (Hong *et al.*, 1994; Marteau *et al.*, 2005), with the rest coming from a number of environmental factors that have also been linked to raised blood pressure, including obesity, insulin resistance, high salt intake, high alcohol intake, stress, aging, sedentary lifestyle, low potassium and low calcium intake (Hashimoto *et al.*, 1989; Sever and Poulter, 1989; Elliott *et al.*, 1990; Andrade *et al.*, 2010; Fujita and Takei, 2010).

An imbalance in the antioxidant/oxidant status especially in the kidney has been proposed as an important pathogenic mechanism in hypertension as well as the progression of kidney disease (Wilcox, 2005; Nistala *et al.*, 2008). In addition to this, maternal influence during early life has also been proposed and might be one of the

risk factors contributing to the adult hypertension (Blizard and Adams, 1992; Ashton, 2000; Davidge *et al.*, 2008). In this regard, several experimental hypertension models e.g. spontaneously hypertensive rats, Dahl's, Milan, Lyon, deoxycorticosterone acetate-salt hypertensive, Sabra and New Zealand strains, differing in the contribution of genetic and environmental factors to the raised blood pressure, have been developed in attempts to understand the pathogenesis of hypertension (Okamoto and Aoki, 1963; Kihara *et al.*, 1993; Johns *et al.*, 1996). The spontaneously hypertensive rat (SHR), the closest animal model that represents human essential hypertension, and its Wistar-Kyoto (WKY) normotensive control were developed in 1963 by Okamoto and Aoki in Japan (Okamoto and Aoki, 1963). SHR exhibits spontaneous hypertension with many features in common with human essential hypertension, which include elevated peripheral resistance, increased cardiac output, elevated sympathetic nervous activity and cardiovascular hypertrophy (Frohlich, 1986; Zicha and Kunes, 1999; Girouard *et al.*, 2004). Furthermore, as in human, its blood pressure is readily lowered with peripheral vasodilators, calcium channel antagonists and blockers of the renin-angiotensin system (Zicha and Kunes, 1999; Polizio and Pena, 2005; Liskova *et al.*, 2010). Numerous sophisticated attempts have been made to modify the natural course of hypertension in adult SHR with established hypertension via various pharmacological and nutritional interventions (Zicha and Kunes, 1999; Nava *et al.*, 2003; Rodriguez-Iturbe *et al.*, 2003; Khanna *et al.*, 2008; Nuyt and Alexander, 2009). In contrast, relatively little attention has been devoted to studies involving pre-weaning or young SHR in a bid to prevent the rise in blood pressure. Modification of pre-weaning maternal environment through cross-fostering of one-day-old SHR offspring to normotensive dams e.g have shown that cross-fostering significantly delayed the development of

high blood pressure in these SHR offspring (Cierpial and McCarty, 1987a; McCarty and Tong, 1995; Di Nicolantonio *et al.*, 2006). Although the precise reason for this delay in the rise in blood pressure was not evident, it was however attributed to differences in quality and quantity of milk delivered to the offspring and perhaps due to exposure of the SHR offspring to a different pattern of maternal behaviour (McCarty and Tong, 1995; Gouldsborough *et al.*, 1998). Whilst the impact of cross-fostering is only temporary, its influence nevertheless needs to be examined further to identify the factor responsible for the hypotensive effect and possibly also the mechanism by which the blood pressure is lowered. In this regard, the effect of cross-fostering on the renal antioxidant/oxidant status in SHR has not been investigated thoroughly and efforts could be made to examine the mechanism and possibly also identify the particular factor in renal antioxidant/oxidant system.

A number of reports over the years have documented a possible link between melatonin and the pathogenesis of hypertension. Decreased melatonin levels have been reported in hypertension (Jonas *et al.*, 2003; Leibowitz *et al.*, 2008), and melatonin supplementation has been shown to successfully ameliorate or reduce the high blood pressure in humans as well as in experimental animal models (Cagnacci *et al.*, 2005; Pechanova *et al.*, 2007). Clinical utility of melatonin in antenatal, parturition and postnatal life has been claimed to result in a wide range of health benefits, improved quality of life and reduction of complications during the neonatal period (Gitto *et al.*, 2009). Maternal melatonin treatment has been reported to reduce the raised blood pressure in offspring of genetically hypertensive animals (Kim *et al.*, 2002). Nevertheless, the association between its hypotensive and antioxidative effects and the regulation of renal antioxidant/oxidant system remains uncertain, particularly when administered during the antenatal, perinatal and postpartum

periods. This study attempts to examine the impact of melatonin supplementation and cross-fostering either alone or in combination on the development of high blood pressure and renal antioxidant system in SHR.

The following literature review focuses on the basic concept of oxidative stress and their harmful effects on cells and tissues; and also the protective role of the antioxidant defence system including antioxidant properties of melatonin in the maintenance of homeostasis of the oxidant and antioxidant status in mammals. In addition, the role of oxidative stress, maternal and environmental factors in the development of hypertension are also reviewed.

1.2 REVIEW OF LITERATURE

1.2.1 Concept of oxidative stress

Metabolism of oxygen by cells generates potentially deleterious reactive oxygen species (ROS). During mitochondrial respiration, electron transport involves a coordinated four electron reduction of oxygen to water in the electron transport chain. However a small number of electrons "leak" prematurely from the mitochondrial respiratory chain enzymes forming the oxygen free radical i.e superoxide anion (Cadenas and Davies, 2000). Measurements of sub-mitochondrial particles suggest a leak of 1-3% of all electrons in the transport chain that go on to generate the superoxide radical (Cadenas and Davies, 2000). Other than mitochondrial electron leak, ROS is also generated by several other mechanism, involving the endogenous respiratory burst, enzyme reactions (especially xanthine oxidase, lipo-oxygenase, prostaglandin synthetase, NADPH oxidase), auto-oxidation reactions, and environmental stress factors like e.g. exposure to pollutants, ultraviolet

light, ionizing radiation, xenobiotics etc (Karbownik and Reiter, 2000; Young and Woodside, 2001).

In a healthy state, there exists a dynamic balance between ROS production and the activity of the antioxidant defence system, which prevents the potential deleterious consequences of ROS. In certain pathogenic situations, however, oxidative stress results when there is an imbalance between the antioxidant activity and ROS production. Oxidative stress occurs when there is an excessive production or diminished detoxification of ROS. Prolonged high-grade oxidative stress leads to tissue damage and is believed to underlie the pathogenesis of a number of diseases (Gutteridge and Halliwell, 1994).

1.2.2 Formation of free radicals and reactive oxygen species

Atoms and molecules are most stable in the ground state when every electron in the valence shell has a complimentary electron that spins in the opposite direction. A free radical is an atom or molecule with at least one unpaired electron in the outermost shell, and is capable of independent existence (Halliwell and Gutteridge, 1984; Fang *et al.*, 2002). The free radical nature of an atom or molecule is usually denoted by a superscript dot (e.g. $\bullet H$, $O_2^{\bullet -}$, $\bullet OH$). Free radicals of different types vary widely in their chemical reactivity, but in general they are more biologically reactive than non-radicals.

When two free radicals meet, their unpaired electrons can join to form a pair, and both radicals are eliminated. However since most molecules present in living organisms do not have unpaired electrons, any free radicals produced will most likely react with non-radicals, thereby generating new free radicals. Hence, free radical reactions tend to proceed as chain reactions. Unpaired electrons can be associated

with many different atoms and molecules and there are several types of radicals that can be produced within the body e.g. reactive nitrogen species (RNS) and ROS (Droge, 2002). RNS includes nitric oxide (NO or NO•), nitrogen dioxide (NO_2•), peroxynitrite ($ONOO^-$), peroxynitrous acid (ONOOH), alkyl peroxynitrite (ROONO) and s-nitrosothiols (RSNO). ROS includes oxygen ions, free radicals and peroxides both inorganic and organic, which includes superoxide anion (O_2•-), hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), hydroperoxyl radical (HO_2•), peroxyl radical (RO_2•), alkoxyl radical (RO•), hypochlorite (HOCl−) and hypochlorus acid (HOCl) (Halliwell and Gutteridge, 1984; Gilbert, 2000). These ROS are generated as intermediate products in the reduction of oxygen to water. They are generally very small molecules and are highly reactive due to the presence of unpaired valence shell electron (Halliwell and Gutteridge, 1984; Gilbert, 2000). The most important free radicals in many disease states are oxygen derivatives, particularly superoxide and the hydroxyl radical. Superoxide radical ($O_2^{\bullet-}$) is produced by the addition of a single electron to oxygen, and several mechanisms exist by which $O_2^{\bullet-}$ can be produced *in vivo* (Halliwell and Gutteridge, 1992). Adrenaline, flavine nucleotides, thiol compounds, and glucose, can oxidize in the presence of oxygen to produce $O_2^{\bullet-}$, and these reactions are greatly accelerated by the presence of transition metals such as iron or copper. The electron transport chain in the inner mitochondrial membrane performs the reduction of oxygen to water. During this process free radical intermediates are generated, which are generally tightly bound to the components of the transport chain. However, there is a constant leak of a few electrons into the mitochondrial matrix and this results in the formation of $O_2^{\bullet-}$ (Becker *et al.*, 1999). There might also be continuous production of $O_2^{\bullet-}$ by vascular endothelium to neutralize nitric oxide (Souchard *et al.*, 1998), production of $O_2^{\bullet-}$ by other cells to

regulate cell growth and differentiation (Masters, 1996), and the production of $O_2^{\bullet-}$ by phagocytes during the respiratory burst (Curnutte and Babior, 1987).

Any biological system generating $O_2^{\bullet-}$ will also produce hydrogen peroxide (H_2O_2) as a result of a spontaneous dismutation reaction. In addition, several enzymatic reactions, including those catalyzed by glycolate oxidase and D-amino acid oxidase, might also produce H_2O_2 directly (Chance *et al.*, 1979; Schroder and Eaton, 2008). H_2O_2 is not a free radical itself, but is usually included under the general heading of ROS because it is a weak oxidizing agent that directly damages proteins and enzymes containing reactive thiol groups. However, its most vital property is its ability to cross cell membranes freely, which $O_2^{\bullet-}$ generally cannot do (Halliwell and Gutteridge, 1990; Schroder and Eaton, 2008). Therefore, H_2O_2 formed in one location might diffuse a considerable distance before decomposing to yield the highly reactive hydroxyl radical, which is likely to mediate most of the toxic effects ascribed to H_2O_2. Therefore, H_2O_2 acts as an agent to transmit free radical induced damage across cell compartments and between cells. In the presence of H_2O_2, myeloperoxidase will generate hypochlorous acid and singlet oxygen, a reaction that plays an important role in the killing of bacteria by phagocytes (Tatsuzawa *et al.*, 1999).

The hydroxyl radical (•OH), or a closely related species, is probably the final mediator of most free radical induced tissue damage (Lloyd *et al.*, 1997). $O_2^{\bullet-}$ and H_2O_2 exert most of their pathological effects by giving rise to •OH formation. The reason for this is that •OH reacts, with extremely high rate constants, with almost every type of molecule found in living cells including carbohydrates, amino acids, lipids, and nucleotides. Although •OH formation can occur in several ways, by far the most important mechanism *in vivo* is likely to be the transition metal catalysed

decomposition of $O_2^{\bullet-}$ and H_2O_2 (Stohs and Bagchi, 1995). The most important transition metals in human disease are iron and copper. These elements play a key role in the production of •OH *in vivo* (Stohs and Bagchi, 1995). H_2O_2 can react with iron II (or copper I) to generate the •OH (Equation 1).

Equation 1:

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

This reaction can occur *in vivo*, but the situation is complicated by the fact that $O_2^{\bullet-}$ (the major source of H_2O_2 *in vivo*) will normally also be present. $O_2^{\bullet-}$ and H_2O_2 can react together directly to produce the •OH, but the rate constant for this reaction in aqueous solution is virtually zero. However, if transition metal ions are present a reaction sequence is established that can proceed at a rapid rate (Equation 2).

Equation 2:

$$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$$

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

net result:

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

The net result of the reaction sequence illustrated in Equation 2 is known as the Haber-Weiss reaction. Although most iron and copper in the body are sequestered in forms that are not available to catalyze this reaction sequence, it is still of importance as a mechanism for the formation of the •OH *in vivo*. The actual reactions, however, may be more complex than those described above and it is possible that other reactive intermediates such as the ferryl and perferryl radicals might also be formed (Halliwell and Gutteridge, 1995).

The rate of generation of ROS appears to be balanced with the status of the antioxidant defence system under physiological conditions. Oxidative stress occurs

when there is excessive production of ROS or a deficiency in the antioxidant system. During oxidative stress, the excess ROS might interact with a variety of macromolecules including lipids, proteins and nucleic acids resulting in cell dysfunction, and apoptosis (Valko *et al.*, 2006). Cytotoxic effect of excessive ROS has been frequently implicated in cardiovascular diseases including in the pathogenesis of high blood pressure and atherosclerosis (Touyz and Schiffrin, 2004; Ward and Croft, 2006; Paravicini and Touyz, 2008).

1.2.3 Oxidative damage to lipid, protein and DNA

Studies *in vitro* reveal that elevated ROS can be an important mediator in causing damage to cell structures. The three main targets of ROS attack are lipid, proteins, and nuclei acids (Valko *et al.*, 2006).

Extensive lipid peroxidation in biological membranes causes alteration in fluidity, decreased membrane potential, increased permeability to hydrogen and other ions, and eventual rupture of the cell. Peroxyl radicals can be rearranged via a cyclisation reaction to endoperoxides (precursors of malondialdehyde) with the final product of the peroxidation process being malondialdehyde (MDA) (Marnett, 1999). The major aldehyde product of lipid peroxidation other than MDA is 4-hydroxy-2-nonenal (4-HNE). MDA is mutagenic in bacterial and mammalian cells and carcinogenic in rats. 4-HNE is weakly mutagenic but appears to be the major toxic product of lipid peroxidation (Esterbauer *et al.*, 1991).

Oxidation of proteins changes their primary structure, including the overall charge, folding, and hydrophobicity. Oxidation of the amino acid residues and/or peptide backbone of proteins results in the generation of protein carbonyl group (Marnett *et al.*, 2003). The process is initiated by hydrogen abstraction from the α-

carbon in a peptide chain. Oxygen molecule attacks the α-carbon-centered radical to form peroxide intermediates leading to rearrangement and subsequent cleavage of the peptide bond to form carbonyl-containing peptides (Dean *et al.*, 1997). It has been demonstrated that the side chains of all amino acid residues of proteins, in particular cysteine and methionine residues of proteins are susceptible to oxidation by the action of ROS/RNS (Stadtman, 2004). Oxidation of cysteine residues may lead to the irreversible formation of mixed disulphides between protein thiol groups (–SH) and low molecular weight thiols, in particular GSH (S-glutathiolation). Oxidatively modified proteins are susceptible to increased aggregation and degradation (Nystrom, 2005). Quantitative measurement of carbonyl concentration is a good indicator of ROS-mediated protein oxidation (Dalle-Donne *et al.*, 2005).

ROS-induced damage of DNA include changes in both DNA structure and chemistry, with the result being strand breakage leading to gene mutations in the event a cell is rendered incapable of DNA repair, or there is miscoding caused by the repair process (Schnackenberg, 2002). The hydroxyl radical is known to react with all components of the DNA molecule, damaging both the purine and pyrimidine bases and also the deoxyribose backbone (Halliwell, 1991). The most extensively studied DNA lesion is the formation of 8-OHdG, which is derived from hydroxyl attack on deoxyguanosine (Shigenaga and Ames, 1991). Irreversible modification of genetic material resulting from these “oxidative damage” incidents represents the first step in mutagenesis, carcinogenesis, and ageing (Valko *et al.*, 2007).

Whether ROS attacks these targets significantly depends upon the delicate balance between the levels of ROS and antioxidants. Under many conditions, an increase in ROS formation also concurrently signals the activation of antioxidant enzymes to aid in the increased metabolism necessary to achieve redox balance.

However, when the amount of ROS produced exceeds the capabilities of the antioxidants, oxidative stress results (Halliwell and Gutteridge, 1984; Young and Woodside, 2001). Cells are normally able to defend themselves against ROS damage through the concurrent existence of the antioxidant defence systems in the cell.

1.2.4 Antioxidant defence systems

The antioxidant system of the cell can be divided into two major groups: enzymatic antioxidants (e.g., superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione S-transferase) and non-enzymatic antioxidant including chain breaking antioxidants (e.g., glutathione, melatonin, vitamin E and C) and transition metal binding proteins (e.g., ferritin, transferrin, lactoferrin, and caeruloplasmin). The first line of antioxidant defence in cell is provided by the antioxidant enzymes (Gutteridge and Halliwell, 1994).

1.2.4 (a) Enzymatic antioxidants

Primary enzymatic antioxidants include superoxide dismutase, catalase and glutathione peroxidase (Chance *et al.*, 1979). A number of enzymes are involved in the supply of substrates and reducing power e.g.NADPH for primary antioxidants such as glutathione reductase and glucose-6-phosphate dehydrogenase, but they do not directly remove ROS. Glutathione S-transferase conjugates reduced glutathione with xenobiotics and toxins that can potentially generate ROS.

(i) Superoxide dismutase

Superoxide dismutase (SOD) protects oxygen-metabolizing cells against harmful effects of superoxide free-radical, $O_2^{\bullet-}$. SOD catalyses the dismutation of superoxide to hydrogen peroxide and oxygen (Equation 3) (Gutteridge and Halliwell, 1994).

Equation 3:

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

SOD, however must work in conjunction with other enzymes that help decompose H_2O_2 like e.g. catalase and glutathione peroxidase. If left unchallenged, H_2O_2 could migrate from its site of formation and form the very reactive ·OH when it comes in contact with iron or copper ions. H_2O_2 also possesses a serious threat to copper-zinc containing SOD and glutathione peroxidase itself as it inactivates these enzymes (Gutteridge and Halliwell, 1994).

In humans, three isoforms of SOD have been described, each with a specific subcellular location and different tissue distribution: (1) copper-zinc containing SOD (Cu/Zn-SOD) is preponderant in the cytoplasm, nucleus, and peroxisomes (2), manganese-containing SOD (Mn-SOD) present in the mitochondria and a small fraction in the cytosol, and (3) extracellular copper-zinc containing SOD (EC-SOD).

Cu/Zn-SOD is principally found in the cytosol and peroxisomes. It is composed of two identical subunits of 32.5kDa in which each subunit contains one copper and one zinc ion bridged by a histamine residue at its active site (McCord and Fridovich, 1969). It deals with $O_2^{\bullet-}$ produced by cytosolic oxidases and from the cytochrome P450 enzymes, which are located in the endoplasmic reticulum of the cell. Cu/Zn-SOD contributes the most to the total SOD activity. It is believed to play a major role in the first line of antioxidant defence system (Marklund, 1982).

Mn-SOD is found in the mitochondria of almost all cells. It is a homotetramer containing one manganese ion per subunit with a molecular weight of 96kDa. Mn-SOD, presumably removes $O_2^{\bullet-}$ produced as a result of electron leakage to O_2 from the mitochondrial electron transport chain and by mitochondria oxidase enzymes (Fridovich, 1982). Mn-SOD has been shown to be greatly induced and depressed by cytokines, but is only moderately influenced by oxidants in human fibroblasts. Cu/Zn-SOD and Mn-SOD are readily distinguished, even in crude extract, since the former is inhibited by cyanide and is stable to treatment by a mixture of chloroform and ethanol, whereas the latter is resistant to cyanide but is denatured by chloroform and ethanol (Gutteridge and Halliwell, 1994; Stralin and Marklund, 1994).

EC-SOD is a secretory copper and zinc containing SOD which is the major SOD detectable in cellular fluids (McIntyre *et al.*, 1999). It is synthesized by only a few cell types, including fibroblasts and endothelial cells. EC-SOD has a molecular weight of 130kDa. It is a homotetramer in which each subunit contains one copper and one zinc ion. EC-SOD is believed to play a role in the regulation of vascular tone, because endothelial derived relaxing factor e.g. nitric oxide is neutralized in the plasma by superoxide (McIntyre *et al.*, 1999).

Unlike most enzymes, SOD lacks Michaelis constant (K_m), and its catalytic activity increases with increasing $O_2^{\bullet-}$ concentration (Chance *et al.*, 1979). Hence, assays of SOD activity are usually based on indirect method that involves inhibiting reaction in which $O_2^{\bullet-}$ is generated (Fridovich, 1982). Therefore, it is not always meaningful to compare reported SOD activities between studies that use different assay methods.

(ii) Catalase

Catalase, (CAT) is a tetrameric enzyme with a molecular weight of 240kDa. Each identical subunit of 60kDa contains a haem group and a molecule of NADPH (Kirkman *et al.*, 1987). It is largely located within cells in peroxisomes, which also contain most of the enzymes capable of generating H_2O_2 (Schrader and Fahimi, 2006). The amount of CAT in the cytoplasm and other subcellular compartments remains unclear, because peroxisomes are easily ruptured during manipulation of cells. It is well distributed in all the cells but the greatest activity is believed to be present in hepatocytes and erythrocytes. Although it has a very large capacity to destroy H_2O_2, its affinity for H_2O_2 is low. It catalyses the two stage conversion of H_2O_2 to water and oxygen in the presence of iron as a cofactor (Equation 4 & 5).

Equation 4 & 5:

$$\text{CAT} - \text{Fe (III)} + H_2O_2 \rightarrow \text{compound I}$$

$$\text{compound I} + H_2O_2 \rightarrow \text{CAT} - \text{Fe(III)} + 2\,H_2O + O_2$$

The rate constant for the reactions described above is extremely high (~107 M/sec), implying that it is virtually impossible to saturate the enzyme *in vivo*. CAT can also oxidize different toxins, such as formaldehyde, formic acid, phenols, and alcohols. In doing so, it uses hydrogen peroxide (Mates *et al.*, 1999) according to the reaction as stated in Equation 6.

Equation 6:

$$H_2O_2 + H_2R \rightarrow 2\,H_2O + R$$

Azide and cyanide are both inhibitors of catalase, an inhibition often used to partition CAT activity from glutathione peroxidase activity in enzymes assays in crude tissue extracts. Caution should be taken with the reported CAT activity, because it is determined not only by the enzyme protein present in the assay medium

but also by the concentration of H_2O_2 used. Therefore, it is not always meaningful to compare reported CAT activities between studies that use different concentration of H_2O_2 (Gutteridge and Halliwell, 1994).

(iii) Glutathione peroxidase

Glutathione peroxidase, GPx catalyzes the reduction of H_2O_2 to H_2O utilizing the reducing equivalents from reduced glutathione (GSH) in which GSH is oxidized to oxidized glutathione, GSSG (Equation 7). In addition, other peroxides including lipid hydroperoxides, can also act as substrates for GPx, which might therefore play a role in repairing damage resulting from lipid peroxidation (Equation 8) (Forstrom *et al.*, 1978).

Equation 7:

$$H_2O_2 + 2GSH \rightarrow GSSG + 2\ H_2O$$

Equation 8:

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

GPx is a homotetramer with each 22kDa subunit bound to a selenium atom existing as a selenocysteine (Forstrom *et al.*, 1978). It requires selenium at the active site for its catalytic function, and deficiency of this enzyme might occur in the presence of severe selenium deficiency (Nakane *et al.*, 1998). There are six isoforms of GPx protein that have been identified to date, which are referred to as GPx-1 to GPx-6. Data from activity assays *in vitro* suggest that all the six isoforms use GSH to catalyze the reduction of H_2O_2 and lipid peroxides. GPx-1 is the most abundant selenoperoxidase and is ubiquitously expressed in almost all tissues (Flohe *et al.*, 1973; Rotruck *et al.*, 1973). GPx-2 expression is most prominent in the gastrointestinal tract (Chu *et al.*, 1997). Expression of GPx-3 is greatest in the

kidney, although this enzyme is also expressed in various other tissues and is secreted into extracellular fluid as a glycoprotein (Takakhashi *et al.*, 1987; Yoshimura *et al.*, 1991). Different from other glutathione peroxidases, GPx-4 or phospholipid hydroperoxide is not a tetramer but rather a monomer, and is the only GPx enzyme that reduces phospholipid hydroperoxides (Thomas *et al.*, 1990). Recently, GPx-6 was identified as a selenoprotein in the human genome by homology search (Kryukov *et al.*, 2003). However, GPx-6 from rodents and GPx-5 from both humans and rodents do not contain selenocysteine or selenium (Kryukov *et al.*, 2003).

The activity of GPx is dependent on the constant availability of GSH (Holben and Smith, 1999). The ratio of GSH to GSSG is usually kept very high (more than 99:1) as a result of the activity of the enzyme glutathione reductase. The NADPH required by this enzyme to restore the supply of GSH is provided by the pentose phosphate pathway. Any competing pathway that utilizes NADPH (such as the aldose reductase pathway) might lead to a deficiency of GSH and hence impair the action of GPx (Meister, 1981).

There is a substrate overlap between GPx and CAT. GPx has a much greater affinity for H_2O_2 at low concentrations, and CAT, with its low affinity is usually recruited at high concentrations. The K_m of CAT (up to 25mM) is >4000-fold higher than the K_m of GPx (6μM) (Carmagnol *et al.*, 1983; Mozaffar *et al.*, 1986). This means at low steady state substrate concentrations, GPx plays a more prominent role in removing H_2O_2, and at high substrate concentrations, CAT plays a greater effective role in removing H_2O_2. Therefore, GPx is thought to be a major defence mechanism in low level of oxidative stress and CAT is effective in high level of

oxidative stress or in the case of limited GPx reaction in protecting cells from H_2O_2 (Wassmann *et al.*, 2004)

(iv) Glutathione reductase

Glutathione reductase, GR is a dimer consisting of two identical subunits of molecular mass 50kDa each, and possesses one flavin molecule at its active site. It is an enzyme that reduces glutathione disulfide (GSSG) to the sulfhydryl form, GSH. GR catalyzes the reduction of one mole of GSSG into two moles of GSH in the presence of one mole of NADPH as electron donor (Fridovich, 1978) (Equation 9). The required NADPH is usually formed by the pentose phosphate pathway (Champe, 2008). GR is essential for the GSH redox cycle, which maintains adequate levels of cellular GSH. A high GSH/GSSG ratio is essential for protection against oxidative stress.

Equation 9:

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

(v) Glutathione S-transferase

Glutathione S-transferase, GST is an important part of the cellular detoxification system and protects cells against reactive oxygen metabolites. GST protects cells against electrophiles and xenobiotics by conjugating them to glutathione, thereby neutralizing their electrophilic sites, and rendering the products more water soluble. The glutathione conjugates are metabolized further to mercapuric acid and then excreted (Boyland and Chasseaud, 1969).

All eukaryotic species possess multiple cytosolic and membrane-bound GST isoenzymes (microsomal GST and leukotriene C4 synthetase), each of which