Effect of palm oil (Elias guinensis) leaf standardized extract on progression of renal dysfunction and arterial stiffness in normal and high fat diet fed Sprague Dawley rats with induced nephrotoxicity.

By

Zaid O. Ibraheem

Thesis submitted in fulfillment of the requirements for the degree of master of science

2011

ACKNOWLEDGEMENT

I would like to pay thanks to Almighty Allah, who gave me strength to survive in this research.

I would like to extend my deepest gratitude to my supervisor Professor Munavvar Zubaid Abdul Sattar and my co-supervisor Dr. Hassaan Rathore for their help and guidance throughout the period of the project. Their patience and care influenced me to work hard and demand success and for this, I am very grateful to them for putting at my disposal every facility that they had which I need during the course of the study.

I wish to extend my thanks to the Dean of the School of Pharmaceutical Sciences and all the administration staff for their kind support and providing an opportunity to finish the task in a nice way. I also wish to thank the Dean of the Institute Postgraduate Studies and his staff who helped me in one way or other. I acknowledge the support given by non-academic staff of school of Pharmaceutical Science.

I would like to pay my special thanks to Puan Yong and Dr. Gurjeet Kaur for their exclusive help in pursuing my study. My thanks also go to my lab mates for their assistance and encouragement throughout the study.

Finally and most importantly, I am indebted to my parents and my friend Tan who stood by me during the most difficult times.

Zaid

TABLE OF CONTENTS

CHAPTER ONE

INTRODUCTION

CHAPTER TWO

MATERIALS AND METHOD

CHAPTER THREE

RESULTS

LIST OF FIGURES

LIST OF TABLES

LIST OF EQUATIONS

LIST OF ABBREVIATIONS

Ad libitum	To be taken as wanted
ADH	Anit-diuretic hormone
AGRP	Agouti related peptide
AI%	Augmentation index
ANP	Anti -natruitic peptide
AT1	Angiotensin receptor
ATP	Adenosin triphosphate
B.W	Body weight
BAT	Brown adipose tissue
BHT	Butylated hydroxyl toleun
CD	Conjugated diens
CETP	Cholesterol ester transfer protein
cGMP	Cyclic guanosine mono phosphate
CHO.	Cholesterol
Cr. Cl.	Creatinine clearance
CSF	Colony stimulating factor
CT	Collecting tubules
DBP	Diastolic blood pressure
DCT	Distal convoluted tubules

DN	Dicrotic notch
DNA	Deoxyribo nucleic acid
DPC	Dextrapropoxyphene
DPPH	1-1-Diphenyl-2-Picryl-Hyrazil
ECF	Extracellular luid
ECV	Extracellular volume
ETC	Electron transport chain
FE	Fractional excretion
Eq.	Equation
$FeCl_3$	Ferric chloride
FFA	Free fatty acids
FRSA	Free radical scavenging activity
g	Gram
g.m.wt	Gram molecular weight
GFR	Glomerular filtration rate
GIT	Gastro-intestinal tract
GN	Glomerulonephritis
GPx.	Glutathione peroxidase enzyme
Gr.	Gluthaion reductase
GSH	Reduced form of glutathion
GSSG	Oxidized frm of glutathione
H & E	Heamatoxylin and eosine
HBSS	Hangs balance salt solution

HDL	High density lipoprotein
HMG-Co-A reductase	3-hydroxy-4-glutaryl-CoA-reductase enzyme
I.P	Intraperitoneal
I.R	Insulin resistance
I.U	International unite
ICAM	Intracellular adhesion molecule
IDL	Intermediate density lipoprotein
IL	Interleukin
IRS-1	Insulin resistance substrate -1
Kcal	Kilo calorie
kg	Kilogram
KI	Kidney index
LCAT	Lecithin cholesterol acyl transferase enzyme
LDL	Low density lipoprotein
MAP	Mean arterial pressure
MDA	Malonyldialdehyde
MDAR	Monohydroascorbate reductase
MIC	Minimum inhibitory concentration
ml	Milliliter
MMps	Metaloproteinase enzyme
MPO	Myeloperoxidase
MSH-α	Melanocyte stimulating hormone
MUSFFAs	Mono-unsaturated free fatty acids

N/S	Normal saline
NADP	Ncotonomide adenine dinucleotide
NF-KB	Natural factor- KB
$NO^{\cdot}$	Nitric oxide
NO_2^-	Nitrite ion
NO_3^-	Nitrate ion
NOS	Nitric oxide synthase enzyme
O.D	Optical density
OGTT	Oral glucose tolerance test
$ONOO^{\cdot}$	Peroxynitrate
P_{BC}	Hydrostatic pressure in Bowman capsule
PCT	Proximal convoluted tubules
PDGF	Platelet derived growth factor
P_{GC}	Glomerular capillary hydrostatic pressure
Pi	Inflection point
PKC-Ǿ	Protein kinase C-theta
PLPT	Phospholipids transfer protein
P.O	Per oral.
POLE	Palm oil leaf extract
PP	Pulse pressure
PPAR	Peroxosome proliferation activating factor
P_{uf}	Ultrafiltartion pressure
PUFFAs	Polyunsaturated free fatty acids

PUSAFFA	Poly unsaturated free fatty acids
PWV	Pulse wave velocity
RAAS	Renin Angiotensine aldosteron system
RBCs	Red blood corpuscles
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPM	Revolution per minute
S.E.M	Standard error of Mean
SAFFAs	Saturated free fatty acids
SBP	Systolic blood pressure
SOD	Super oxide dismutase
SREBP	Sterol regulatory element binding protein
STD	Standard
T.G	Triglyceride
TBA	Thiobarbituric acid
TCA	Tri-chloro acetic cid
TNF	Tumor necrosis factor
UCP	Uncoupling protein
U_{cr}	Concentration of creatinine in urine
UFFAs	Un-esterified free fatty acids
UFR	Urine flow rate
UK^{+}	Concentration of potassium in urine
UNa^{+}	Concentration of sodium in urine

USAFFAs	Unsaturated free fatty acids
$U_{vol.}$	Urine volume
VCAM	Vascular cell adhesion molecule
VD	Volume of distribution
VLDL	Very low density lipoprotein
WAT	White adipose tissue
$\prod_{BC}$	Oncotic pressure in Bowman capsule
$\prod_{GC}$	Oncotic pressure in glomerular capillary
µg	Microgram
4-HHE	4- Hydroxyhexenal
4-HNE	4-Hydroxynonenal

Kesan ekstrak daun sawit (pechan etanol terstandard) terhadap kemajuan kegagalan ginjal dan kekakuan arteri dalam tikus Sprague-Dawley yang normal dan yang diberi diet lemak tinggi melalui kenofrotolsikan

ABSTRAK

Asid lemak bebas tepu (saturated free fatty acids, SAFFA) mengaruh kesan mudarat mereka melalui aruhan laluan inflamatori intrasel yang berkaitan dengan rintangan insulin. Dalam bidang bioperubatan, keadaan ini dikenali sebagai sindrom metabolisme. Gentamicin merupakan suatu antibiotik aminoglikosid yang digunakan dengan meluas. Tindakan nefrotoksiknya dicirikan oleh tubul berlingkar proksimal dan kerosakan pada membran glomerular. Mekanisme kenefrotoksikan berkaitan rapat dengan stres oksidatif dan penjanaan radikal bebas.

Dewasa ini ditemui bahawa ekstrak daun sawit mempunyai beberapa kepentingan bioperubatan kerana polifenol yang terkandung di dalamnya boleh bertindak balas bagi mengurangkan stres oksidatif.

Kajian kami bertujuan menentukan impak daripada menggantikan elemen diet penting dalam makanan yang diperkaya dengan SAFFA terhadap kemajuan kerosakan ginjal dan kaitannya dengan perubahan hemodinamik dan metabolisme dalam model tikus yang kenefrotoksikan diaruh dengan gentamicin. Kajian ini juga menilai kesan

profilaktik dalam ekstrak daun sawit (palm oil leaf extract POLE) terhadap kemajuan gangguan ini.

Ketidakfungsian ginjal dinilai melalui ukuran serum kreatinin, klearans kreatinin, pecahan dan kumuhan / eksresi mutlak daripada natrium dan kalium. Malonildialdehid (suatu biopenanda bagi stres oksidatif) dalam homogen ginjal dan kumuhan protein urin. Keputusan menunjukkan bahawa terdapat insidens kenefrotoksikan yang amat tinggi dalam tikus yang diberi diet yang diperkaya dengan SAFFA dibandingkan dengan tikus yang diberi diet yang standard. Kenefrotoksikan digred sebagai sedikit bagi kumpulan gagal-ginjal yang diberi diet yang standard. Sebaliknya, digred sebagai sederhana hingga teruk, bagi yang diberi diet yang diperkaya dengan SAFFA. Dos POLE yang tinggi boleh membataskan kemajuan kenefrotoksikan dan perkembangan stres oksidatif dalam tisu ginjal. Dari sudut lain, sindrom metabolisme dinilai melalui ujian tolerans oral-glukos (oral-glucose tolerance test, OGTT), ukuran profil lipid dan indeks obesiti / kegemukan. Kajian menunjukkan kemerosotan dalam sindrom metabolisme pada tikus yang diberi diet yang diperkaya dengan SAFFA. Pemberian gentamicin bersama diet ini menyebabkan kemerosotan yang amat sedikit dalam tolerans glukosa tanpa impak yang ketara terhadap profil lipid. Sindrom metabolisme yang diaruh dengan SAFFA dikaitkan dengan perubahan kardiovaskular yang dicirikan oleh hipertensi dan kekakuan yang amat tinggi pada arteri. Selepas pemberian gentamicin bersama diet SAFFA, didapati tekanan semakin berkurangan dan kekakuan kekal. Sebaliknya, injeksi gentamicin pada tikus yang diberi diet standard, tidak menunjukkan sebarang perubahan kardiovaskular. Kemajuan sindrom metabolisme dan perubahan kardivaskular terbatas sedikit selepas pemberian POLE.

Secara keseluruhan, sindrom metabolisme yang diaruh SAFFAs mempercepat kemajuan kenefrotoksikan dalam mekanisme yang berkaitan untuk penjanaan radikal bebas yang tinggi. POLE sebagai suatu produk makanan kesihatan menghasilkan kesan propilaktik yang amat sedikit terhadap impak ini. .

Effect of palm oil (Elias guinensis) leaf standardized extract on progression of renal dysfunction and arterial stiffness in normal and high fat diet fed Sprague Dawley rats with induced nephrotoxicity.

ABSTRACT

Saturated free fatty acids (SAFFAs) trigger their deleterious effects through inducing the intracellular inflammatory pathway related to insulin resistance. This results in a constellation of biomedical disorders known as metabolic syndrome. Gentamicin is a widely used aminoglycoside antibiotic. Its nephrotoxic action is characterized by both proximal convoluted tubules and glomerular membrane damage. The mechanism of nephrotoxicity is closely related to oxidative stress and free radicals generation.

It is found nowadays that palm oil leaf extract has got some biomedical importance due to its content of polyphenols which act to counteract the oxidative stress.

Our study aimed to find the impact of replacing the essential dietary elements in food by SAFFAs enriched fats on progression of renal damage and its associated hemodynamic and metabolic changes in rat's model of gentamicin induced

nephrotoxicity. The study also evaluated the prophylactic effect of palm oil leaf extract (POLE) on progression of these disorders.

Renal dysfunction was assessed through measuring serum creatinine, creatinine clearance, fractional and absolute excretion of both sodium and potassium, malonyldialdehyde (a biomarker of oxidative stress) in renal homogenate and the urinary protein excretion. The results showed a higher incidence of nephrotoxicity in the rats fed with the diet enriched with SAFFAs as compared to those fed with the standard diet. The nephrotoxicity was graded as mild for the renal failure group fed with the standard diet and moderate to severe for those fed with the SAFFAs enriched diet. High dose of POLE could have limited the progression of nephrotoxicity and oxidative stress development in the renal tissue. Metabolic syndrome was assessed through performing oral-glucose tolerance test (OGTT), measuring the lipid profile and obesity indices. The study showed deterioration in metabolic syndrome after feeding the rats with the diet rich in SAFFAs. Gentamicin co-administration along with this diet deteriorated the glucose tolerance without producing a noticeable impact on lipid profile. The metabolic syndrome induced by the SAFFAs was associated with cardiovascular changes characterized by hypertension and higher arterial stiffness. The blood pressure was reduced after gentamicin co-administration along with the SAFFAs enriched diet while the stiffness remained comparable. Moreover, gentamicin injection to the rats fed with the standard diet did not produce any cardiovascular change. Progression of metabolic syndrome was limited after POLE co-administration.

Overall, metabolic syndrome induced by SAFFAs hastens the progression of nephrotoxicity in a mechanism related to higher free radicals generation. POLE as a health product produced a prophylactic effect against these impacts.

Chapter One

Introduction

1.1 Physiology of kidneys

Kidneys are bean shaped organs, located in the abdominal cavity. They have a contact with the diaphragm and the posterior abdominal muscles and are surrounded by both the 11^{th} and 12^{th} ribs. Both kidneys are surrounded by the epirenal adipose connective tissue and some visceral organs. (Kinne, 1989).

1.1.1 General structure

Kidneys are oval shaped organs. They have two parallel borders; the concave border which is directed toward the body's midline and the convex border which is directed laterally. There is a slit in the middle of the concave border called helium (the site where the kidney receives the renal artery and both the renal vein and ureter emerge (Lote, 2000).

Renal tissue is made up of two layers; cortex and medulla. It is surrounded by a layer of loose connective tissues, called renal capsule. The cortex appears as a red colored spotted area due to the presence of numerous capillary bundles that represent Malpighian corpuscles. The saggital section of the kidney shows the presence of 8-18 pyramidal triangular areas whose bases are on the cortex and their apices are inside the

medulla. These areas are known as renal pyramids, they are marked by fine converging lines known as medullary rays which represent the collecting ducts. Renal pyramid apices are known as renal papillae where the urine that is formed in conducting tubules leave the pyramids through some small orifices into a space called the renal sinus. Then it drains to the renal pelvis and ureter (Kinne, 1989; Lot, 2000).

1.1.1.1 Nephrons

Nephrons are the basic functional units of renal tissue. There are about one million nephrons in each kidney. They are composed of the filtration unit that is known as the glomerulus and a tubular system; responsible for re-absorption and secretion of biochemical entities. Each renal glomerulus (Malpighian corpuscle) is made of a globular bundle of capillaries surrounded by a crescent shaped chamber (Bowman's capsule). The glomerular capillary is made of separate loops of capillaries but it appears as a tangled mass of vessels. Bowman's capsule is made up of a single layer of flat cells known as podocytes (Kinne, 1989; Sherwood, 2008).

The tubular system of nephron is made up of four distinct zones; proximal convoluted tubule (PCT), loop of Henle, distal convoluted tubule (DCT) and collecting tubule (CT) that deliver urine into the orifices present on renal papillae. Both PCT and DCT are the twisted and coiled portions located in the renal cortex. Loop of Henle & CT constitute the converging lines which mark the medullary renal pyramid (Sherwood, 2008; Kinne, 1989).

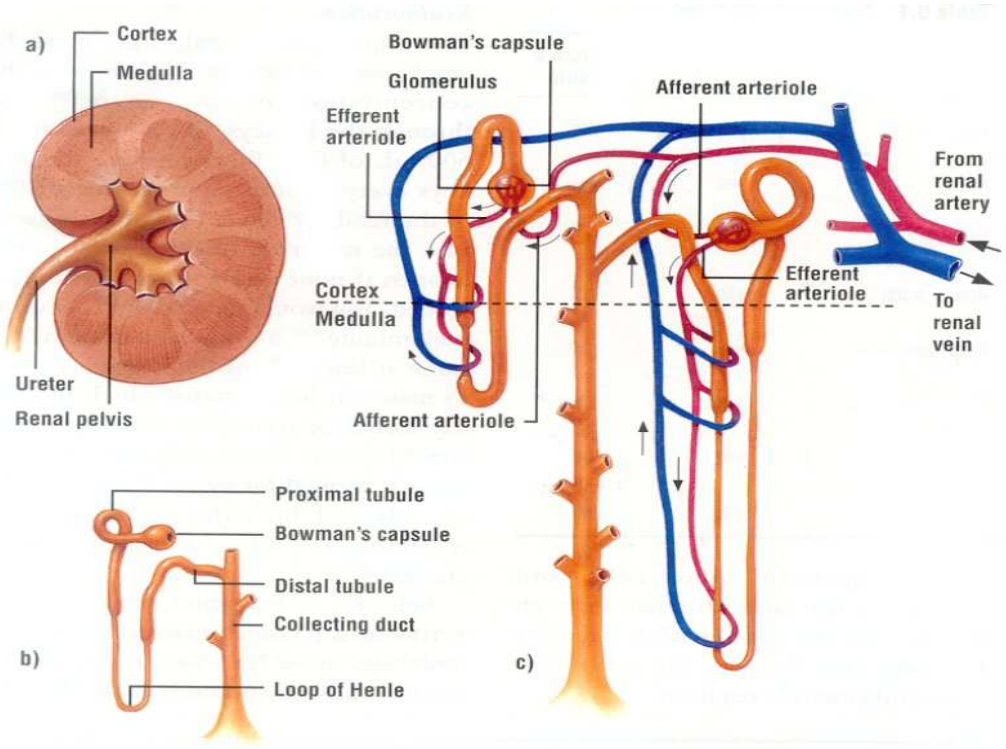


Figure 1.1 a) Saggital structure of kidney, **b)** General structure of nephron **c)** Detailed outline of renal tubules. (Adapted from http://www.google.com.my/images?hl=en&q=kidney+structure+image).

1.1.1.2 Renal glomeruli

Renal glomeruli are made up of a capillary bundle surrounded by a crescent shaped chamber, known as Bowman's capsule. Blood enters the glomerular capillary tuft through the afferent arteriole and leaves it through the efferent arterioles. Along its passage through the capillary tuft, some of the plasma passes into the lumen of Bowman's capsule through the glomerular membrane (Kinne, 1989; Sherwood, 2008).

The glomerular membrane is made up of the wall of glomerular capillary loop and the alignment of epithelial cells called podocytes which constitute the wall of Bowman capsule. The glomerular membrane sieve is made of three filtration layers; the first is represented by the fenestrae distributed among the capillary endothelial cells. It is a coarse filter layer which retains high gram molecular weight (g.m.wt) proteins, fat globules and blood cells. Underneath the capillary endothelium, there is another filtration unit. It is represented by an alignment of negatively charged glycoprotein and glycolipid molecules. It hinders penetration of the negatively charged proteins across the glomerular membrane and permits penetration of molecules with radii exceeding 60 °A. The third unit is represented by podocytes. Podocytes are irregularly shaped and possess a number of intermingled primary and secondary processions leaving slits of rectangular pores measuring 140 x 40 °A, providing the most physically restrictive filter (Ota, et al, 1980).

1.1.1.3 Proximal convoluted tubules PCT

PCT is a joining segment between Bowman's capsule and loop of Henle. It is lined by simple cuboidal epithelial cells which are uniform in all parts of the tubule with some ultra-structural differences. Their plasma membrane plays an important role in their function. The site of the membrane that faces the tubular lumen is called the apical membrane while the one which faces the interstitial fluid space is called the basolateral membrane. The apical membrane is covered by a border made of densely packed microvilli, called brush border which increases the surface area upon which re-

absorption takes place while the basolateral one contains proteins related Na^{+}-K^{+} pump (Kinne, 1989). Their cytoplasm is densely packed, acidophilic in nature due to plenitude of mitochondria. Under the light microscope there is no obvious discrete margin among cells except for lateral interdigitations near the apical membrane creating a tight junction between the neighboring cells and leaving a space (called the intercellular space) between them at the site of basolateral membrane (Moffat, 1975). Most of the biochemical entities cross the PCT wall into the interstitial space either by trans-cellular pathway (crossing both the apical and basolateral membranes) or by crossing the tight intercellular junction into the intercellular space and then to interstitial fluid (para-cellular pathway) (Neumann and Rector, 1979).

According to the gross appearance, PCT is divided into; *pars convoluta & pars recta. Pars convoluta is* the initial convoluted portion while *recta* are the straight descending second portion. PCT is subdivided also according to the functional difference into **S1** and **S2** segments or according to ultra structural difference into **S1, S2** and **S3** (Tisher and Osborn, 1969 ; Barrett and Heidger, 1975).

1.1.1.4 Loop of Henle

It is the joining segment between PCT and DCT. It is made up of two parallel tubes penetrate deep into medulla where they connect in a hair pin like manner. It is surrounded by an anastomosis of capillary system called *vasa recti renis*. Both loop of Henle and *vasa recta renis* play an important role in countercurrent multiplication

mechanism that is responsible for water re-absorption from the collecting tubules (Moffat, 1975; Kinne, 1989).

1.1.1.5 Distal convoluted tubules

It is the joining segment between loop of Henle and the collecting tubular system. It is lined by simple cuboidal epithelial cells, similar to that in PCT with some minor differences as paucity of brush border, making their lumen wider than that of PCT. Their cytoplasm is less acidic and less densely packed than that of PCT due to their lower content of mitochondria. DCT is responsible for re-absorption of some ions as chloride, sodium, potassium and calcium and fine adjustment of tubular fluid and PH. A part of DCT gets near the afferent arteriole; its epithelial cells are more tightly packed and specialized. It is called *macula densa* that is responsible for blood osmolality monitoring (Moffat, 1975; Kinne, 1989).

1.1.1.6 Collecting tubules system

A series of tubules drain the nephronal tubular fluid from DCT into renal papilla. The lining epithelium of the CT system participates in electrolyte homeostasis through potassium ion secretion and sodium re-absorption, pH adjustment and the anti-diuretic hormone (ADH) mediated water re-absorption (Haas, et al., 1979).

1.1.2 Renal glomerular filtration and tubular function

Plasma free fluid transverses the three abovementioned sieving layers of the glomerulus into Bowman capsule. Rate of transfer is determined by Starling's hypothesis which states that the filtration pressure of any capillary system is the algebraic sum of the opposing hydrostatic and oncotic pressures (Renkin and Robinson, 1974).

$P_{UF} = (P_{GC} + \prod_{BC}) - (P_{BC} + \prod_{GC}) \longrightarrow$ Eq. 1.1

P_{UF}, P_{GC}, P_{BC}, $\prod_{BC}$, $\prod_{GC}$ are the ultra-filtration pressure, glomerular capillary hydrostatic pressure, hydrostatic pressure in Bowman's capsule, oncotic pressure in Bowman's capsule and oncotic pressure in glomerular capillary, respectively. $\prod_{BC}$ is mostly set to be zero due to virtual exclusion of proteins in the glomerular filtrate (Sherwood, 2006).

The ultra-filtration pressure is higher at the afferent end of glomerular capillary; it is about 12 mmHg and drops to zero at the efferent end of glomerular capillary as the blood transverses the length of glomerular capillary. This drop is attributed to the slight decrease in hydrostatic pressure due to resistance to flow caused by glomerular capillary and to the increase of intra-glomerular capillary oncotic pressure that result from the filtration of the protein free plasma, a factor that offsets the hydrostatic pressure and drops the ultra-filtration pressure fall to zero (Renkin and Robinson, 1974).

Kidney has a system of glomerular filtration auto-regulation which relies on regulation of the caliber and resistance of both the afferent and efferent arterioles, in such a way that the intra-glomerular capillary hydrostatic pressure is balanced

according to the requirement (Christensen, et al., 2003). Normally, the caliber of afferent arteriole is bigger than that of efferent, a disproportionality that elevates intra-glomerular hydrostatic pressure and increases vascularity of the glomerular bed (Sherwood, 2006).

Auto regulation mechanism is controlled by renal sympathetic nerve fiber and a series of systemic and local autacoids as angiotensin II, catecholamines, prostaglandins, thromboxans, adenosine, dopamine, nitric oxide ($NO^{\cdot}$), endotheline and ANP (Anti-natriutic peptide). Angiotensin II, catecholamines, thromboxans, adenosine and endotheline act as afferent and efferent arterioles vasonstrictors leading to reduction of glomerular filtration rate while the rest have apposite function afferent and efferent arterioles. Auto regulation of glomerular filtration is accomplished by one of the two mechanisms; the *myogenic* where vasoconstriction incurs as a response to any stretch or the *tubulo-glomerular feedback* mechanism, in which, the increase in sodium delivered to DCT triggers *macula densa* to activate the juxta-glomerular apparatus to release some autacoids as angiotensin II and adenosine which induce afferent arterioles vasoconstriction (Salman, et al., 1999; Vallon, 2003; Christensen, et al., 2003).

Renal tubular function is associated with the re-absorptive and secretory job of the ductile system that starts from the beginning of PCT and ends up in ducti of Billini (Thies, 1995).

PCT absorbs about 67% of electrolytes and water and almost all filtered glucose and amino acids. Sodium ion plays an important role in PCT's function, since most of the sym-porters and anti-porters require sodium for proper functioning. Sodium re-

absorption creates the required osmotic gradient for water re-absorption. Mechanism of sodium re-absorption in the early segment is different from that in the late segment. In the early segment, it is coupled with bicarbonate, phosphate and a number of organic molecules, such as; glucose, amino acids and lactate while it is coupled with chloride in the late segment. It is so avid that these solutes are almost completely cleared from tubular fluids in this segment leading to a great change in tubular fluid composition between the first and second halves. Most of the water soluble electrolytes can follow para-cellular route down their electrochemical gradient potential by a process called solvent drag. PCT cells can excrete both organic cations and anions through specific symporters and antiporters present on both the apical and basolateral membrane (Beck, et al., 1973; Neuman and Rector, 1976).

Tubular fluid leaves PCT towards loop of Henle where the counter current multiplication mechanism takes place in cooperation with *vasa recta renis*. This mechanism aims to increase osmolality of the renal interstitium at the deep inner medullary portion; an action that facilitates reabsorption of water from the medullary CT toward renal interstitium by the aid of ADH (anti diuretic hormone). Unlike the descending limb, thick ascending limb of loop of Henle is impermeable to water and permeable to ions. Its apical membrane is endowed with lots of frusemide sensitive Na^+-K^+-Cl^- symporters which can actively extrude these ions from the tubular fluid. Like other tubular epithelia, basolateral membrane is endowed with lots of Na^+-K^+ ATPase pump mechanism which extrude Na^+ toward the interstitium. The reabsorbed K^+ can be recycled again to tubular fluid and translocated into the DCT.

Accumulation of sodium in the interstitium elevates the interstitial osmolality and makes water to transfer from descending limb of the loop of Henle into the interstitium according to the osmolar gradient. Repetition of this process leads to a high osmotic pressure at the tip of the loop. *Vasa recta renis* acts to wash the reabsorbed fluid into the deep intra-medullary interstitium towards the cortical region (Layton and Weinstein, 2002).

DCT is the site where Na^+ is reabsorbed by the aid of Na^+-Cl^- co-transporters in the apical membrane and Na^+-K^+ ATPase pump in the basolateral membrane of the lining epithelium. Accordingly other electrolytes are re-absorbed by solvent drag mechanism along with reabsorbed water through the para-cellular shunt. Inside the CT ducti system urine volume and electrolyte composition are regulated. The system is rich in two types of cells; principle and intercalated cells. Principle cells are responsible for sodium re-absorption in replacement of potassium ion. It is facilitated by basolateral Na^+-K^+ ATPase pump, apical membrane Na^+ and K^+ channels and the electrochemical gradient that push potassium to CT lumen. Intercalated cells are found mainly in the medullary region. They transfer some K^+ and reabsorbed water in response to ADH from the medullary CT toward renal interstitium (Thies, 1995).

1.2 Aminoglycosides induced nephrotoxicity

1.2.1 Aminoglycosides

Aminoglycosides are a group of structurally complicated antimicrobial compounds, composed of modified amino-sugar moieties linked by O-glycosidic linkage. They are

extracted from some species of *Streptomyces* bacteria or *Micromonospora* fungi and chemically modified for a stronger antimicrobial activity or to enervate its toxicity (Mitsuhashi, 1975). Their antimicrobial action is attributed to their ability to indispose bacterial protein synthesis and dismount the cell wall integrity (Lutwyche, et al., 1998). Toxicity of aminoglycosides is demarcated by being an oculo-, vestibulo-, cochlear and nephrotoxicity due to the specific cellular uptake mechanisms that epithelia of these organs possess for molecules having a cationic nature like aminoglycosides (Arya, 2007; Mingut-Leclerq, et al., 1999). Leading to some notorious clinical signs and symptoms, such as oscillopsia (bouncing vision), blurred vision, tinnitus, vertigo, difficulty in balance, mental fogginess, short term memory lapse, tiredness and change in urine volume (Kelly, 2006). Hairy cells of vestibulocochlear apparatus in the middle ear, the oculo-retinal cells, the lining epithelium of renal proximal convoluted tubules, the neuromuscular junctions and the renal glomerular basement membrane of renal glomeruli are the main targets of aminoglycosides (Arya, 2007; Mingut-Leclerq, et al., 1999).

1. 2.2 Aminoglycosides *induced nephrotoxicity*

Aminoglycosides induced nephrotoxicity is a multi-step process, characterized by prominent changes in renal glomeruli and PCT (Rougier, et al., 2003).

1.2.2.1 Aminoglycosides induced tubular injury

It commences with the uptake of aminoglycosides by the simple cuboidal epithelial cells of *pars recti* segment of PCT. Due to their cationic nature, aminoglycosides can easily bind to acid phosphates that incrust the brush border as a precursory step for their subsequent intracellular internalization. Then they bind to megalin (a trans-membrane protein that binds to polybasic compounds like aminoglycoside and internalize them into the cytosol. The selectivity of aminoglycosides induced toxicity on PCT epithelium, hairy cells of vestibulocochlear apparatus and retinal epithelia is attributed to the megaline on their surface) (Nagai, et al., 2001; Pruiska, et al., 1997; Kanig et al., 2008). As soon as they access the cytosol, they are engulfed by endosomes which provide an endocytic membrane transport shunt from plasma membrane to lysosomes (Sandoval, et al., 1998) where the environment is more acidic (PH=5), rendering aminoglycosides to be more cationic and increase their potency to bind to lysosomal membrane acid phosphates (that constitute about 5-20% of the membrane), leading to a cellular change known as lysosomal phospholipidosis (Schank, et al., 1992). In this case, lysosomal phospholipase and sphingomylinase enzymes lose their ability to break down phospholipids molecules. This in turn leads to a conspicuous accumulation of myeloid bodies in the form of intracellular aggregations of phospholipids, cholesterol and proteins (Kacew, 1987). When the critical threshold of intralysosomal concentration of aminoglycoside is reached, It bursts out to the cytosol inducing destructive changes in mitochondria and both the apical and basolateral membranes (Guirgea-Marion, et al., 1986; Giuliano, et al., 1987). Inside the mitochondria, aminoglycosides bind with iron to form a complex with Fe^{++} which initiates lipid peroxidation and affects membrane

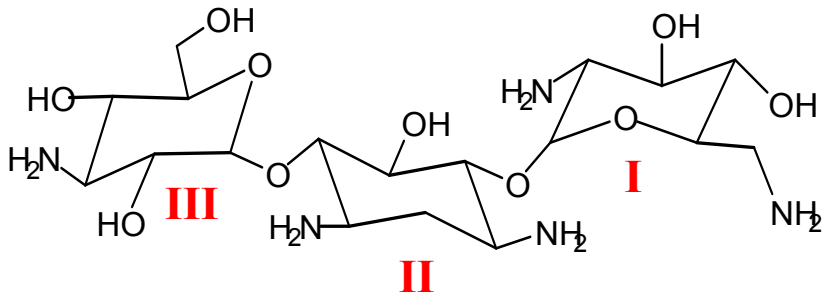
integrity that in turn leads to damping of mitochondrial internal respiration and decrease in ATP synthesis (Msastrasinh, et al., 1982; Zorov, 2010). The passive effect of gentamicin on apical and basolateral membranes integrity is attributed to lipid peroxidation and inhibition of ATP dependent transport mechanisms (Blias, et al., 1993).

Hydropic degeneration may inflict tubular cells due to a decrease in ATP that is required for the active Na^+-K^+ pump, leading to Na^+ retention and accumulation of an equimolar amount of water inside the cytoplasm as cytoplasmic vacuoles (Solez, 1986). If the destructive action pursues, necrosis may inflict injury to the cells. It is characterized by loss of cellular outlines and nuclear changes (karyopyknosis, karyorrehix and karyolysis) (Turton and Hooson, 1998). Progression of these events is controlled by chemical structure of the aminoglycoside which affects the ability of the drug to bind into the negatively charged phospholipids that incrust the brush border (Malis, 1984) and mode of the drug administration, as dosing and time interval (nephrotoxicity pattern differs if the drug is given in low or high dose and if the drug is given once a day, multiple doses or by continuous infusion). The single dosing system is less nephrotoxic as compared with the multiple dosing systems, as it creates just one peak of the drug plasma level while the multiple dosing systems create more (Reiner, 1987). This is due to the nature of the cellular uptake of aminoglycosides which is a saturable process such that a finite quantity of drug transposes into the intracellular compartment after surpassing a certain critical threshold (Giuliano, et al., 1986).

There are many approaches that have been proposed to halt aminoglycosides induced nephrotoxicity. One of the approaches proposed using agents that make a complex with

aminoglycosides extracellularly or compete with the aminoglycosides at the binding sites. (For example; the series of polyaspartic acid derived compounds, like daptomycin) (Williams, 1985). Changing the mode of drug administration is another way to ameliorate the nephrotoxic effect of gentamicin (by switching the patient into the single dosing system, as mentioned above) (Reiner, et al., 1978).

Recently a series of aminoglycosides with a modified structure have been evolved. They have lower ability to bind to brush border or lysosomal phospholipids, e.g. amikacin, arbekacin and isepamicin (Carlier, et al., 1983). Another approach proposed is by using antioxidants and iron chelators. They intervene with the cascade sequential processes associated with intracellular aminoglycosides induced lipid peroxidation (Walker , et al., 1987).



.

Figure 1.2:- The structure of kanamycin (The standard chemical structure of aminoglycoside molecule. IUPAC name (2-(aminomethyl)- 6-[4,6-diamino-3- [4-amino-3,5-dihydroxy-6-(hydroxymethyl) tetrahydropyran-2-yl]oxy- 2-hydroxy-cyclohexoxy]- tetrahydropyran- 3,4,5-triol). Adapted from http://chemistry.about.com/od/factsstruc -tures/ig/Chemical-Structures K/Kanamycin.htm).

1.2.2 Gentamicin as an example of nephrotoxic aminoglycoside

Gentamicin is an aminoglycoside with methyl-N-substitution. Its chemical formula is $C_{21}H_{43}N_5O_7$ and its g.mwt is 488 gm/mole. It is a water soluble crystalline white to yellow colored powder. It is obtained from *Micromonospora* species; a genus of gram positive bacteria, disseminated widely in nature. It has a broad spectrum of bactericidal action against an array of gram negative bacteria, such as pseudomonas, niesseria, legionella and various coliform bacteria (Mitsuhashi, 1975). Gentamicin is more toxic as compared to other aminoglycosides due to its stronger ability to bind into acid phosphates (the targets of aminoglycosides-induced toxicity). This deterrent characteristic makes physicians more cautious while prescribing it (Carlier, 1980).

Bioavailability of gentamicin after oral administration is very poor while it is higher if it is given parenterally. Its hydrophilicity and molecular size allows it to permeate easily through the capillary endothelium (Segal, et al., 1988). Its Vd (volume of distribution) is affected by changes in total volume of extra-cellular fluids. In edema, the concentration decreases due to the increase in VD while in dehydration, the concentration goes up (Kelman, et al., 1984). Its binding to plasma proteins is weak and reaches 10-30%. Gentamicin is eliminated primarily by kidneys. Gentamicin has a very short half life which is higher in infancy as compared to higher ages. This difference may be due to the difference of GFR and renal function with different age groups. (Vozeh, et al., 1979).

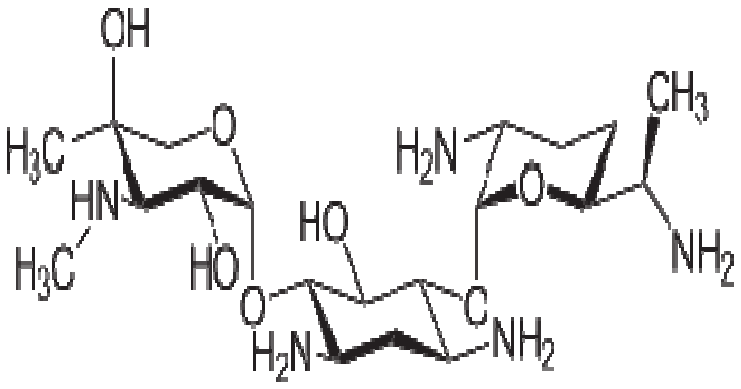


Figure 1.3:- Chemical structure of gentamicin. IUPAC name (diamino-3 3-amino-6-1-(methylamino)ethyl]oxan-2-yl]oxy}-2-hydroxycyclohexyl]oxy}-5-methyl-4-(methylamino)oxane-3,5-diol) (Mitsuhashi, 1975).

Therapeutic plasma concentration of gentamicin ranges from 2 to 12 µgm/ml. The minimum value represents the MIC (minimum inhibitory concentration) for bacteria while the highest one represents the critical threshold concentration. So as an antibiotic, it should be given in a dose that keeps the concentration between the two thresholds. In human, the therapeutic dose that maintains it within the abovementioned range is about 4-7 mg/kg(B.W)/ day. Higher doses make the concentration higher than this range. In rats, the dose that maintains the concentration within the therapeutic level is about 10-20 mg/Kg (B.W)/day. It is documented that nephrotoxicity in rats is induced at a dose exceeding 40 mg/ Kg (B.W)/day. In one study, after a single injection of 100 mg/kg gentamicin, the plasma level rose to168 µgm/ml (Stahlmann, et al., 1988).

1.3 Oxidative stress

Oxidative stress is evolved due to constant interaction of the body with oxygen during intracellular respiration resulting in unleashing of highly reactive molecules, known as free radicals. Cells are endowed with a redox system, responsible for mopping free radicals out by scavenging them (Sies, 1991). Free radical molecules hareve an atom with an unpaired electron in its outer shell, making them highly reactive species with a tendency to attack various biomolecules. It is formed due to heterophilic breakdown of covalent bonds during cellular metabolism (Nonhebel and Walton, 1974).

When the free radical is formed, a series of chain propagation reactions are triggered. They are called electron transport chain (ETC). In these reactions, each newly formed radical looks to return into its original state by stealing another electron with antiparallel spin from the surrounding environment. This in turns evolves more new radicals. ETC mostly happens inside the mitochondria where oxygen is used to generate energy and acts as an electron acceptor (Gropper, et al, 2008).

Free radicals target double bonds in poly unsaturated free fatty acids (PUFFAs) resulting in a heterophilic breakdown of C-H bonds attached to the double bonds. PUFFAs contain one or more methelene groups between the double bonds. They are highly reactive to oxidizing agents and can easily lose their hydrogen after forming carbon centered radicals (Gropper, et al, 2008). In an effort to stabilize it, molecular rearrangement occurs which converts the molecule into more stable form called conjugated diene (CD). CDs tend to react with other oxygen molecule to form

peroxyradicals which look to attack other PUFFAs' double bonds, leading to continuous repetition of this process (radical chain propagation reaction) (Gropper, et al, 2008).

After the formation of peroxy radicals, either they are reduced to hydroperoxides which are relatively more stable or they suffer sequence of reactions associated with intramolecular cyclization, leading to generation of a broad range of products (Mittler, 2002) (Figure 1.4).

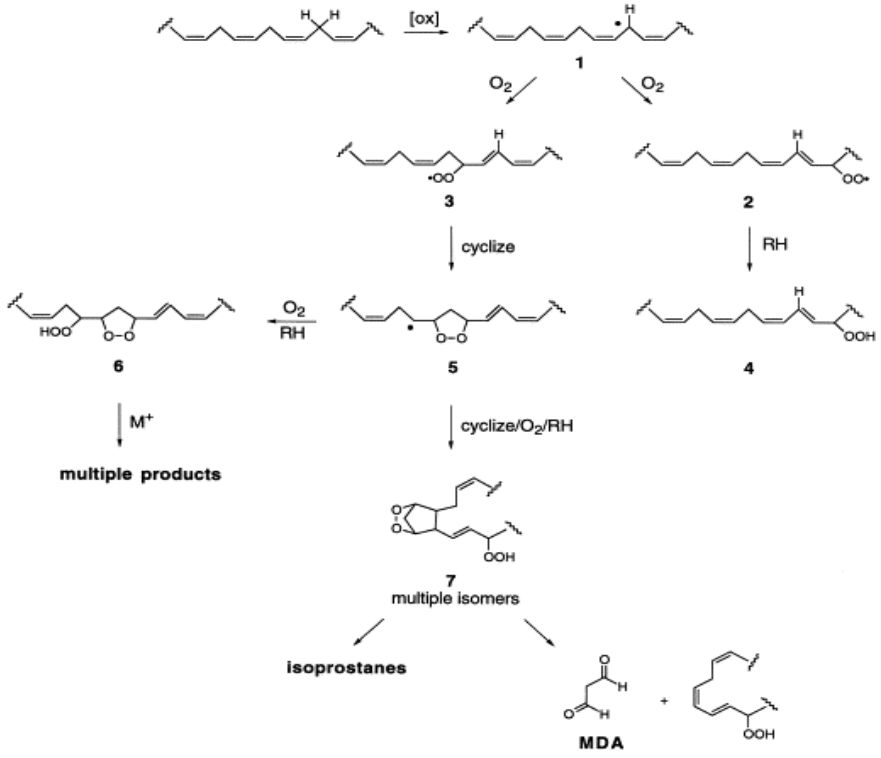


Figure 1.4:-Lipid peroxides fate pathways (adapted from Mittler, 2002).

Final products of lipid peroxidation are either aldehydes, like formaldehyde, acetyladehyde, acrolien, malonyldialdehyde (MDA),4-hydroxyhexenal (4-HHE) and 4-hydroxnonenal (4-HNE), oxaloaldehydes such as glyoxal and methyl glyoxal, ketones

such as; acetone and butanone and some alkanes such as; hexane, cyclohexane and heptanes (Mittler, 2002). MDA is the most convenient biomarker of lipid peroxidation among them because of its plain reaction with thiobarbituric acid (TBA) to form TBA-MDA adduct that is easy to be detected calorimetrically (Akhgari, et al., 2003). Recently, F_2- isoprostane is used as a marker as well (Tianying, et al., 2004). Aldehyde products of lipid peroxidation are relatively less harmful than the free radicals, although they have a tendency to form Schiff bases with lysine, histidine and cysteine residues of proteins and nitrogen bases of DNA backbone leading to conformational changes of the proteins along with loss of their function and some genotoxic and mutagenic effects due to DNA binding (Cederbaum, 2001; Cohen , et al., 1984; Kharbanda , et al., 2002).

1.3.1 Types of free radicals

Free radicals are either reactive oxygen species (ROS) or reactive nitrogen species (RNS) depending on whether oxygen or nitrogen is centered in (Nonhebel and Waton, 1974).

1.3.1.1 Reactive oxygen species

Reactive oxygen species (ROS) include; superoxide anion ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), alkoxy radicals ($RO^{\bullet}$), peroxy radicals ($ROO^{\bullet}$) and hypochlorous acid (HOCl). Oxygen has a tendency to be reduced in sequential univalent processes releasing intermediates such as $^{\bullet}O_2^-$, H_2O_2 and $^{\bullet}OH$ (Uday, 1999).

Leakage of electrons during ETC reaction in mitochondria results in oxygen reduction. ETC reaction accompanies steps associated with oxidation of hydroxyacids icas those in citric acid cycle, amino acids or fatty acids. Nicotinamide adenine dinucleotide (NADPH) acts as a source of reducing equivalents in these reactions. It donates electrons to cytochrom P450 via flavoproteins. Cytochrom P450 reduces oxygen to water without the formation of $^{\bullet}O_2^-$. Any disturbance in mitochondrial function reaction leads to leakage of electrons and release of $^{\bullet}O_2^-$.

As soon as $^{\bullet}O_2^-$ is released, it is dismutated spontaneously or by the aid of super oxide dismutase enzyme (SOD) forming H_2O_2. $^{\bullet}O_2^-$ is rather inactive and has very poor penetration through plasma membrane while hydrogen peroxide is more active and especially ferrous and cuprous ions induce release of $^{\bullet}OH$ by a reaction called Fenton reaction (Uday, et al., 1999). These metal ions are not found freely and mostly are bound to a tissue protein called metalloprotein. They are released during cellular degeneration and tigger Fenton reaction (Aruoma, 1989). $^{\bullet}OH$ is extremely active and due to its low diffusion capacity, it damages any molecule in its vicinity (Uday, et al., 1999).

Inside the body, there are two defensive mechanisms to mop ROS out; the primary one which is represented by antioxidant enzymes and the secondary one which is represented by the antioxidants which exist in dietary supplements. SOD, catalase and glutathione system represent the enzymatic system that mop out ROS. SOD is a metalloprotein. It contains copper and zinc as cofactor ions. It dismutates $^{\bullet}O_2^-$ to H_2O_2. Catalase enzyme is present in peroxosomes. It is a hem-protein that catalyzes conversion of H_2O_2 to water and oxygen.

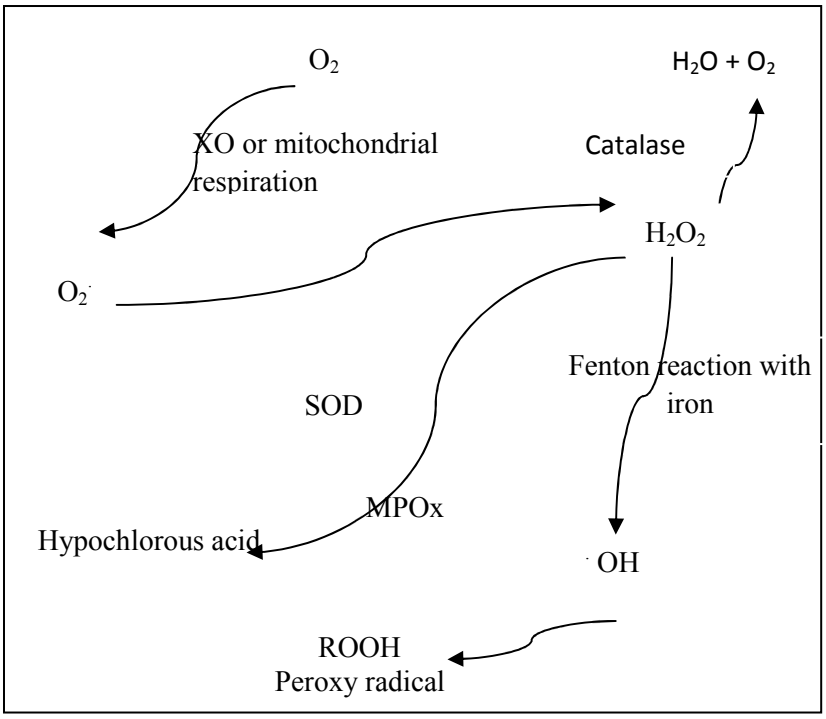


Figure 1.5: - Cascade sequential reaction of ROS formation after superoxide free radical generation. O_2^-:- singlet oxygen or superoxide radical, SOD:- superoxide dismutase enzyme, H_2O_2:- hydrogen peroxide, OH:- hydroxyl radical and MPOx:- myeloperoxidase enzyme (Thorp, et al., 2004).

Glutathione system is made up of glutathione, glutathione peroxidase enzyme and glutathione reductase enzyme. It works along with ascorbic acid to shuttle electrons from NADPH to hydrogen peroxide or other hydroperoxides as illustrated in Fgure1.6 (Thorp, et al., 2004; May, et al., 1996).

Furthermore, immune system lays an important role in triggering oxidative stress. Neutrophiles produce H_2O through NADPH oxidase enzyme and hypochlorous acid through action of meyloperoxidase enzyme on H_2O_2 (Gropper, et al., 2008).

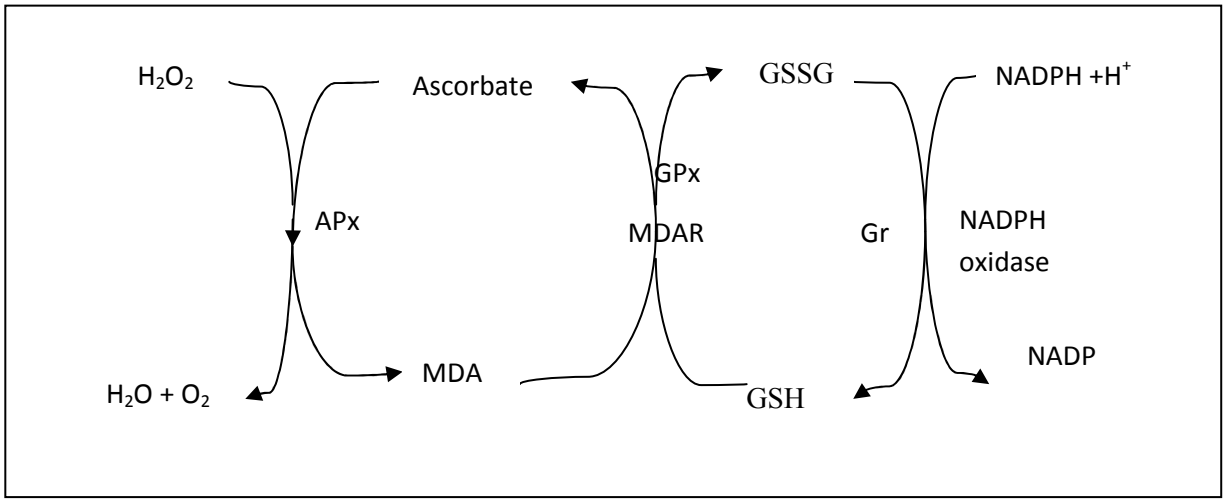


Figure 1.6:- Glutathion-ascorbate cycle to detoxify H_2O_2. Apx:-Ascorbate peroxidase enzyme, MDA: - Monohydroascorbate, MDAR: - Monohydroascorbate reductase, GPx:- glutathione peroxidase enzyme, Gr:- glutathione reductase enzyme, GSH:- reduced form of glutathione, GSSG:-Oxidized form of glutathione (May, et al., 1996).

1.3.1.2 Reactive nitrogen species RNS

Nitric oxide ($NO^{\cdot}$) and peroxynitrate (ONOO) are the RNS forms of free radicals. $NO^{\cdot}$ is produced extensively by endothelial cells as a short lived paracrine physiological transmitter that mediates vascular smooth muscles dilation and inhibition of platelets aggregation through activation of cGMP and decreasing intracytosolic calcium concentration. Furthermore, it inhibits endothelial function by acting as a gene modulator preventing the expression of surface adhesion molecules as VCAM-1 and ICAM-1which are required for leukocyte adhesion and development of atherosclerotic events (Van der, et al., 1999; Walker, et al., 2001).

During oxidative stress, $NO^{\cdot}$ reacts with superoxide radical generating peroxynitrate ($ONOO^{\cdot}$). Peroxynitrate is a strong free radical. It induces free radical chain propagation reaction and nitrosation of tyrosine residues of proteins and nitrogen bases of DNA

leading to cellular dysfunction and mutation (Van der, et al., 1999; Walker, et al., 2001). The human body fends this pathway through triggering nitrate-nitrite-nitric oxide pathway. When the amount of $NO^{\cdot}$ exceeds the threshold, $NO^{\cdot}$ is oxidized to nitrite (NO_2^-) and then to nitrate (NO_3^-) by aid of multicopper oxidase system, cerruloplasmin and oxyhaemoglobin which converts to methaemoglobin (Van der, et al., 1999; Walker, et al., 2001).

Conversion of $NO^{\cdot}$ to NO_2^- and NO_3^- is a bidirectional process which occurs either spontaneously after disturbance of the chemical balance or triggered by enzymes. On the other hand, $NO^{\cdot}$ could be evolved from NO_2^- and NO_3^- by aid of nitrate reductase enzyme produced by gastrointestinal commensal bacteria (Lundberg, et al., 2008). Regulation of protein function through nitrosylating its thiol groups by $NO^{\cdot}$ is another mechanism that the body is endowed to discard excessive $NO^{\cdot}$ radical (Walker, et al., 2001).

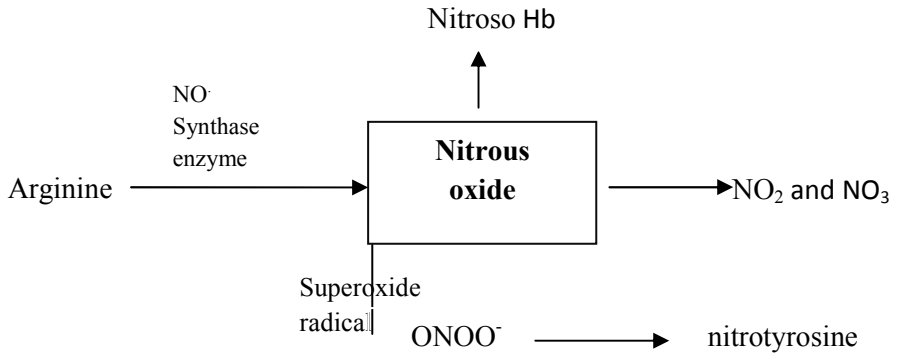


Figure 1.7:-Fate of nitric oxide inside the body (Walker, et al., 2001).

1.3.2 Dietary antioxidants

Diet provides plenty of biochemical compounds able to counteract oxidative stress and enforce the defensive processes against free radicals generation. These compounds are either vitamin products , as vitamin A, vitamin E and vitamin C which are found in animal and plants products such as some carotenoid derivatives (zeaxanthine, lycopene and lutein) and polyphenols (Shahidi, 1997).

Polyphenols are water soluble chemical compounds that bear more than one phenolic groups. Phytochemical polyphenols are classified into:-tannins, flavonoids and phenylpropanoids (Shahidi, 1997).

Palm oil leaf extract used in our study, contains polyphenols with catechin and ferrulic acid constituting the majority. Catechin is a tricyclic flavonoid derivative. It possesses an antioxidant activity due to the presence of phenolic groups attached to benzene ring (Chumbalov, et al., 1995). Ferrulic acid is a phenylpropanoid derivative, found in plant cell wall as a covalent side chain attached to arabinoxylan and cellulose of the cell wall. Inside the plant, it serves to crosslink lignine to polysaccharides adding some rigidity to the cell wall. Previous studies reveal that after ingestion, ferrulic acid is absorbed in gastrointestinal tract after cleavage of its linkage with lignines by pancreatic acids (Pan, et al., 1999). It has better bioavailability in plasma as compared to other polyphenols (Scheliner, 1968). Ferrulic acid has a unique antioxidant power due to the presence of carboxyl group in its structure which acts as a free fatty acid anchor in membranous structures resulting in higher anti lipid peroxidation effect (figure 1.8) (Kanski, et al., 2002).