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Biochemistry of Free Radicals and Oxidative Stress

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ABSTRACT

Oxidative stress is caused by free radicals, Reactive Oxygen Species (ROS) which damage DNA, biomembrane lipids, proteins and other macromolecules. The primary source of ROS is leakage of electron from the respiratory chain during the reduction of molecular oxygen to water generating superoxide anion. ROS can be classified into oxygen centered radicals and oxygen centered non radicals. The oxygen centered radicals are superoxide anion ($O^{\bullet-}_2$), hydroxyl radicals ($OH^{\bullet}$) and alkoxyl radicals ($RO^{\bullet}$) and peroxyl radicals ($ROO^{\bullet}$). Oxygen centered non radicals are hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). Other radicals species are nitrogen species such as nitric oxide ($NO^{\bullet}$), nitric dioxide ($NO^{\bullet}_2$) and peroxynitrite ($OONO^-$). ROS can be scavenged by the use of antioxidant system including non enzymatic components and a series of antioxidant enzymes. Non enzymatic components include glutathione, selenium, vitamin C and E. The antioxidant enzymes include glutathione peroxidase, catalase and superoxide dismutase which are the most major antioxidant enzymes that are capable to minimize oxidative stress in the organelles. The degree of lipid peroxidation is often used as an indicator of ROS mediated damage and the concentration of Malonaldehyde (MDA) in blood and tissues are generally used as biomarkers of lipid peroxidation. The mechanism of action of most of natural products and chemical drugs is done through the antioxidant properties of these drugs by reducing the lipid peroxidation and stimulation of enzymatic and non enzymatic antioxidant system within the organism. For instance, antioxidant properties of different natural product such as black cumin seed, curcumin, canola oil and plant combination can be evaluated by estimation of enzymatic and non enzymatic antioxidant level. Also, oxidative stress parameters as biomarker of metabolic diseases in equine whereas the preservation condition of spermatozoa in camel was also evaluated by determination of antioxidant capacity of the epididymal fluid. Importantly, many studies were exhibited in the oxidative stress era. However, this field of study still needs additional future researches at the molecular level.

Key words: Reactive oxygen species, oxidative stress biomarkers, antioxidants, metabolic diseases

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INTRODUCTION

Reactive Oxygen Species (ROS) or free radicals are generated in biological systems by prooxidative enzyme systems, lipid oxidation, irradiation, inflammation, air pollutants and glycoxidation[1,2]. The generation of these free radicals induced oxidative stress which associated with many degenerative diseases, including atherosclerosis, vasospasms, cancers, trauma, stroke, asthma, hyperoxia, arthritis, heart attack, age pigments, dermatitis, cataractogenesis, retinal damage liver injury[3,4] and induction of apoptosis[5]. In animals, free radicals are also associated with metabolic disorders as Rhabdomyolysis in Arabian horses[6], diabetes in bitches[7] and infectious diseases as theileria in Egyptian buffaloes[8,9]. In the contrary there are some benefits of free radicals have been reported. These benefits are the activation of nuclear transcription factors, gene expression and destructive effect to tumor cells and microorganisms[5]. Superoxide radicals ($O^{\bullet-}_2$) serve as a cell growth regulator[1] further; it can attack various pathogens inducing physiological inflammatory response[10]. Nitric oxide ($NO^{\bullet}$) is signaling molecules participating in cellular and organ function as a neurotransmitter and a mediator of the immune responses[11]. However, we can say that their deleterious effects are more than the beneficial one. Chemical antioxidants were used to ameliorate the harmful effect of ROS in animal model. Waheed[12] reported that sodium pyruvate, bovine serum albumin, zinc chloride and sodium thiosulfate were more effective for improving sperm viability and acrosine amidase activity of chilled stallion spermatozoa. Waheed[13] used the level of lipid peroxidation and antioxidants as biomarkers for evaluation of the preservation of epididymal spermatozoa in dromedary camel. El-Deeb[14] concluded that transportation were significantly enough to trigger changes in oxidative stress biomarkers in buffalo calves. Most recently, Waheed[13] concluded that season and

stallion age could affect the antioxidant defense systems in stallions' seminal plasma. The same authors added that, the impairment of seminal antioxidants and osteopontin can lead to low fertility in Arabian horses. In addition, many trials have been done to alleviate the oxidative stress harmful effect in animal tissues successfully by using antioxidants in medicinal plants which lay under broad strategy named back to nature. Salama[15] demonstrated that, generation of oxidative stress is one of the plausible mechanisms for cadmium induced cellular dysfunction and curcumin is a promising natural drug against cadmium toxicity. El-Bahr[16] concluded that iron overload induced oxidative stress to rat tissues and curcumin was a powerful antioxidant agent. The present article aimed to gain information about (1) classification of free radicals, (2) generation and pathways of free radicals, (3) antioxidant system, (4) effect of free radicals on lipid, protein and DNA and (5) biomarkers of oxidative stress.

CLASSIFICATION OF FREE RADICALS

Although oxygen is vital for aerobic bioprocesses up to 5% of inhaled oxygen is converted into reactive oxygen species (ROS[17]). ROS can be classified into oxygen centered radicals and oxygen centered non radicals (Fig. 1). The oxygen centered radicals are superoxide anion ($O^{\bullet -}_2$), hydroxyl radicals ($OH^{\bullet}$) and alkoxyl radicals ($RO^{\bullet}$) and peroxyl radicals ($ROO^{\bullet}$). Oxygen centered non radicals are hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). Other radicals species are nitrogen species such as nitric oxide ($NO^{\bullet}$), nitric dioxide ($NO^{\bullet}_2$) and peroxynitrite ($OONO^-$)[18,15]. In biological systems, ROS are related to free radicals, while $^1O_2$ and H_2O_2 are non radical compounds.

RS (reactive species), ROS (reactive oxygen species), $O^{\bullet -}_2$ (superoxide anion),.OH (hydroxyl radicals), $RO^{\bullet}$ (alkoxyl radicals), $ROO^{\bullet}$ (peroxyl radicals), H_2O_2 (hydrogen peroxide), $^1O_2$ (singlet oxygen), $NO^{\bullet}$ (nitric oxide), NO_2 (nitric dioxide), $OONO^-$(peroxynitrite).

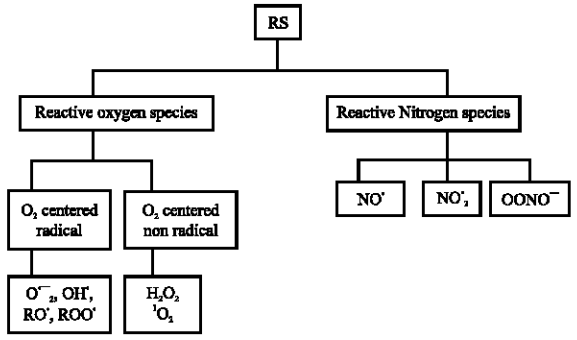

Fig. 1: Classification of free radicals

RADICAL GENERATION AND PATHWAYS FREE RADICALS

There are two unpaired electron of parallel spin in oxygen and thereby it can behave like a diradical but due to its quantum mechanical restriction, it does not exhibit such reactivity[19]. Its electronic structure results in formation of water by reduction with four electrons:

$$O_2+4H^++4e^-\rightarrow 2H_2O$$

However, in the sequential univalent process by which O_2 undergoes reduction, several reactive intermediates are formed such as $O^{\bullet -}_2$, H_2O_2 and $OH^{\bullet}$ as fellow:

$$O_2 \xrightarrow{+e} O_2^{\bullet -} \xrightarrow{+e} H_2O_2 \xrightarrow{+e} OH^{\bullet} \xrightarrow{+e} H_2O$$

$O^{\bullet -}_2$: Initial free radical: It has been shown that, the cell subjected to aerobic bioprocesses are always affected by production of reactive species as under normal physiological condition, it is estimated that up to 1% of mitochondrial electron flow leads to the formation of superoxide ($O^{\bullet -}_2$), the primary oxygen free radical produced by mitochondria during electron transport chain (respiratory chain; oxidative phosphorylation). Univalent reduction described above of oxygen in respiratory cells is restricted by cytochrome oxidase of the mitochondrial electron transport chain which reduces oxygen by four electrons to water without releasing either $O^{\bullet -}_2$ or H_2O_2. However, leak of single electron at the specific site of the mitochondrial electron transport chain resulting in electron reduction of oxygen to $O^{\bullet -}_2$[20,21]. While these partially reduced oxygen species can attack iron sulfur centers in a variety of enzymes, $O^{\bullet -}_2$ is rapidly converted within the cell to hydrogen peroxide (H_2O_2) by the Superoxide Dismutase (SOD), however (H_2O_2) can react with reduced transition metals via the Fenton reaction (Fig. 2) to produce the highly reactive hydroxyl radicals ($OH^{\bullet}$) a far more damaging molecule to the cell. Beside its role in the formation of H_2O_2, $O^{\bullet -}_2$ can rapidly react with nitric oxide (NO) generating a cytotoxic peroxynitrite anion ($OONO^-$) which can react

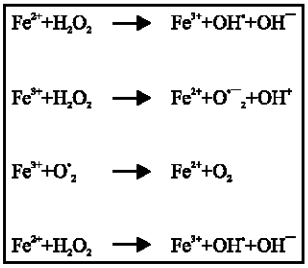

Fig. 2: Fenton reaction

with CO_2 leading to protein damage via. the formation of nitrotyrosine and lipid peroxidation[22].

Production of H_2O_2: The least reactive free radicals: The accumulated H_2O_2 as discussed above can generate the $OH^{\bullet}$ in the presence of metal ions and $O^{\bullet-}_2$[1]. It can produce $^1O_2$ through reaction with $O^{\bullet-}_2$ or with HOCl or chloroamines in living systems[2,10]. H_2O_2 can degrade certain heme proteins, such as hemoglobin, to release iron ions. The followings are examples of enzymes and some compounds that participate in H_2O_2 generation: Peroxisomal oxidase, Falvoprotein oxidase, D-aminoacid oxidase, L-hydroxyacid oxidase, fatty acid oxidase, Cytochrom P_{450}, Cytochrom P_{450} reductase, xanthin oxidase, phagocytic cell such as neutrophils, Spontenious dismutation of oxygen at neutral pH or dismutation by SOD and for details check review of Ramasarma[23].

Production of $OH^{\bullet}$: The most reactive free radical: Away from $OH^{\bullet}$ production during abnormal exposure to ionizing radiation, the production of $OH^{\bullet}$ *in vivo* requires the presence of trace amount of transition metals like iron or copper. This is clear by Fenton reaction.

The redox active free iron or cupper do not exist in biological system as these transition metal ions remain bound to proteins, membranes, nucleic acids and ATP[24]. However, during ischemic condition and cellular acidosis, transition metal ions may be released from some metaloproteins resulting in generation of $OH^{\bullet}$ as demonstrated above. This is an interpretation of the use of chelating therapy for control of myocardial infarction.

$^1O_2$: Excited non radicals: Breakdown of phosphatidylcholine hydroperoxides *in vivo* produced $^1O_2$[25]. The $^1O_2$ is the result of reaction of H_2O_2 with $O^{\bullet-}_2$ in tissues[2]. Compared with other ROS, $^1O_2$ is rather mild and nontoxic for mammalian tissue[2]. $^1O_2$ is involved in cholesterol oxidation[26].

$ROO^{\bullet}$ and $RO^{\bullet}$: Direct reaction between alkyl radicals ($R^{\bullet}$) and O_2 produced peroxyl radicals ($ROO^{\bullet}$), as reported in the reaction involved between lipid radicals and oxygen. However, $RO^{\bullet}$ and $ROO^{\bullet}$ can be produced by decomposition of alkyl peroxides (ROOH). UV rays or transition metal ions can produce the same action on ROOH. $ROO^{\bullet}$ and $RO^{\bullet}$ are good oxidizing agents and can abstract hydrogen from other molecules with lower standard reduction potential. This reaction is frequently observed in the propagation stage of lipid peroxidation. $RO^{\bullet}$ formed from this reaction can react with oxygen to form another peroxyl radical, resulting in chain reaction. Some peroxyl radicals break down to liberate superoxide anion or can react with each other to generate singlet oxygen[27].

$NO^{\bullet}$ and $NO^{\bullet}_2$: Nitric Oxide ($NO^{\bullet}$) is not a very free radical, with a single unpaired electron, formed from L-arginine by NO synthase[11]. $NO^{\bullet}$ overproduction is involved in ischemia reperfusion and other diseases. $NO^{\bullet}$ initiate lipid peroxidation and deplete the concentration of ascorbic acid and uric acid[28]. Reaction of $ROO^{\bullet}$ and NO generate $NO^{\bullet}_2$[29]. $NO^{\bullet}_2$ do the same effect of $NO^{\bullet}$ concerning lipid, ascorbic acid and uric acid[30].

OONO: $OONO^-$ is formed from the reaction of NO and $O^{\bullet-}_2$. $OONO^-$ is a oxidizing agent for Low-density Lipoprotein (LDL) and tissues[1]. It is involved in various neurodegenerative disorders[31]. It is high sponsored it as a biological oxidant[31].

ANTIOXIDANT SYSTEM

Now, good question is arisen, the question is how aerobic organisms survive its presence? The answer is simply only because they contain antioxidant defense[32]. Antioxidants can be synthesized *in vivo* or taken in diet[33]. Antioxidants can be efficiently removing ROS thereby, protecting cells from adverse effect. The generation of ROS in normal cell occurred under tight homeostatic control by antioxidants, however, when ROS levels exceed the antioxidant capacity of the cell, a deleterious condition known as oxidative stress occurs. Halliwell[33] define antioxidants as any substance or action that minimize exposure to oxygen and this definition worked well in food manufacturers who exploit this strategy when they seal foods under nitrogen or in vacuum packs. By the way, in healthy aerobic organisms, production of free radicals is approximately balanced with antioxidant defense system[32]. Thereby, we can say that antioxidants control the level of reactive species rather than eliminate them[33]. The aerobic organism's posses a multi-leveled ROS defense network of enzymes and non enzymatic antioxidants. Enzymatic antioxidants act as primary defense whereas non enzymatic antioxidants act as secondary against ROS[19].

Enzymatic antioxidants defense: Superoxide Dismutase (SOD), catalase and peroxidases are the main enzymes incorporated in defense mechanism against ROS. SOD dismutates $O^{\bullet-}_2$ while catalase and peroxidases detoxify H_2O_2.

Superoxide dismutase (SOD): The first line of defense against ROS is the SOD which is a metalenzyme found in prokaryotic and eukaryotic cells[34,35]. The iron and manganese are the main prosthetic groups of SOD in

prokaryotes whereas in eukaryotes, the prosthetic groups of cytosolic SOD are copper and zinc. Beside the cytosolic SOD, eukaryotic mitochondrial SOD is also present and containing manganese as a prosthetic group[34]. The level of $O^{\bullet-}_2$ regulates the rate of SOD biosynthesis.

Glutathione peroxidase (GPx): Glutathione peroxidase is mainly cytosolic selenoenzyme and attack hydroperoxides with the aid of reduced glutathione (GSH) to form oxidized glutathione (GSSG) and the reduction product of the hydroperoxide[19]. Mitochondrial glutathione peroxidase is also present[36].

Catalase: Catalase is hemoenzyme catalyze decomposition of H_2O_2 to water and O_2 protecting the cell against oxidative stress induced by H_2O_2 or consequently formed $OH^{\bullet}$[37]. Enzyme is peroxisomal or microperoxisomal in origin[21].

Non-enzymatic antioxidants defense: Free radical scavengers: Secondary defense against ROS is induced by small molecules which react with radicals to produce a lesser harmful radical species α-tocopherol (vitamin E), ascorbic acid (vitamin C) and reduced Glutathione (GSH) may acts as cellular antioxidants. α-tocopherol, present in the cell membrane and plasma lipoproteins, functions as a chain-breaking antioxidant[38]. Once the tocopherol radical is formed, it can migrate to the membrane surface and is reconverted to α-tocopherol by reaction with ascorbate or GSH. The resulting ascorbate radical can regenerate ascorbate by reduction with GSH which can also directly scavenge ROS and the resulting GSSG can regenerate GSH through NADPH-glutathione reductase system. In addition, to mentioned above some medicinal plants has antioxidant activity and used extensively nowadays as back to nature. *Curcumin*, the active ingredient from the spice turmeric is a potent antioxidant against oxidative tissue damage [16,15,39]. It can significantly inhibit the generation of ROS both *in vitro* and *in vivo*[40]. Salama[15] reported that, cadmium induced testicular damage in albino rats which reflected as significant increase in lipid peroxidation, Malonaldehyde (MDA) and catalase with decrease in reduced glutathione and glutathione-S-transferase. However, curcumin administration ameliorate the worth effect and restored the antioxidants value around normal. El-Bahr[16] demonstrated that iron overload caused many adverse effects reflected the significant increase of all serum iron profile, tissue iron deposition and tissue lipid peroxidation. Iron overload also caused a significant decrease of GPx activity while GST activity and GSH level were significantly increased in all tissues. In the contrary administration of turmeric alone induced a significant decrease of serum and tissue iron profile. The powerful antioxidant effect of turmeric was reflected on the marked increase of GPx activity, GST activity and reduced glutathione level in examined tissues. A comparison between four antioxidants namely, sodium pyruvate (0.5 mg mL^{-1}), sodium thiosulfate (STS, 1.0 mg mL^{-1}), bovine serum albumin (BSA, 5.0 mg mL^{-1}), zinc chloride (0.15 mg mL^{-1}) and a mixture of them was studied in a chemically-defined stallion semen extender (Tris-egg yolk) at 5°C[12]. The comparison was based on sperm viability, acrosin amidase activity and changes in the levels of extracellular alanine aminotransferase (ALT). The researchers demonstrated that, sodium pyruvate and the mixture of antioxidants were most effective for improving viability and acrosin amidase activity of stallion spermatozoa. Black cumin seed (*Nigella sativa*) is herbaceous plant which is a member of the Ranunculocea family. The black cumin seed showed high antioxidant activity[41]. The same authors added that, the antioxidant activity of black cumin seeds was attributed to the capability of plants active principles (polyphenols and thymoquinone) to scavenge ROS (hydroxyl and peroxide radicals) and thus inhibits radical mediated lipid peroxidation. However, beneficial effect of medicinal plants fluctuated according to species, age and source of plants, dose and duration of experiment[42].

EFFECT OF FREE RADICALS ON LIPID, PROTEIN AND DNA

Lipid: The phospholipid bilayers of the cell membranes are the site of lipid oxidation[43]. Decrease in thermal denaturation resistance and lipid molecular mobility with increased lipid surface charge and protein oligmers are the consequence of increased lipid peroxidation. Malonaldehyde (MDA), is one of the most lipid oxidation products. When it reacts with the free amino group of proteins, phospholipid and nucleic acids induce dysfunction of immune systems. The increases of lipid oxidation products are found in diabetes, atherosclerosis and liver disease. Oxidative modification of LDL has been reported to be involved with the development of atherosclerosis and cardiovascular disease[44]. Oxidized cholesterol or fatty acid in the plasmatic LDL can develop atherosclerosis[40,43,45].

Protein: Methionine sulfoxide, 2-oxohistidine and protein peroxides considered biomarkers of oxidative stress of protein (structural modification). Initiation of protein modification started by hydroxyl radicals, leading to the oxidation of amino acid side chains, protein cross linkage and finally protein fragmentation[46,47]. The availability of oxygen, superoxide anion and its protonated form (HO_2^-) determines the pathways of protein oxidation processes. Berlett[46] reported that,

induction of 3-chlorotyrosine from tyrosine by hypochlorous acid, the oxidization of histidine to 2-oxohistidine in the metal binding site of proteins, the oxidation of thiol groups and the generation of carbonyl derivatives of amino acids are some examples of protein modifications. Malonaldehyde and 4-Hydroxy-2-nonenal from lipid oxidation reacts with protein amino groups. $NO^{\bullet}$ and $ONOO^{-}$ acts as oxidizing agents of protein.

DNA: Mitochondrial DNA is susceptible to oxidative damages because of the lack of protective protein, histones of nuclear DNA and close locations to the ROS producing systems. Hydroxyl radical oxidizes guanosine or thymine to 8-hydroxy-2-deoxyguanosine and thymine glycol, respectively which changes DNA and leads to mutagenesis and carcinogenesis[48] and used as a biological marker for oxidative stress[49]. If oxidative stress is too great, the DNA repair system using glycosylase is not enough and mutagenesis and/or carcinogenesis can be induced.

Oxidative stress biomarkers: It is well known that, increment of ROS leads to elevation of the levels of oxidized DNA (8-hydroxydeoxyguanosine), oxidative damaged proteins with carbonyl modifications, loss of protein-SH group, reduced glutathione: Oxidized glutathione ratio, NADPH:$NADP^{+}$ ratio and NADH:NAD^{+} ratio [1,18,50]. El-Deeb[9] demonstrated a significant decrease in the level of reduced GSH, SOD, Catalase, total antioxidant capacity and nitric oxide in Egyptian buffaloes infected with theileria annulata. The decrease in antioxidants was attributed to the consumption of these antioxidants to counteract sever oxidative stress of buffaloes tissues induced by theileria infection. El-Deeb[9] reported that, the level of reduced GSH, SOD, catalase, total antioxidant capacity and nitric oxide were decreased in rhabdomyolysis diseased Arabian horse. The same authors demonstrated a significant increase in tumor necrosis factor alpha (TNF-α), malondialdehyde (MDA), interleukin 6 and prostaglandin $F_2\alpha$ ($PGF_2\alpha$) in rhabdomyolysis diseased horse. These findings indicated that rhabdomyolysis induced oxidative stress to Arabian horse and subsequently increased level of lipid peroxidation product (MDA) and proinflammatory cytokines[51,52]. Waheed[13] designed a study to investigate the enzymatic antioxidants activity and non-enzimatic antioxidants levels in seminal plasma of stallions and to relate them with season, age and fertility of Arabian stallions. The results demonstrated that season and stallion age could affect the antioxidant defense systems in stallions' seminal plasma. The same authors added that, the impairment of seminal antioxidants and osteopontin can lead to low fertility in Arabian horses. El-Deeb[12] concluded that transportation were significantly enough to trigger changes in oxidative stress biomarkers in buffalo calves.

CONCLUSION

The generation of ROS in normal cell occurred under tight homeostatic control by antioxidants, however, when ROS levels exceed the antioxidant capacity of the cell, a deleterious condition known as oxidative stress occurs. Excessive ROS can lead to the destruction of cellular components including lipids, protein and DNA and ultimately cell death via apoptosis or necrosis. Molecular study concerning the blocking of the expression of genes involved in inflammatory responses and tissue injury should be encouraged in the Future. Investigation of medicinal plants ingredient in this field could be of great importance for controlling the ROS-mediated pathogenesis.

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