CHARPTER II

LITERATURE AND RESEARCH

Cadmium

Cd is a toxic heavy metal for humans, animals and plants (Wagner, 1993). The mechanisms involved in Cd toxicity have been demonstrated with reference to oxidative burst in many animal cells (Lopez, et al., 2006; Pathak and Khandelwal, 2006). According to Stohs and Bagchi (1995), Cd induced changes of the antioxidant status either by enhancing superoxide radical production and lipid peroxidation, or by reducing the enzymatic and nonenzymatic antioxidants. The accumulation of reactive oxygen species (ROS) such as H_2O_2 and superoxide radical ($^{\cdot}O_2^-$) will damage the cellular components such as DNA, proteins and lipids (Lopez, et al., 2006).

Cadmium, in spite of not being a Fenton metal, is capable of inducing oxidative stress in cell culture models (Joseph, et al., 2001; Shih, et al., 2004) and in experimental animals (Nigam, et al., 1999). Approximately 10,000 adults who participated in the third U.S. National Health and Nutrition Survey were examined for associations between urinary Cd levels and oxidative as γ-glutamyltransferase (GGT), vitamin C, carotenoids and vitamin E (Lee, et al., 2006). Results of this study demonstrated graded associations of urinary Cd levels, positive with serum GGT and inverse to serum vitamin C, carotenoids and vitamin E, among the participants of the survey. The strong association observed between the urinary Cd levels and the markers of oxidative stress warrants that oxidative stress be considered in the pathogenesis of Cd-related diseases, including cancer. The ability of Cd to deplete GSH to inhibit the mitochondrial electron transport chain so as to cause the generation of superoxide anion radical, and to modulate the cellular level of proteins involved in the generation/detoxification of various ROS are thought to contribute to the oxidative stress observed in response to Cd exposure. Exposure of cultured cells and animals to Cd can cause in the generation of ROS (Bolduc, et al., 2004; Shih, et al., 2004). Similarly, the expression of various detoxification enzymes that are known to play a key role in the cellular response to the generation of ROS and the resulting toxicity,

are affected by exposure to Cd (Casalino, et al., 2006). Additional evidence to support the capability of Cd to induce oxidative stress comes from the observations of increased lipid peroxidation (Jurczuk et al., 2004), depletion of glutathione (Nigam, et al., 1999) and induction of several stress response genes (Badisa, et al., 2008) in response to Cd exposure.

Generation of ROS lead to oxidative stress is believed to play a prominent central role in Cd carcinogenesis. Many of the cellular and molecular events taking place in the cells that have relevance to Cd induced carcinogenesis are mediated, directly or indirectly, by oxidative stress. As mentioned above, Cd is very weak with respect to its ability to cause genotoxicity and most of the genotoxic events observed in cells in response to Cd exposure are mediated mainly by the generation of ROS. The genotoxicity induced by Cd exhibits hallmarks of oxidative stress and scavengers/modulators of ROS are capable of significantly reversing the Cd-induced genotoxicity (Bertin and Averbeck, 2006). Antioxidant enzymes such as superoxide dismutase and catalase were able to block the DNA strand breaks (Ochi and Ohsawa, 1985; Liu and Jan, 2000), chromosomal aberrations (Ochi and Ohsawa, 1985) and gene mutations (Yang, et al., 1996) induced by Cd. Similarly, Cd-induced oxidative stress is known to play a major role in its potential to inhibit DNA damage repair and to induce apoptosis. (Xie and Shaikh, 2006)

Cadmium accumulates and proves to be very toxic in many organs, such as kidney, liver, lung, testis, bone and the blood system. The two main storage organs of Cd are the kidney and the liver which contain about 50% of the total body burden. The highest concentrations of Cd are found in the kidney which is considered as the critical organ whatever the source and portal of entry of Cd. The renal accumulation of Cd takes place mainly in the proximal tubules where from a certain stage the metal gives rise to a dose-dependent toxicity. Other segments of the nephron can also be affected but usually at more advanced stage of the intoxication than the proximal tubules.

Cadmium is a serious environmental toxicant. The mining of zinc, lead and copper ores naturally also removes cadmium, which often finds itself in the earth extracted during mining but later restored to the surrounding environment and washes used in the ore extraction process. These both lead to chronic soil contamination. Cadmium is also present as a pollutant in phosphate fertilizers, adding to the increased uptake in crops. Cadmium compounds are used commonly in re-chargeable nickel–cadmium batteries, and their disposal causes soil contamination. Cadmium-containing products are rarely re-cycled and so end up in waste that is often incinerated and placed back in the soil. The outcome is increased cadmium uptake into crops supplied by contaminated soil and water and grown for human consumption. Cigarette smoking is also a major source of cadmium exposure. Although it is often claimed that this contributes little to total cadmium body burden, the high chronic exposure to cadmium as a result of tobacco smoking may contribute to cardiovascular diseases like hypertension and these diseases have close links with chronic kidney disease (CKD).

The critical targets of cadmium binding are the thiol groups of proteins. Thiol proteins are important in cellular anti-oxidant defences and redox signaling and it is thought that ROS cause selective thiol oxidation. The relative sensitivities of different cell proteins and critical targets are not well characterized. Most of the cadmium penetrates into cells combined in metallothionein complexes. Cadmium–metallothionein complexes cause endocytosis of brush-border transporters in rat renal proximal tubules. This may lead to a loss of cell membrane function resulting in reabsorptive and secretory defects that occur in cadmium induced nephrotoxicity. Some of the cadmium–metallothionein complexes have been identified in the cytosol of renal proximal tubular epithelial cells with secondary evidence that the main target organelles are the mitochondria. Cadmium penetration of the mitochondria has been identified with the outcome of significant inhibition of mitochondrial function, increased ROS production and eventual apoptotic cell death. The mechanisms behind these changes are the subject of much research but they still need further definition. Regarding effects on the mitochondrial electron transport chain heavy metals in mitochondria most likely inhibit the activity of complexes II and III more than that of the other complexes. Since the principal site of ROS production seems to reside in

complex III, its dysfunction may increase ROS production beyond the neutralizing ability of normal anti-oxidants that also reside in mitochondria.

In the kidney, the ability of toxic heavy metals like cadmium to elicit "oxidative stress" and to alter metal homeostasis may lead to augmentation of the defence mechanism involving heme oxygenase-1(HO-1) as well as metallothionein. HO-1 is induced by its substrate heme and by oxidant stress in conjunction with the ability of HO-1 to protect against oxidative insult. The cell protective (anti-oxidant) effects of increased HO-1 arise from its capacity to degrade the hememoiety from destabilized heme proteins and to generate biliverdin and bilirubin which are products of HO-1 that possess potent antioxidant properties. Since accumulation of cadmium occurs in the proximal tubular epithelium of the kidney, this may trigger the host responses mediated by HO-1 in an attempt to protect the kidney against injurious cadmium-induced oxidative stress and eventually the development of CKD. These findings suggest that HO-1 may be a target for therapies in the alleviation of cadmium-induced nephrotoxicity. (Glenda Gobe and Denis Crane, 2010)

Since free radical-induced lipid peroxidation is a complex process and occurs in multiple stages there are many techniques available for the detection and measurement of lipid peroxidation products. Peroxidation of lipids can be assessed by measurement of the loss of unsaturated fatty acids, generation of primary peroxidation products or secondary degradation products. (Halliwell and Chirico, 1993)

Free radical is atoms or molecules that contain one or more unpaired electrons. This atom or molecules can form covalent bond with others atom or molecule gathering. (Halliwell, et al., 1995) For example of free radical is $O_2^{\cdot}$. When the O_2 oxidized it can be cause the formation of reactive oxygen species both radical and non radical particularly hydroxyl radical ($OH^{\cdot}$), superoxide anion radical ($O_2^{\cdot\cdot}$) and hydrogen peroxide (H_2O_2). (Halliwell, et al., 1995) This free radical can react with others molecules in several ways if free radicals react together they combine their unpair electrons and become non-radical. When a radical gives electron to or takes electron from non-radical, these non-radicals become radical (Halliwell and Chirico, 1993). A feature of reaction of free radicals with nonradicals usually proceeded as chain reaction including lipid peroxidation is the most biological relevant of free radical chain reaction. (Halliwell and Chirico, 1993)

Major compounds of cell membranes are phospholipid, and their polyunsaturated fatty acid (PUFAs) because of their conjugated double bonds are the first target of free radical. One of the main forms of damage resulting from oxidative stress is lipidperoxidation (Arab, Steghens, 2004). Free radicals for example, hydroxyl, lipoxyl and lipid peroxyl are oxidized by unsaturated covalent bond from PUFAs to be PUFA radical ($L^{\cdot}$) and decomposition to conjugated diene for more stable or add with others radical to nonreactive complex. When lipid radical ($L^{\cdot}$) decomposition to conjugate diene then add with O_2 become lipid peroxide ($LOO^{\cdot}$) and damage unsaturated covalent bond of PUFAs and produce lipid hydroperoxide (LOOH) and final product of lipid peroxidation is malondialdehyde (Walter, et al., 2008). Lipid peroxidation can perturb membrane function and contribute to loss of cellular functions for example, lost of flexibility, change function of enzyme and receptor and change ion channel and permeability of Ca^{2+}. (Aruoma, 1998)

Unstable free radical species attack cellular components causing damage of lipids, protein and DNA which can be initiated a chain of events resulting in the onset of a variety of diseases. In living organisms, they have developed the antioxidant system for converting free radicals into non-toxic molecules. The cellular antioxidant can be divided into 2 major groups, enzymatic and non-enzymatic (Gate, et al., 1999). Enzymatic system including superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) and nonenzymatic including albumin, celuloplasmin, ferritin, ascorbic acid, α-tocopherol, β-carotene, reduce glutathione, uric acid and bilirubin. The cooperation among different antioxidant provides greater protection against cellular damage by reactive oxygen or nitrogen species. Thus, the overall of antioxidant capacity may provide more information compared to that obtained by the measurement of individual components. (Ghiselli, et al., 2000)

The methods available for evaluation of oxidative stress and antioxidant may divided into three main categories: (1) based on the peroxidation product such as lipid hydroperoxide, antioxidant, malondialdehyde and reactive oxygen species production, (2) based on the oxidative or reductive potency of a biological fluid and (3) based on the susceptibility of various component of body fluids or their components to ex-vivo oxidation (Dotan, Lichtenberg, and Pinchuk, 2004). Phospholipid is major component of cell membrane and is the first target of the attack or free radical as describe above.

Thus, the most used criteria of oxidative stress were determination of the concentration of lipid peroxidation product and the susceptibility of lipid to peroxidation induced ex vivo. (Dotan, et al., 2004)

Free radical

Free radical may in simple terms be defined as any species capable of independent existence that contains one or more unpaired electrons. The reactivity of free radicals caused from the present of unpaired electrons. (Baynes, 1991; Halliwell, 1994; Halliwell and Chirico, 1993; Halliwell, Murcia, et al., 1995; C Rice-Evans and Burdon, 1993; Slater, 1984), an unpaired electron being one that is alone in an orbital(indicated in the text by •) (Aruoma, 1998), but electrons usually associate in pairs in orbitals of atoms and molecules. Then Free radicals are generally more reactive than non-radicals due to their unpaired electron (Aruoma, 1998; Halliwell, 1994; Halliwell and Chirico, 1993; Halliwell, Murcia, et al., 1995; C Rice-Evans and Burdon, 1993; Slater, 1984). Free radical will grasp electron from other molecules because they require another electron to fill the orbital and become stable (J. K. Willcox, et al., 2004). The simplest free radical is an atom of the element hydrogen. The hydrogen atom contains one proton and a single unpaired electron, but different types of free radicals vary widely in their reactivity. (Aruoma, 1998; Halliwell, 1994; Halliwell and Chirico, 1993; Halliwell, Murcia, et al., 1995; C Rice-Evans and Burdon, 1993; Slater, 1984)

Radicals can react with other molecules in several ways (Halliwell and Chirico, 1993; Halliwell and Gutteridge, 1985; C Rice-Evans and Burdon, 1993; Slater, 1984). When two free radicals react together, they will share their unpaired electrons and both radicals are lost. When a radical gives one electron to takes one electron from or simply adds on to a non-radical, that non-radical becomes a radical. In living organisms, most molecules are non- radicals then any free radical produced in the body will most react with non-radical and generate a new radical. Hence, free radical reactions in the body tend to proceed as chain reactions.

Radical react with radical

$$R'\bullet + R''\bullet \longrightarrow R\text{-}R$$

Radical react with non radical to gives electron from non-radical

$$R'\bullet + R''\text{-}R''' \longrightarrow R' + R''\bullet + R'''$$

To takes electron from non-radical

$$R'\bullet + R''\text{-}R''' \longrightarrow R' + R''\bullet + R'''\bullet$$

Major free radical

Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are chemically-reactive molecules containing oxygen. Examples include oxygen ions and peroxides. Reactive oxygen species are highly reactive due to the presence of unpaired valence shell electrons under normal biological conditions, oxygen does manage to steal away electrons from other molecules by non-enzymatic auto oxidations. Because it cannot accommodate a spin-matched pair, it must settle for stealing electrons one at a time. This breaking of electron pairs results in free radical formation. Radicals derived from oxygen represent the most important class of radical species generated in living systems (Jensen, 2003; Miller, et al., 1990) in metabolic processes or following oxygen activation by physical irradiation if the one-electron reduction product of oxygen is the superoxide radical, $O_2^{\bullet -}$. Superoxide anion, is considered the primary ROS, and if two electrons are transferred the product is secondary ROS(for example, hydrogen peroxide, H_2O_2) (M. Valko, et al., 2005). Hydrogen peroxide is not a radical. It is nonetheless still eagerly receptive of two more electrons, causing hydrogen peroxide to be a cytotoxic oxidant. Certain chelates of ferrous iron and cuprous copper are capable of transferring a third electron to hydrogen peroxide, causing lysis of the O-O bond. One fragment is reduced to the state of water; the other fragment is the hydroxyl free radical, $OH^{\bullet}$, one of the most potent oxidants known (McCord, 2000).

Table 1 Reactive oxygen species

Type of free radical	
Reactive oxygen species	H_2O_2 ozone O_3
Superoxide anion $O_2^{\bullet-}$	Hypobromous acid, HOBr
Hydroxyl, $HO_2^{\bullet}$	Hypochlorous acid, HOCl
Peroxyl, $RO_2^{\bullet}$	Singlet oxygen
Alkoxyl, $RO^{\bullet}$	Organic peroxides, ROOH

Reactive nitrogen species (RNS)

Reactive nitrogen species (RNS) are a family of antimicrobial molecules derived from nitric oxide ($NO^{\bullet}$) and superoxide ($O_2^{\bullet-}$) produced via the enzymatic activity of inducible nitric oxide synthase 2 (NOS2) and NADPH oxidase respectively. Nitric oxide ($NO^{\bullet}$) is an abundant reactive radical that acts as an important oxidative biological signaling molecule in a large variety of diverse physiological processes, including neurotransmission, blood pressure regulation, defend mechanisms, smooth muscle relaxation and immune regulation (Bergendi, et al., 1999) Overproduction of reactive nitrogen species will act together with reactive oxygen species (ROS) to damage cells, causing nitrosative stress (Klatt and Lamas, 2000; Ridnour, et al., 2005; Marian Valko, et al., 2007). Therefore, these two species are often collectively referred to as ROS/RNS. Nitrosative stress may lead to nitrosylation reactions that can alter the structure of proteins and so inhibit their normal function. Cells of the immune system produce both the superoxide anion and nitric oxide during the oxidative burst triggered during inflammatory processes. Under these conditions, nitric oxide and the superoxide anion may react together to produce significant amounts of a much more oxidatively active molecule, peroxynitrite anion ($ONOO^-$), which is a potent oxidizing agent that can cause DNA fragmentation and lipid oxidation (Carr, et al., 2000)

Table 2 Reactive nitrogen species

Type of free radical	
Nitric oxide, $NO^{\bullet}$	Nitrous acid, HNO_2
Nitrogen dioxide, $NO_2^{\bullet}$, $NO_2^{\bullet-}$	Nitrosyl cation, NO^+
	Nitroxyl anion
	peroxynitrite

Lipid peroxidation

Dietary fats, after being digested and absorbed, are transported through the body via lipoproteins. The more cholesterol rich lipoprotein, LDL, contains approximately 2700 fatty acids molecule, about half of which are PUFAs, that are very sensitive to oxidation (H Esterbauer, et al., 1991) because of an adjacent double bond weakens the energy of attachment of the hydrogen atoms present on the next carbon atom. Therefore, the greater the number of double bonds in a fatty acid chain, the easier the removal of a hydrogen atom, that is why PUFAs are more susceptible to peroxidation (Halliwell and Chirico, 1993). Polyunsaturated fatty acids are also found in cell membranes where their side chains mainly determine cell membrane fluidity. Membrane fluidity is essential for the proper function of biological membranes, including the action of many important receptors. The fluidity of cell membranes decreases with lipid peroxidation. (Arora, et al., 2000)

The lipid peroxidation chain reaction

Like all chain reaction, lipid peroxidation has three stages: initiation, propagation, and termination.(Feillet-Coudray, et al., 1999; Girotti, 1998; Gutteridge, 1995; Gutteridge and Halliwell, 1990; Halliwell and Chirico, 1993; Horton, et al., 1987; Niki, 2009; Oranje and Wolffenbuttel, 1999; Pacifici, et al., 1994) Initiation of lipid peroxidation is caused by attack of any species that has sufficient reactivity to abstract a hydrogen atom from a methylene group ($-CH_2-$) upon a PUFA (de Zwart, et al., 1999; Gutteridge, 1995; Gutteridge and Halliwell, 1990; Halliwell and Chirico, 1993). Species that can abstract the first hydrogen atom include the hydroxyl radical ($OH^{\bullet}$), alkoxyl radical ($RO^{\bullet}$), peroxyl radical ($ROO^{\bullet}$), and possibly $HO_2^{\bullet}$ but not H_2O_2 or O_2^{-} (Gutteridge, 1995) Since a hydrogen atom in principle is a free radical with a

single unpaired electron, its removal leaves behind an unpaired electron on the carbon atom to which it was originally attached. The carbon-center radical is stabilised by a molecular rearrangement to form a conjugated diene, followed by reaction with oxygen to give a peroxyl radical. Peroxyl radicals are capable of abstracting a hydrogen atom from another adjacent fatty acid side-chain to form a lipid hydroperoxide, but can also combine with each other or attack membrane proteins. When the peroxyl radical abstracts a hydrogen atom from a fatty acid, the new carbon-centred radical can react with oxygen to form another peroxyl radical, and so the propagation of the chain reaction of lipid peroxidation can continue. Hence, a single substrate radical may result in conversion of multiple fatty acid side chains into lipid hydroperoxides. The length of the propagation chain before termination depends on several factors e.g. the oxygen concentration and the amount of chain-breaking antioxidants present. Hydroperoxides are fairly stable molecules, but their decomposition can be stimulated by high temperatures or by exposure to transition metal ions (iron and copper ions). Decomposition of hydroperoxides generates a complex mixture of secondary lipid peroxidation products such as hydrocarbon gases (e.g. ethane and penthane) and aldehydes (e.g. malondialdehyde (MDA) and 4-hydroxynonenal).

Lipid peroxidation can proceeds not only non enzymatic free radical induced pathways, but also through processes that are enzymatically catalysed (Gutteridge, 1995; Halliwell, 1994; Halliwell and Chirico, 1993). Enzymatic lipid peroxidation may be referred only to the generation of lipid hydroperoxides achieved by insertion of an oxygen molecule at the active centre of an enzyme (Gutteridge, 1995; Halliwell, 1994; Halliwell and Chirico, 1993). Free radicals are probably important intermediates in the enzymatically-catalysed reaction, but are localized to the active sites of the enzyme. Cyclooxygenase (COX) and lipoxygenase full fill the definition for enzymatic lipid peroxidation when they catalyse the controlled peroxidation of various fatty acid substrates. The hydroperoxides and endoperoxides produced from enzymatic lipid peroxidation become stereospecific and have important biological functions upon conversion to stable active compounds. Both enzymes are involved in the formation of eicosanoids, which comprise a large and complex family of biologically active lipids derived from PUFAs with 20 carbon atoms. Prostaglandins are formed by COX

catalyzed the peroxidation of arachidonic acid (Samuelsson, et al., 1975). COX exists in at least two isoforms (Mitchell, et al., 1993; Vane, 1994). COX-1 is present in cells under physiological conditions, whereas COX-2 is induced in macrophages, epithelial cells and fibroblasts by several inflammatory stimuli leading to release of prostaglandins. (Fu, et al., 1990; Mitchell, et al., 1993; Vane, 1994; Xie, et al., 1991)

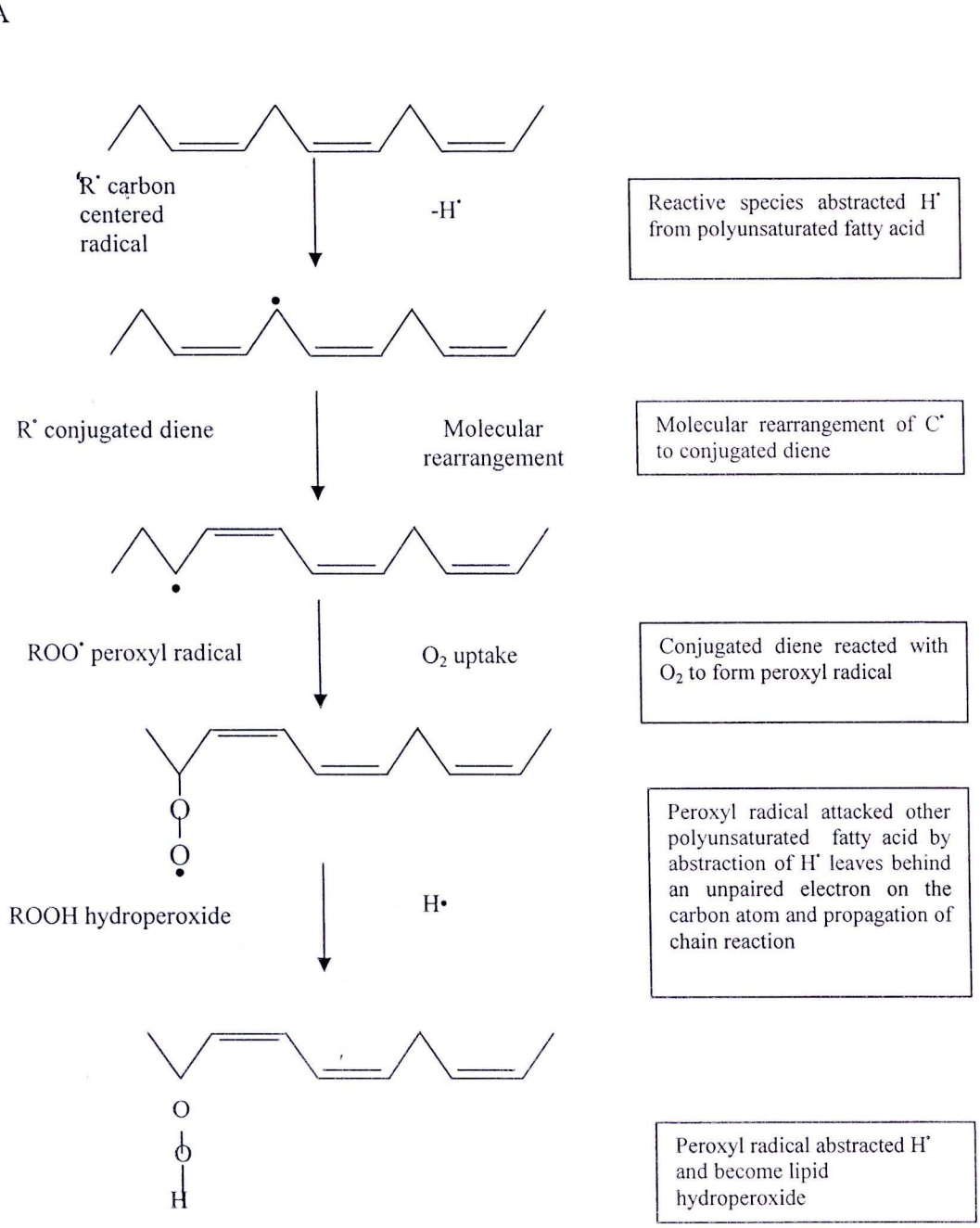

Figure 1 Lipid peroxidation process

Antioxidant

An antioxidant has been defined by Halliwel and Gutteridge as "any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substate" (Joye, K., Willcox, et al., 2004). Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions that damage cells. Living organisms have evolved antioxidant defend systems and repair systems for protection against free radicals and free radical damages at different sites (Chaudiere and Ferrari-Iliou, 1999; Gate, et al., 1999; Gutteridge, 1995; Halliwell, 1997; C Rice-Evans and Burdon, 1993; Slater, 1984). There are a number of antioxidants present in the body and derived from the diet. Based on their location in the body, they can be divided into enzymatic and non-enzymatic antioxidants. (Chaudiere and Ferrari-Iliou, 1999; Gutteridge, 1995; C Rice-Evans and Burdon, 1993)

Enzymatic antioxidants

Intracellular antioxidants include low molecular weight scavengers of oxidizing species, and enzymes which degrade superoxide and hydroperoxides (Chaudiere and Ferrari-Iliou, 1999). Some of the antioxidant enzymes exist in several forms for example, membrane, cytosolic, and plasma forms. The level and locations of these antioxidants must be tightly regulated for cell survival (Willcox, et al., 2004). Several antioxidant enzymes exist that convert ROS into less noxious compounds, for example, superoxide dismutase (SOD), catalase, thioredoxin reductase, peroxiredoxin and glutathione peroxidase (GPx) (Arnér and Holmgren, 2000; Chaudiere and Ferrari-Iliou, 1999; Hayes and McLellan, 1999; Mates, et al., 1999; Talalay, 2000). Collectively, these enzymes provide a first line of defense against superoxide and hydrogen peroxides.

Table 3 Major enzymatic antioxidant

enzyme	action
Superoxide dismutases (SOD)	Converse of two superoxides into hydrogen peroxide and oxygen
Catalase	Degrades hydrogen peroxide to water and oxygen
Glutathione peroxidase	Like catalase, degrade hydrogen peroxide.

Non-enzymatic antioxidant

The major non-enzymatic antioxidant defense system includes ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione (GSH) and β-carotene (Kharrazi, et al., 2008) Non enzymatic antioxidants are exert their protective effect by quenching potentially damaging oxidants to a slow reacting radical, or is converted to a new non-reactive product before damage to cellular components (Halliwell and Gutteridge, 1990; Palace, et al., 1999)

Table 4 Major non-enzymatic antioxidant

enzyme	action
Vitamin E	Protect membrane from oxidative damage
Vitamin C	It also appears to participate in recycling vitamine E radicals. Interestingly, vitamin C also functions as a pro-oxidant under certain circumstances.
Glutathione	May well be the most important intracellular defense against damage by reactive oxygen species. It is a tripeptide (glutamyl-cysteinyl-glycine). The cysteine provides an exposed free sulphydryl group (SH) that is very reactive, providing an abundant target for radical attack. Reaction with radicals oxidizes glutathione, but the reduced form is regenerated in a redox cycle involving glutathione reductase and the electron acceptor NADPH.

In addition of major non enzymatic antioxidant, uric acid, bilirubin, were scavenging antioxidant. Ubiquinone is chain breaking antioxidant. Chelating agent, transferin lactoferrin ceruloplasmin albumin are inhibit fenton reaction.

Under normal conditions, there is a balance between both the activities and the intracellular levels of these antioxidants. This balance is essential for the survival of organisms and their health if this balance is perturb, it's lead to oxidative stress and damage to DNA, lipids and protein. The cooperation among different antioxidant provides greater protection against cellular damage by reactive oxygen or nitrogen species. Thus, the overall of antioxidant capacity may provide more information compared to that obtained by the measurement of individual components. The measure of cooperation of antioxidant is call total antioxidant capacity

Total Antioxidant capacity

A number of assays have been introduced for the measurement of the total antioxidant activity of body fluids (Halliwell and Chirico, 1993), food extracts (Evans and Burdon, 1993). Two types of approach have been taken, namely, the inhibition assays in that the extent of the scavenging by hydrogen- or electron-donation of a pre-formed free radical is the marker of antioxidant activity, as well as assays involving the presence of antioxidant system during the generation of the radical. From the ABTS [2, 29-azinobis-(3-ethylbenzothiazoline- 6-sulfonic acid)] radical generation as the cation form that the basis of the spectrophotometric methods that have been applied to the measurement of the total antioxidant activity of solutions of pure substances. The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS [2, 29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] to $ABTS^{\cdot}+$ by metmyoglobin. The amount of $ABTS^{\cdot}+$ produced can be monitored by reading the absorbance at 405 nm color is reverse correlated to total antioxidant (Roberta, et al, 1999).