J Med Allied Sci 2011; 1 (2): 53-60
www.jmas.in
Print ISSN: 2231 1696 Online ISSN: 2231 170X
Journal of Medical & Allied Sciences

Review

Free radicals, oxidative stress and importance of antioxidants in human health

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Article history

Received 13 March 2011
Revised 04 May 2011
Accepted 14 June 2011
Early online 01 July 2011
Print 31 July 2011

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Abstract

Reactive oxygen species (ROS) is a collective term used for oxygen containing free radicals, depending on their reactivity and oxidizing ability. ROS participate in a variety of chemical reactions with biomolecules leading to a pathological condition known as oxidative stress. Antioxidants are employed to protect biomolecules from the damaging effects of such ROS. In the beginning, antioxidant research was mainly aimed at understanding free radical reactions of ROS with antioxidants employing biochemical assays and kinetic methods. Later on, studies began to be directed to monitor the ability of anti-oxidants to modulate cellular signaling proteins like receptors, secondary messengers, transcription factors, etc. Of late several studies have indicated that antioxidants can also have deleterious effects on human health depending on dosage and bioavailability. It is therefore, necessary to validate the utility of antioxidants in improvement of human health in order to take full advantage of their therapeutic potential.

Key words: Reactive oxygen species, oxidative stress, antioxidant supplementation

Reactive oxygen species (ROS) is a collective term used for a group of oxidants, which are either free radicals or molecular species capable of generating free radicals. Intracellular generation of ROS mainly comprises superoxide ($O_2^{\bullet-}$) radicals and nitric oxide ($NO^{\bullet}$) radicals. Under normal physiologic conditions, nearly 2% of the oxygen consumed by the body is converted into $O_2^{\bullet-}$ through mitochondrial respiration, phagocytosis, etc[1]. ROS percentage increases during infections, exercise, exposure to pollutants, UV light, ionizing radiation, etc. $NO^{\bullet}$, is an endothelial relaxing factor and neurotransmitter, produced through nitric oxide synthase enzymes. $NO^{\bullet}$ and $O_2^{\bullet-}$ radicals, are converted to powerful oxidizing radicals like hydroxyl radical ($^{\bullet}OH$), alkoxy radicals ($RO^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$), singlet oxygen ($^1O_2$) by complex transformation reactions. Some of the radical species are converted to molecular oxidants like hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl). Sometimes these molecular species act as source of ROS.

For example, H_2O_2 is converted to $^{\bullet}OH$ radicals by Fenton reaction and HOCl through its reaction with H_2O_2 can be converted to $^1O_2$. $ONOO^-$ at physiological concentrations of carbon dioxide becomes a source of carbonate radical anion ($CO_3^{\bullet-}$)[1]. The various pathways involved in the generation of ROS are given in fig 1.

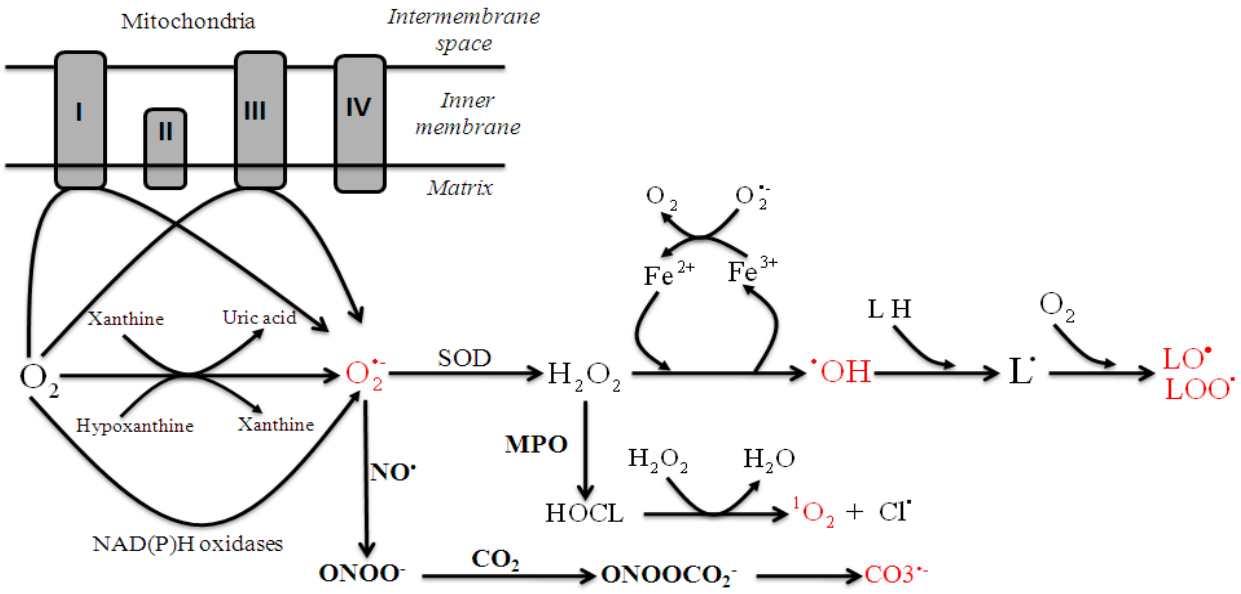

Fig 1. Production of free radicals via different routes

ROS in normal physiology

Typically, low concentration of ROS is essential for normal physiological functions like gene expression, cellular growth and defense against infection. Sometimes they also act as the stimulating agents for biochemical processes within the cell[2]. ROS exert their effects through the reversible oxidation of active sites in transcription factors such as nuclear factor-kappa B (NF-kB) and activator protein-1 (AP-1) leading to gene expression and cell growth[3]. ROS can also cause indirect induction of transcription factors by activating signal transduction pathways[3]. One example of signal transduction molecules activated by ROS is the mitogen activated protein kinases (MAPKs). ROS also appear to serve as secondary messengers in many developmental stages. For example, in sea urchins ROS levels are elevated during fertilization. Similarly prenatal and embryonic development in mammals has also been suggested to be regulated by ROS[3]. Apart from these; ROS also participate in the biosynthesis of molecules such as thyroxin, prostaglandin that accelerate developmental processes. It is noteworthy that in thyroid cells, regulation of H_2O_2 concentration is critical for thyroxine synthesis, as it is needed to catalyze the binding of iodine atoms to thyroglobulin[3]. Finally ROS are also used by the immune system. For example, ROS have been shown to trigger proliferation of T cells through NF-κB activation. Macrophages and neutrophils generate ROS in order to kill the bacteria that they engulf by phagocytosis. Furthermore, tumor necrosis factor (TNF-α) mediates the cytotoxicity of tumor and virus infected cells through ROS generation and induction of apoptosis[2,3].

ROS induced oxidative damages

Depending upon their nature, ROS (for e.g. •OH radicals) reactions with biomolecules such as lipid, protein and DNA, produce different types of secondary radicals like lipid radicals, sugar and base derived radicals, amino acid radicals and thiyl radicals. These radicals in presence of oxygen are converted to peroxyl radicals. Peroxyl radicals are critical in biosystems, as they often induce chain reactions[1]. The biological implications of such reactions depends on several factors like site of generation, nature of the substrate, activation of repair mechanisms, redox status among many others[4].

For example, cellular membranes are vulnerable to the oxidation by ROS due to the presence of high concentration of unsaturated fatty acids in their lipid components. ROS reactions with membrane lipids cause lipid peroxidation, resulting in formation of lipid hydroperoxide (LOOH) which can further decompose to an aldehyde such as malonaldehyde, 4-hydroxy nonenal (4-HNE) or form cyclic endoperoxide, isoprotans, and hydrocarbons. The consequences of lipid peroxidation are cross linking of membrane proteins, change in membrane fluidity and formation of lipid-protein, lipid-DNA adduct which may be detrimental to the functioning of the cell[5].

Proteins can undergo direct and indirect damage following interaction with ROS resulting in to peroxidation, changes in their tertiary structure, proteolytic degradation, protein-protein cross linkages and fragmentation[5]. The side chains of all amino acid residues of proteins, in particular tryptophan, cyste-

ine and methionine residues are susceptible to oxidation by ROS. Protein oxidation products are usually carbonyls such as aldehydes and ketones.

Although DNA is a stable, well-protected molecule, ROS can interact with it and cause several types of damage such as modification of DNA bases, single and double strand DNA breaks, loss of purines (apurinic sites), damage to the deoxyribose sugar, DNA-protein cross-linkage and damage to the DNA repair system[5]. Not all ROS can cause DNA damage and •OH radical is one of the potential inducers of DNA damage. A variety of adducts are formed on reaction of •OH radical with DNA. The •OH radical can attack purine and pyrimidine bases to form •OH radical adducts, which are both oxidizing and reducing in nature. This induces base modifications and sometimes release of bases. Some of the important base modifications include 8-hydroxydeoxyguanosine (8-OHdG), 8 (or 4-, 5-)-hydroxyadenine, thymine peroxide, thymine glycols and 5-(hydroxymethyl) uracyl[5]. Free radicals can also attack the sugar moiety, which can produce sugar peroxyl radicals and subsequently inducing strand brakeage. The consequence of DNA damage is the modification of genetic material resulting in to cell death, mutagenesis, carcinogenesis and ageing.

Antioxidants and natural defense from ROS induced damages

Uncontrolled generation of ROS can lead to their accumulation causing oxidative stress in the cells. Therefore, cells have evolved defense mechanisms for protection against ROS mediated oxidative damage. These include antioxidant defenses to keep a check on the generation of ROS. An antioxidant is a substance that is present at low concentrations and significantly delays or prevents oxidation of the oxidizable substrate[6]. Antioxidants are effective because they can donate their own electrons to ROS and thereby neutralizing the adverse effects of the latter. In general, an antioxidant in the body may work at three different levels: (a) prevention - keeping formation of reactive species to a minimum e.g. desferrioxamine (b) interception - scavenging reactive species either by using catalytic and non-catalytic molecules e.g. ascorbic acid, alpha-tocopherol and (c) repair - repairing damaged target molecules e.g. glutathione[6]. The antioxidant systems are classified into two major groups, enzymatic antioxidants and non enzymatic antioxidants. Enzymatic antioxidants present in the body include superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) that act as body's first line of defense against ROS by catalyzing their conversion to less reactive or inert species (Fig 2)[7].

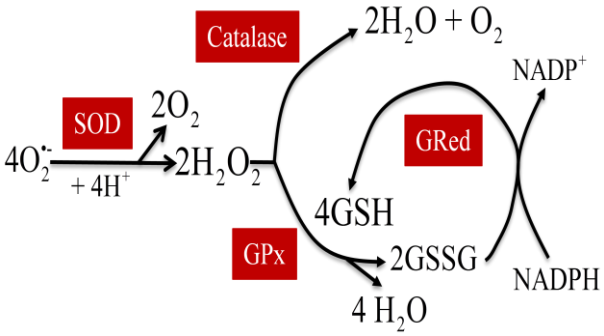

Fig 2. Removal of different reactive oxygen species by antioxidant enzymes

Several low molecular weight molecules present inside the cell provide secondary defense against free radicals. A few examples of such molecules include glutathione (GSH), α-tocopherol, ascorbate, bilirubin, etc[6]. These agents either scavenge the ROS directly or prevent the production of ROS through sequestration of redox active metals like iron and copper.

Redox state and oxidative stress

All forms of life maintain a steady state concentration of ROS determined by the balance between their rates of production and their rates of removal by various antioxidants. Thus each cell is characterized by a particular concentration of reducing species like GSH, NADH, FADH, etc. stored in many cellular constituents which determines the redox state of a cell[6]. By definition redox state is the total reduction potential or the reducing capacity of all the redox couples such as GSSG/2GSH, NAD+/NADH, $Asc^{\bullet-}$/$AcsH^-$, etc found in biological fluids, organelles, cells or tissues[8]. Redox state not only describes the state of a redox pair, but also the redox environment of a cell. Under normal conditions, the redox state of a biological system is maintained towards more negative redox potential values. However, with increase in ROS generation or decrease in antioxidant protection within cells, it is shifted towards less negative values resulting in the oxidizing environment (Fig 3). This shift from reducing status to oxidizing status is referred as oxidative stress[6,8]. During elevated oxidative stress, there is loss of mitochondrial functions, which results in to ATP depletion and necrotic cell death, while moderate oxidation can trigger apoptosis. There are a few recent reports have shown evidence that the induction of apoptosis or necrosis during oxidative stress is actually determined by the redox state of cell[8]. For example it has been reported that an increase in reduction potential of +72 mV in HL-60 cells (i.e., from -239 ± 6 to -167 ± 9 mV) or an increase of +65 mV in murine hybridoma cells (i.e., from -235 ± 5 to -170

± 8 mV) would cause induction of apoptosis[8]. Oxidative stress has been implicated in a number of human diseases like cancer, atherosclerosis, diabetics, neurological diseases such as Alzheimer's disease, Parkinson's disease, etc. as well as in the ageing process.

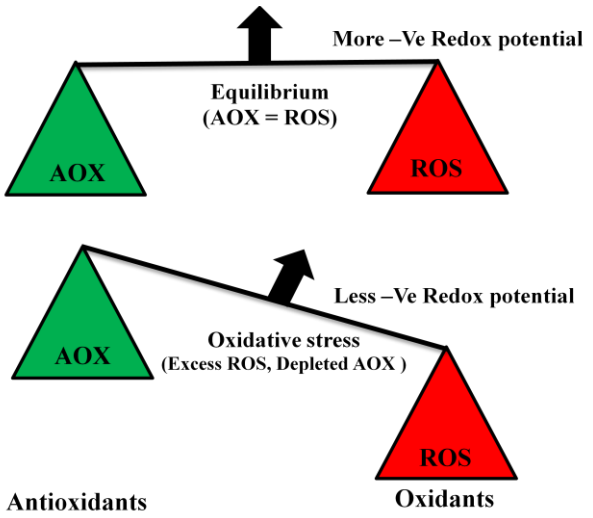

Fig 3. Balance between oxidant and antioxidant defines oxidative stress

Antioxidant supplementation

Although cells are equipped with an impressive repertoire of antioxidant enzymes as well as small antioxidant molecules, these agents may not be sufficient enough to normalize the redox status during oxidative stress[9]. Under such conditions supplementation with exogenous antioxidants is required to restore the redox homeostasis in cells. Recent epidemiological studies have shown an inverse correlation between the levels of established antioxidants (vitamin E and C) / phytonutrients present in tissue / blood samples and cardiovascular disease, cancer and with mortality due to these diseases[10-12]. Since several plant products are rich in antioxidants and micronutrients, it is likely that dietary antioxidant supplementation protects against the oxidative stress mediated disease development. Therefore, to maintain optimal body function, antioxidant supplementation has become an increasingly popular practice. Researchers are now attempting to develop new antioxidants either of natural or synthetic origin.

Natural products as antioxidants

A variety of dietary plants including grams, legumes, fruits, vegetables, tea, wine etc. contain antioxidants. The prophylactic properties of dietary plants have been attributed to the antioxidants / polyphenols present in them. Polyphenols with over 8000 structural variants are secondary metabolites of plants and represent a huge gamut of substances having aromatic ring(s) bearing one or more hydroxyl moieties[13]. Polyphenols are effective ROS scavengers and metal chelators due to the presence of multiple hydroxyl groups. Examples of polyphenolic natural antioxidants derived from plant sources include vitamin E, flavonoids, cinnamic acid derivatives, curcumin, caffeine, catechins, gallic acid derivatives, salicylic acid derivatives, chlorogenic acid, resveratrol, folate, anthocyanins and tannins[13]. Apart from polyphenols there are also some plant derived non-phenolic secondary metabolites such as melatonin, carotenoids, retinal, thiols, jasmonic acid, eicosapentaenoic acid, ascopyrones and allicin that show excellent antioxidant activity[14,15]. Vitamin C, the water soluble natural vitamin, plays a crucial role in regenerating lipid soluble antioxidants like vitamin E[6]. Both vitamin E and C are used as standards for evaluating the antioxidant capacity of new molecules[6]. As an example, the antioxidant activity of curcumin has been discussed in some detail in the following section.

Curcumin a well-known natural antioxidant

Curcumin is a yellow pigment, the major constituent of turmeric. It is a diferuloyl methane having an unsaturated β-diketone, and phenolic groups. It exhibits a variety of pharmacological properties such as anti-inflammatory, anti-carcinogenic, anti-microbial, neuro-protective, cardio-protective, thrombo-suppressive and anti-diabetic actions[16,17]. The compound is considered as a potent anti-cancer agent and is currently being evaluated in different stages of clinical trials against a variety of cancers[16].

Curcumin is also a potent antioxidant. Studies from our laboratory as well as others have shown it to be an excellent scavenger of ROS such as $O_2^{\bullet-}$ radicals, lipid peroxyl radicals, $^{\bullet}OH$ radicals and nitrogen dioxide radicals, whose production is implicated in the induction of oxidative stress[18,19]. Its free radical scavenging ability is comparable to well known antioxidants like vitamins C and E[19]. It has been shown to inhibit lipid peroxidation in a variety of *in vitro* models such as rat brain homogenates, rat liver microsomes, erythrocytes, liposomes, and macrophages, where peroxidation is induced by Fenton reagent, H_2O_2, radiation and 2,2-azo-bis(2-amidinopropane) hydrochloride (AAPH)[19]. It has also been reported to inhibit singlet oxygen-stimulated DNA cleavage in plasmid pBR322 DNA, H_2O_2 and AAPH induced hemolysis of erythrocytes[19,20]. In epithelial cells, curcumin has been shown to increase GSH levels which, in turn lead to lowered ROS production[21]. It also mediates its antioxidative effects by elevating the levels of phase II enzymes such as

NAD(P)H:quinone reductase (QR) and antioxidant enzymes like SOD, GPx and hemeoxygenase (HO)[21,22]. For example our own studies have found that curcumin induces the expression of SOD, GPx and HO-1 in RAW 264.7 (murine macrophage) cells contributing to its antioxidant effects[22]. Similarly, the *in vitro* incubation of bovine aortic endothelial cells and human proximal renal tubular cells with curcumin has been reported to result in dose and time dependent increase of *HO-1* mRNA, protein expression and enzymatic activity[23]. The postulated mechanism for these actions involves the activation of PKC pathways and antioxidant response element (ARE) mediated transcriptional induction. Curcumin has also been shown to inhibit oxidative damage in different animal models. For example, it inhibited lipid degradation and decreased ischemia-induced biochemical changes in heart in the feline model. In a focal cerebral ischemia model of rats, it offered significant neuroprotection through inhibition of lipid peroxidation, increase in endogenous antioxidant defense enzymes and reduction in peroxynitrite formation[24]. Further, studies on the mechanistic aspects of antioxidant activity revealed that phenolic hydroxyl groups of curcumin play a significant role in its diverse antioxidant activity[25]. Some reports suggested that both hydroxyl and diketone groups exert antioxidant properties. The phenolic hydroxyl groups give ROS scavenging ability and the diketone structure is considered to be responsible for its ability to bind to metals. The ability of curcumin to act as an antioxidant in the presence of metals arises mainly by preventing the Fenton chemistry within cells through chelation of free metal ions such as Cu^{+2}, Fe^{+2}, etc[26]. There are some reports which indicate that stable metal complexes of curcumin exhibit higher antioxidant activity as compared to native curcumin molecule. The manganese complexes of curcumin were found to show greater SOD activity, hydroxyl radical scavenging activity, and nitric oxide radical scavenging activity than the parent molecules[27]. Similarly, our group has reported that copper complex of curcumin also exhibits antioxidant, superoxide-scavenging and SOD enzyme mimicking activities superior to those of curcumin itself[28]. These copper curcumin complexes were found better than curcumin in preventing the γ-radiation induced oxidative stress in splenic lymphocytes. The associated mechanisms responsible for above effects were identified as activation of cytoprotective signaling components like protein kinase C delta (PKCδ) and nuclear factor-κB (NF--κB) in temporally relevant manner[29]. Thus, curcumin exhibits a variety of antioxidant effects and appears to have a significant potential in the treatment of multiple diseases that are mediated through oxidative stress.

Interestingly, reports are now appearing about apparently contradictory pro-oxidative effects of curcumin. For example, curcumin induced DNA fragmentation and base damage in the presence of copper and isozymes of cytochrome p450 (CYP) that are present in lung, lymph, liver, and skin[30]. The authors hypothesized that the damage was the result of CYP-catalyzed O-demethylation of curcumin, leading to the formation of an O-demethyl curcumin radical, which, in the presence of copper, formed a DNA-damaging Cu(I)-hydroperoxo complex. DNA damage was attenuated when concentrations of curcumin exceeded those of copper, presumably due to the chelation of copper by curcumin. Copper dependent formation of 8-hydroxy-deoxyguanosine in response to curcumin was also reported (Yoshino et al., 2004) and linked to apoptotic cell death in HL60 cells[31]. Similarly, curcumin-mediated DNA damage was also reported in mouse lymphocytes. In agreement with these reports we also observed that although curcumin inhibited that AAPH induced lipid peroxidation and hemolysis in erythrocytes, it could not prevent the leakage of K^+ ions. Rather curcumin itself induced K^+ ion release and GSH depletion at higher concentration suggesting its pro-oxidant nature[20]. Further, our group has reported that curcumin induced the ROS generation and GSH depletion in RAW 264.7 cells in a concentration and time dependant manner[22]. Of late, several reports have emerged demonstrating pro-oxidative nature of curcumin, in view of its ability to promote oxidative stress in transformed cells in culture. These effects have been correlated with enhanced ROS production, alteration of the cellular redox homeostasis (e.g., the depletion of glutathione), and disruption of the mitochondrial functions e.g., dissipation of mitochondrial inner transmembrane potential[32-35]. The enhancement of oxidative stress by curcumin in transformed cells ultimately results in mitochondrial-mediated apoptosis, and this has been considered as one of the mechanisms responsible for the anticancer activity of curcumin[32-35]. The mechanism by which curcumin mediates its pro-oxidant effects is not completely understood. However, some reports suggest that curcumin irreversibly binds to mitochondrial thioredoxin reductase, and modifies its activity in to NADPH oxidase through alkylation of cysteine residue present in the catalytically active site of the enzyme[35]. This leads to the production of ROS, which according to few others is due to the α,β-unsaturated carbonyl moiety of curcumin[19]. The pro-oxidant property is also believed to be due to the generation of phenoxyl radicals of curcumin by heme peroxidase-H_2O_2 system. These phenoxyl radicals could be repaired by cellular GSH or NADH. In this process, the resulting $GS^{\bullet}$ radical forms

$GSSG^{\bullet-}$ radical and this may further reduce O_2 to form $O_2^{\bullet-}$ radical leading to elevated ROS levels[36].

In short, all these published reports support that curcumin may switch from antioxidant to pro-oxidant depending on cell type, redox environment and dosage. A few reports also suggest that curcumin acts as an antioxidant in normal cells while showing preferable pro-oxidant behavior in tumor cells. It is this differential property of curcumin, which makes it a potent anti-tumor agent. The chemical structure of curcumin and its reported antioxidant and pro-oxidant mechanisms has been shown in fig 4.

Synthetic compounds as antioxidants

The use of synthetic compounds possessing antioxidant activity for the preservation of cosmetic, pharmaceutical and food products has been a common practice. The most commonly used synthetic antioxidants in the food industry are butylated 4-hydroxytoluene (BHT) and butylated 4-hydroxyanisole (BHA)[37]. However, the use of synthetic antioxidants in the health industry has been fraught with concerns about the toxicity associated with synthetic compounds[16]. There are numerous reports indicating that polyphenols which are the major constituent of most of the natural antioxidants are poorly bio-absorbed and the concentrations achieved in the target tissues are sub-therapeutic *in vitro*[38]. These findings have shifted the attention of researches towards the development of synthetic, water soluble, stable and nontoxic compounds with potent antioxidant activity and therapeutic application. Many different antioxidants and antioxidant compositions have been developed over the years based on their mechanism of action.

One group of such antioxidants includes molecules that prevent the production of ROS through metal ions sequestration, free radical scavenging or by inhibiting the ROS producing enzymes. For example, desferrioxamine an iron chelator have been tested for preventing ROS formation in a myocardial stunning model system following hemorrhagic and endotoxic shock[39]. The allopurinol and other pyrazolopyrimidines, which are inhibitors of xanthine oxidase, have also been tested under similar disease model system and have been found to be very effective. Several congeners of GSH have been used in various animal models to attenuate ROS induced injury. For example, *N*-2-mercaptopropionylglycine has been found to confer protective effects in a canine model of myocardial ischemia and reperfusion and N-acetylcysteine (NAC) has been used to treat endotoxin toxicity in sheep. Dimethyl thiourea (DMTU) and butylphenylnitrone (BPN) are believed to scavenge hydroxyl radical, and have been shown to reduce ischemia reperfusion injury in rat myocardium and in rabbits[40].

Another important group of synthetic antioxidants includes molecules that act as antioxidant enzyme mimic and catalytically remove the ROS. For example, the complex formed between the chelator, desferroxamine and manganese possesses SOD activity and has shown some activity in biological models, but the instability of the metal ligand complex apparently precludes its pharmaceutical use. Porphyrin-manganese and curcumin-transition metal complexes have also shown SOD activity and are under development as SOD mimetic drugs[27]. Ebselen an organoselenium compound exhibits GPx activity and has been tested in clinic as anti-inflammatory drug[41]. Recently our group has also been engaged in the development of aliphatic water-soluble selenium compounds as antioxidants. One such compound diseledipropionic acid showed significant antioxidant activity and potent *in vivo* radioprotection against exposure to lethal dose of γ-radiation[42].

Based on these studies, it is clear that a need exists for antioxidant agents, which are efficient in removing ROS, inexpensive to manufacture, stable, and possess advantageous pharmacokinetic properties, such as the ability to cross the blood-brain barrier and penetrate tissues. Such versatile antioxidants would find use as pharmaceuticals and possibly as neutraceuticals.

Limitations of antioxidant supplementation

The primary concern regarding antioxidant supplementation is their potentially deleterious effects on ROS production (pro-oxidant action) especially when precise modulation of ROS levels are needed to allow normal cell function[43]. In fact, some negative effects of antioxidants when used in dietary supplements (flavanoids, carotenoids, vitamin C and synthetic compounds) have emerged in the last few decades[11,12,44]. Mechanistic investigation has revealed that antioxidants may exhibit pro-oxidant activity depending on the specific set of conditions. Of particular importance are their dosage, redox conditions and also the presence of free transition metals in cellular milieu[36,44]. For example, ascorbate, a well-known antioxidant in the presence of high concentration of ferric iron is a potent mediator of lipid peroxidation. Recent studies suggest that ascorbate sometimes increases DNA damage in humans. Similarly β-carotene also can behave as a pro-oxidant in the lungs of smokers. Of note, natural antioxidant compounds have relatively poor bioavailability. It is therefore necessary to take into cogni-

zance the bioavailability and differential activities of natural and synthetic antioxidant compounds before considering

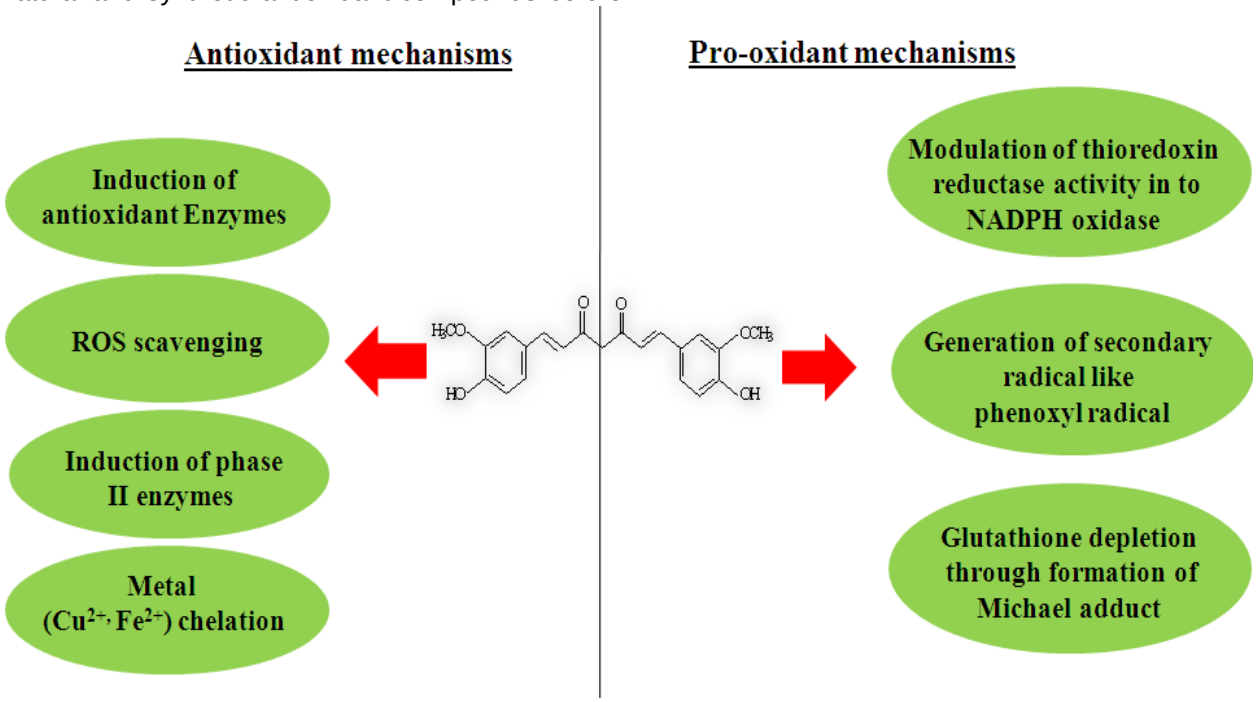

Fig 4. Important factors controlling the antioxidant and pro-oxidant activities of curcumin

them as therapeutic or pharmacological agents.

Conclusion

In this regard it is worth mentioning that at present several natural as well as synthetic compounds are available in the market as antioxidant supplements in different formulations like capsules, tablets, etc. with a direction to be consumed under specific diseased condition. However, as a caution it is advised to undertake the consumption of such supplements only under a strict medical supervision in order to avoid the dosage related negative effects.

Acknowledgments

The authors are also grateful to Dr. S.K. Sarkar, Head, RPC Division and Dr. T. Mukherjee, Director, Chemistry Group, BARC for encouragement.

Conflict of interest: None

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