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PERSPECTIVE

Strategies to Target Mitochondria and Oxidative Stress by Antioxidants: Key Points and Perspectives

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ABSTRACT For several decades, many antioxidants studies have emphasized the marked disparity between the beneficial effect of the antioxidants shown in preclinical studies and their inability to show beneficial effects in clinical trials. Besides, it is not uncommon to find highly contradictory clinical results, which may explain why consumers are less enthusiastic for antioxidant uses. This perspective article aims to highlights the critical role of Reactive Oxygen Species (ROS) and antioxidants, the potential mechanisms that might account for these discrepancies in clinical trials and some strategies to target oxidative stress and mitochondria by antioxidants. We need urgently to set up standard methods to evaluate antioxidants and oxidative stress in human and in particular at mitochondria level. The determination of what the basal level of ROS is in normal human may be used to identify pathologic ROS levels in patients and ultimately guide antioxidants treatment.

KEY WORDS antioxidant · mitochondria · oxidative stress · standardization methods

INTRODUCTION

Oxygen and nitrogen reactive species are considered as potential cell toxins. Even if it is now taken for certain that they play a crucial role in major pathologies, recent discoveries have broadened the perspective. In fact, these reactive species have physiological functions and are part of numerous cellular signalling pathways (1,2). Many reactive oxygen species (ROS) are second messengers that trigger complex cellular events (cytokine production, activation of AP-1 and NF-B pathways). Current research has to deal with free radicals as toxics, as much as signal transduction agents or as gene regulators. Now, it is established that physiological and pathological functions of oxygen and nitrogen reactive species are often complex and polyvalent (2).

The superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), thioredoxin reductase, glucose-6-phosphate dehydrogenase and glutathione (GSH) act as the endogenous antioxidants defense system. However, they require the effect of exogenous antioxidants such as vitamin C, vitamin D, carotenoids, polyphenols and trace elements such as selenium and zinc for eliminating the ROS (1–4).

When the generation of ROS exceeds the counterbalance of antioxidant cellular mechanism, it results in oxidative stress, finally leading to many pathological outcomes. The implementation of antioxidants through foods and medications can enhance the redox balance giving resistance to overcome the pathological effects of ROS (4,5). However, the doses of antioxidant matters, at high concentrations they exhibit prooxidant activities leading to disruption of cellular pathways.

This review aims to examine the double-edged effects of ROS and antioxidants, the potential mechanisms that might account for these discrepancies and ineffectiveness in clinical trials and provides some strategies to target oxidative stress and mitochondria by antioxidants.

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Sources of Cellular ROS

ROS are oxygen-containing molecules comprising superoxide anion, hydroxyl radicals, hydrogen peroxide, hypochlorous acid and singlet oxygen which act as acceptors or donors of free electrons. They are highly unstable and react with other molecules leading to generation of more reactive molecules. The complex chain of ROS begins with the reduction of molecular oxygen, leading to production of superoxide O_2^-. The superoxide molecule is unstable and undergoes reduction to hydrogen peroxide. The hydrogen peroxide is relatively stable and can capable of affecting important cellular molecules resulting in more free radicals (hydroxyl radicals) which accompanies the chain reaction. The chain reaction stops when the substrates get depleted and milieu becomes anaerobic or when chain gets broken up by the antioxidants (3). The sources of ROS are listed in Table I. We can observe three sources of ROS: environmental, physiological and mitochondrial**.** The metabolism of alcohol, therapeutic agents, radiations, inflammation and iron overload favors oxidative stress (4).

Environmental Factors

Certain agents stimulate the cellular ROS production, which can be either environmental or physical. The environmental factors such as UV radiations, microbes, allergens, increased ozone, cigarette smoke; polycyclic aromatic hydrocarbons, photochemic smog, industrial chemicals, metabolism of xenobiotics enhance the volume of ROS in the body (2). Exhaustive exercises, stressful conditions, sleep deprivation, emotional depression, and diets excess of fat and carbohydrate also stimulates the enormous production of toxic free radicals (1,2).

Mitochondria in Aerobic Metabolism Principle Source of ROS

One of the major ROS sources under physiologic conditions is the mitochondria. The electron transport chain for generation of adenosine tri-phosphate (ATP) in the inner membrane of mitochondria permanently generate superoxide ion, It is estimated that 1–2% or even 4% of oxygen consumption undergoes transformation to superoxide (5,6). The mitochondrial outer membrane contain the monoamine oxidase, which catalyses the oxidative deamination of organic amines, producing large amounts of hydrogen peroxide in the mitochondrial matrix and cytosol. The P-450 cytochrome oxidase, heme containing enzyme present in mitochondria catalyses the hydroxylation of Vitamin D, retinoic acid and metabolize xenobiotics. It follows a multi step transfer of two electrons to substrate where part of the oxygen is converted as superoxides (7).

Physiological Factors

The NADPH oxidase in the neutrophils constantly produce trace amounts of superoxide free radical associated with their regulatory function. Under stimulation the production of NADPH oxidase is lower playing an important role in pathological states of reperfusion, hypertension and atherosclerosis (8,9). The xanthine oxidase (XO) which uses molecular oxygen for metabolic process results in the accumulation of ROS when the antioxidant system is incomplete (10). Under

Table I Sources of ROS

Environmental factors	Physiological factors	Mitochondrial factors
UV radiations	Production of NADPH oxidase	Electron transport chain for generation of ATP
Microbes	Production of Xanthine oxidase	Oxidative deamination of organic amines
Allergens	Metabolism of arachidonic acid by cyclooxygenase, lipooxygenase and cytochrome P-450	
Increased ozone	Auto-oxidation of haemoglobin	
Cigarette smoke		
Polycyclic aromatic hydrocarbons		
Photochemic smog		
Industrial chemicals		
Metabolism of xenobiotics		

physiologic conditions xanthine oxidoreductase (XOR) is present as xanthine dehydrogenase (XD). XD is transformed to XO releasing hydrogen peroxide and superoxide ion. XOR converts nitrates to nitrites and NO which combines with superoxide forming highly reactive peroxynitrite.

The ROS are generated during metabolism of arachidonic acid by cyclooxygenase, lipooxygenase and cytochrome P-450. The electron transport system in the lysosomes promotes three electron reduction of oxygen resulting in hydroxyl ions. Myeloperoxidase in neutrophils and eosinophils catalyse the reaction of hydrogen peroxide with other substrates leading to release of hypochlorous acid (11).

The red blood cells hemoglobin serves as significant source for ROS generation. The hemoglobin gets autooxidised by electron transfer during bonding between heme and oxygen of oxygenated hemoglobin. This results in formation of methemoglobin and superoxide ion which turns to hydrogen peroxide and singlet oxygen (12).

Physiological *vs.* Pathological ROS: The Subtle Gap

ROS play a critical role in many physiological functions including cellular signaling, gene expression, regulation of immune response and antioxidative defense mechanism. ROS affect cellular redox status and intracellular signalling pathways which controls the expression of more than 40 genes (2,3,13,14).

The differences between physiological and pathological ROS are shown in Table II. The role of ROS in biological system is stringent and biphasic. ROS at high concentrations exhibit many pathological effects like lipid peroxidation, and oxidation of proteins and DNA. Cells enter an oxidative stress due to loss of equilibrium between the oxidant and antioxidant production further leading to cellular dysfunction, gene expression, protein expression, cell signaling, membrane fluidity, and potentiality resulting in cell death (15).

Oxidative Stress and Glycation: The Vicious Cycle

The reaction of glycation is observed during Maillard Reaction. This reaction leads to the formation of complex compounds, the Advanced Glycation End products (AGEs), which alter structure and functions of proteins. It has been shown that the formation of AGE *in vivo* contributes to several pathophysiologies associated with aging and diabetes mellitus, such as chronic renal insufficiency, alzheimers disease, nephropathy, neuropathy and cataract (16). Glycation and oxidative stress are closely linked, and both phenomena are referred to as glycoxidation. All steps of glycoxidation generate oxygen free radical production, some of them being common with lipidic peroxidation pathways. Besides, glycated proteins activate membrane receptors such as Receptor for Advanced Glycation End Products (RAGE) through AGEs, and induce an intracellular oxidative stress and a pro-inflammatory status (17,18). Glycated proteins may modulate functions of cells involved in oxidative metabolism and induce inappropriate responses. Finally, some oxidative products (reactive aldehydes such as methylglyoxal) or lipid peroxidation products (malondialdehyde) may bind to proteins and amplify glycoxidation generated lesions. The knowledge of glycoxidation mechanisms may lead to new therapeutic approaches of oxidative stress and its implication in many chronic diseases (16–18).

AGEs Mediated by ROS Formation

AGEs bind to the cell surface receptor (RAGE). This binding triggers cellular events through p38 MAP Kinase, NF-B, P21 Ras and Jak/STAT pathways. NF-kB is a transcription factor that regulates many cellular mechanisms. It is predominant in the inflammatory phenomena (inflammatory cytokines secretions). It regulates positively or more often, negatively, the apoptosis dependant on the cell type. AGEs formation keeps oxidative stress going. During an oxidative stress and reaction of glycation, NF-B will be activated. This activation will hyperactivate TNF production. TNF will produce more and more ROS. We

Table II Difference Between Physiological and Pathological Role of ROS

Physiological role of ROS	Pathological role of ROS
Moderate ROS levels constitute normal physiological pathways in the regulation of cellular functions, including signaling cascades and transcriptional/post-transcriptional control of gene expression.	Severe oxidative stress induces cellular damage that can lead to apoptosis or necrosis.
Direct modification of factors or indirect mechanisms through change in the oxidative and reductive status inside/outside cells.	Damage to cell structures, including lipids and membranes, proteins, and DNA.
Play essential role in signal transduction, gene expression, apoptosis and aging.	At high doses lead to pathogenesis of cancer, cardiovascular disease, atherosclerosis, hypertension, ischemia/reperfusion injury, diabetes mellitus, neurodegenerative diseases, rheumatoid arthritis, and ageing.

will observe a vicious cycle between ROS, AGE and NF-B (16–18).

Defense Against ROS: Antioxidant Defense Systems

The major defense systems against the ROS are the antioxidants. Antioxidants are substances that at lower concentrations prevent the oxidative damage of biological molecules and cells caused by ROS (2,19). ROS at higher concentration are deleterious causing various pathological effects however at lower concentration; they are beneficial for effective physiological functions. Schematic representation of antioxidant defense system in humans is tabulated in Table III. The primary antioxidant system is the endogenous system which prevents the ROS formation by inhibiting the ROS precursors and the catalysts like SOD, CAT and GPx. Exogenous antioxidant system plays a secondary role by reacting with the ROS. The endogenous antioxidants work actively in combination with the exogenous antioxidants to prevent the damage caused by ROS (4,15,19). The antioxidants can exert their activity only when they attain soluble form and are taken up by the epithelium. The activity also depends on the bioavailability factors like absorption by the gut, transport to the site of action, formation of phase I and phase II metabolites and excretion. The primary antioxidative action of natural compounds is when antioxidants directly act on free radicals ($R^{\cdot}$) characterized by the donation of hydrogen atoms or electrons. The secondary action of the antioxidants is by absorption of UV radiation or by intervention in anti-oxidation processes, acting as deactivators of singlet oxygen ($^1O_2$) or by conversion of hydroperoxides (ROOH) to non-radical species (4,19).

Biopeptides as Exogenous Antioxidants

The peptides from protein molecules act as effective antioxidants. Antioxidant peptide contains 5-16 amino-acids; they are safe and healthy, less expensive, low molecular weight, simple structure, more stable, and readily absorbed. The antioxidant property depends on the composition, structure and hydrophobicity of the peptides. Tyr, Trp, Met, Lys, Cys, and His amino acids cause antioxidant activity as these amino acids with aromatic residues donate protons to electron deficient radicals, thereby scavenging the free radicals (20). The order of aminoacids in a peptide, structural features of the peptide, peptide bond and its conformation, configuration of the peptides and correct positioning of imidazole group also serves key role in antioxidant activity. The exact mechanism of the biopeptides antioxidant activity remains to be understood, however, studies have demonstrated their role as inhibitors of lipid peroxidation, scavengers of free radicals and chelators of transition metal ions. Moreover peptides keep cell safe from negative effects of ROS by induction of protective genes? The operational conditions applied to isolate proteins, degree of hydrolysis, type of protease, peptide structure and peptide concentrations are the other factors influencing antioxidant properties (20).

Mitochondrial Antioxidant Defense

Many antioxidants play a central role in the mitochondria antioxidant defense system, The mitochondria control the effects of ROS through small antioxidant molecules, GSH, vitamin C and E. The antioxidant enzyme Mn-SOD converts superoxide ion to hydrogen peroxide and this free

Table III Antioxidant Defense System

In human, the antioxidant defense system includes endogenous and exogenous antioxidants. Dietary intake serves as the source of exogenous antioxidants.

Non-enzymatic Antioxidants	Endogenous Antioxidants	Exogenous Antioxidants
Glutathione	Enzymes as antioxidants:	Vitamins: Vitamin C, Vitamin E
Coenzyme Q	Superoxide dismutase (SOD) detoxifies superoxide radicals	Polyphenols: quercetin, catechins, epicatechins, gallic acid, epigallocatechins-3 gallate, butylated hydroxyanisole, butylated hydroxytoluene, tert-butyl hydroquinone, propyl, octyl and dodecyl gallates
	Catalase (CAT) detoxifies peroxides	
NADPH	Glutathione peroxidise (GPx) detoxifies peroxides	Carotenoids: carotene
		Exogenous biopeptides
	Glutathione reductase regenerates glutathione	Plants: *Rosmarinus officinalis*
	Hemeperoxides detoxifies hydrogen peroxide	

radical is detoxified by mitochondrial GSH peroxidase and peroxiredoxin III. In the phospholipid bilayer of mitochondria the fat soluble antioxidants vitamin E and Coenzyme Q prevents the lipid peroxidation. Vitamin C is one of the molecules that form mitochondrial antioxidative defenses. It enters mitochondria through facilitate glucose transporter 1 (Glut 1) in its oxidized form (21).

EVALUATION OF OXIDATIVE STRESS AND ANTIOXIDANTS METHODS: LACK OF STANDARDIZATION

The Concept to Evaluate Antioxidants Activities: Multiplicity of Methods

A great multiplicity of protocols has been used to evaluate the activity of antioxidants by using a wide variety of free radical generating systems, different methods of inducing oxidation, and measuring end points of oxidation (22–24). Unfortunately, variable and confusing results have been reported depending on the protocols, methods, and conditions used to test the antioxidant activity. We already discussed many controversies observed in many clinical trials with antioxidants (14,33), more than 53 methods to assess the antioxidant activity for both food and biologically relevant substrates, using a wide variety of initiators, substrates, and end point measurements (22–24). More than four inhibition assays, measuring inhibition at a fixed time point, reaction rate, or lag phase.

For antioxidant *in vivo* protocols, many different methods have been used to test the protective activity of these compounds. Unfortunately, many of these protocols have been based on questionable methodology to accurately measure oxidative damage and to assess relevant changes in biological targets. To determine the real effects of polyphenolic antioxidants, it is important to obtain specific information on the type of oxidation products that are inhibited and their biological source(s). Several specific assays are needed to elucidate products causing oxidative damage in biological tissues. Until comparative results of the various methods can be related to significant influence in biological systems, the results must be questioned (22–24).

The antioxidant potency of polyphenols evaluated by a number of *in vitro* and *in vivo* tests is commonly based on their activity using artificial azo initiators of free radical oxidation, ABTS and AAPH; radicals produced by oxidation of linoleic acid; or reducing ferric ions in plasma (24). Antioxidant methods that commonly use these artificial radical model systems provide no information on what biological targets are protected. These initiating processes are also not relevant to either food or biological systems, and the damage caused cannot be used to estimate the *in vitro* and *in vivo* activity of natural antioxidants.

One example is the evaluation of antioxidants activities and concentration after supplementation with exogenous antioxidants. These analyses can be confounded by ROS and oxidative stress that causes an up-regulation of antioxidant enzymes such as Mn-SOD. Protocols for biological antioxidant using *in vivo* systems have been especially controversial because the complex mechanisms of their protection are not well-understood (22,24).

Techniques to Evaluate ROS and Oxidative Stress: Physiological *vs.* Pathological ROS

Many analytical methods directly measure the accumulation and generation of free radicals and ROS. Because of a wide divergence of results, more rigorous guidelines and assay protocols and standardization are urgently required to bring order and credibility (14,22).

Our understanding of the effects of antioxidant supplementation can only be improved if more specific methodology is used to clearly identified the gap between physiological *vs.* pathological ROS. It had become inevitable to differentiate ROS physiologic from ROS pathologic, which necessitate and motivate the development of better evaluation methods (7,19,22,25).

We cannot set up strategies to use antioxidants in human, if we can not differentiate the physiologic level of ROS from the pathologic one, which necessitate and motivate the development of better evaluation methods.

The Perfect Example: ROS and Male Infertility

Oxidative stress is a common pathology seen in approximately half of all infertile men. ROS are generated by sperm and seminal leukocytes within semen and produce infertility by two key mechanisms. First, they damage the sperm membrane, decreasing sperm motility and its ability to fuse with the oocyte. Second, ROS can alter the sperm DNA, resulting in the passage of defective paternal DNA on to the conceptus (26,27). Despite the different methods available to measure ROS, currently no standard exists for estimating oxidative stress. Allamaneni and colleagues defined the basal levels of ROS in normal donors in whole, unprocessed semen specimens and in mature and immature spermatozoa. They demonstrated that the determination of what the basal level of ROS is in human semen may be used to identify pathologic ROS levels in infertile men and ultimately guide treatment (27).

STRATEGY TO REINFORCE THE ANTIOXIDANT DEFENSE SYSTEM

Targeting Mitochondria by Antioxidants

Mitochondria play a vital role in cell signaling resisting cell against oxidative stress and development of pathological conditions such as cardiovascular, neurodegenerative, diabetes and atherosclerosis (28). The available antioxidants are not effective as they do not target and reach specific site of ROS generation, especially when the source is the mitochondria itself. Mitochondria act as a major source of ROS observed in ischemia, reperfusion injury etc., by detoxifying the oxygen species in its compartment, mitochondria limit ROS excessive production which may damage biological membrane (29). One practical example is diabetes. Hyperglycaemia is responsible for a rise in the mitochondrial production of ROS. Moreover, mitochondria of cells are involved in glucose-triggered insulin secretion. Hyperglycaemia causes formation of AGEs, activation of the polyol pathway, increased hexosamine pathway flow and activation of some isoforms of protein kinase C. All these events have been related to superoxide genesis by mitochondria dysregulation (28–30). Again, in the context of hyperglycaemia, mitochondria are quickly fragmented in a fission process. The inhibition of this change in mitochondrial morphology prevented the ROS overproduction following exposure to high glucose concentrations (31). Thus, the targeting of mitochondrial fission/fusion mechanism may be of interest to prevent ROS associated disorders in hyperglycaemic states. Targeting antioxidants to mitochondria is thus an interesting therapeutic perspective, as well as the development of safe uncoupling agents (32).

Therapeutic Limits of Untargeted Antioxidants

Over and above, in human, many clinical trials involving antioxidants have brought disappointing results (14,33). Their targeting to oxidative stress sources has been put into question. Targeting antioxidants to mitochondria may be of particular interest, since the effects of untargeted antioxidants have sometimes been described as paradoxical. Opposite effects of green tea and antioxidant vitamins on cellular and extracellular oxidative events have been reported in diabetic rats (34). Antioxidants may affect the balance from oxidative to carbonyl stress in the extracellular environment. Nevertheless, green tea has been described as protective against the alteration of mitochondrial proteins caused by carbonyl stress. A protective property of green tea on renal mitochondrial respiration was demonstrated in diabetic rats (34).

A Shift in the Perception of the Role of Antioxidants

Antioxidants have largely been considered as chain-breaking molecules, able to interrupt free radicals reactions involving initiation, propagation and termination. A classical chain breaking antioxidant is α-tocopherol. Secondly, antioxidant properties have been associated with enzymatic activities, for example those of Mn-SOD, catalase, glutathione peroxidase. As mitochondria have been recognized as the most significant source of ROS, some antioxidants have been shaped to specifically reach these organelles. Mitochondria targeted antioxidants have recently been reviewed. Three strategies have been set out (35). The first is the targeting of the negative potential of the matrix by resorting to cationic carriers. The second is the targeting of specific mitochondrial enzymes that free the active form of a drug from its prodrug form. The third consists in targeting mitochondrial specific transporters. MitoQ, mitoVit-E, and mitoperoxidase are molecules known as antioxidant coupled with a triphenylphosphonium group (35).

Another powerful antioxidant used is glutathione. Choline esters of glutathione have been devised to be accumulated in mitochondria, because of its pronounced negative potential. In this case, the repletion of mitochondrial glutathione stocks is sought (33,35).

ANTIOXIDANT-BASED CLINICAL STUDIES: EXPLANATION FOR LOW HEALTH BENEFITS

A balance between the oxidants and the antioxidants has been found crucial in maintaining healthy biological systems. The antioxidants at higher doses act as prooxidants and also scavenge the ROS present at physiological concentrations required for normal cellular functions, disrupting the normal cellular activities (19). Antioxidants such as vitamin C, vitamin E and carotenoids in our diet act as scavengers of free radicals at micromolar and millimolar concentrations, while the polyphenols works well at nanomolar concentrations (36). It has been demonstrated that antioxidative natural compounds exhibit a double edged effects on inflammatory reactions. A list of diet or supplement based clinical studies in humans and their observations were listed in Table IV.

It has been evidenced that high doses of the polyphenol, quercetin (>50μM) initiates the production of superoxide radical, decrease the cell survival and viability, thiol content, total antioxidant capacity and hinder the activities of SOD, CAT and glutathione S-transferase (37). At low concentrations (10–25 μM), flavonoids have been found to fight against hydrogen peroxide induced cytotoxicity, DNA strand breakage and cell death (38). At concentrations

Table IV Human Clinical Studies with Dietary Intervention

Dietary invention trials in humans	Author	Findings
Fruits and vegetables	Reviewed by Halliwell 2002 (48)	Decreased levels of oxidative DNA damage in healthy human volunteers
Plant-based foods such as olive oil, cereals, legumes, nuts and vegetables	Reviewed by Lairon 2007 (49)	Reduced several CVD risk factors in subjects at risk
Carotene supplementation	Reviewed by Goralczyk 2009 (50)	No negative effects
Vitamin A and-carotene	Beta-Carotene and Retinol Efficacy Trial Yeh 2009 (51)	Negative impact in patients with lung cancer and CVD
Vitamin E and-carotene	Lepp 2000 (52)	Failed to show beneficial effects on stroke incidence
Vitamin E and Vitamin C	Prieme 1997 (45)	Failed to reduce oxidative damage in smokers
Vitamin C	Podmore 1998 (44)	increased oxidative lymphocyte DNA damage in healthy individual on regular administration for 6 weeks

above 50 μM flavonoids cause cytotoxicity, DNA damage and apoptosis. Flavonoids at high concentrations generate ROS by autoxidation and redox-cycling (39). The epigallocatechin-3-gallate (EGCG), dietary antioxidant in green tea shows resistance against oxidative stress at low doses whereas at higher doses exhibit cytotoxicity (40).

The presence of metal ion plays a major role in diminishing the antioxidant property of the polyphenols. The EGCG in presence of transition metal ions causes oxidative damage to DNA (41). The reducing power of antioxidants was also affected by presence of metal ions, iron and copper inducing the ability to form reactive hydroxyl radicals. Due to this property of antioxidants the condition is worse in organisms with iron overload (42).

The effect of pH influences the activity of phenolics. At pH 7.4 phenolics exhibited prooxidant activities and at pH 5.8 it displayed antioxidant activity (43).

Synergestic action of antioxidants plays a vital role to exhibit their effective antioxidant capacity. The supplementation of isolated forms of vitamin C, vitamin E and carotene in high doses increased the oxidative damage of lymphocytic DNA (44), failed to reduce DNA damage in smokers (45). Individual components do not have beneficial effects, the natural antioxidants such as fruits and vegetables act as better antioxidants as whole. The oxidative stress depletes the vitamin C and E when given as individual supplements further increasing the production of free radicals. In order to overcome this; combination of vitamin C and vitamin E can be given in synergism to resist the cells from oxidative stress (46).

STRATEGIES TO IMPROVE CLINICAL STUDY ON ANTIOXIDANTS

Antioxidants prevent and repair oxidative stress, a process that damages cells in the body and has been linked to the development of cancer, heart disease, Alzheimers disease, and Parkinsons disease. A balance between the oxidants and the antioxidants is required for maintaining normal physiological functions. The oxidants are vital for regular functioning of metabolic pathways and cellular functions.

Under normal conditions, the endogenous antioxidants suppress the effects caused by free radicals; however in certain conditions require the exogenous antioxidants to make their antioxidant activity effective against oxidative stress. This brings the need for natural or synthetic antioxidants supplemented in diet or medications to combat the oxidative stress.

Synthetic antioxidants are cost effective but they are hazardous than the natural oxidants present in our foods such as fruits and vegetables. The dose and mixture of antioxidants that are given may also be critical. In the initial observational dietary studies that demonstrated strong apparent benefit from antioxidant vitamins, vitamins were obtained from fruits and vegetables. Naturally occurring antioxidant vitamins differ in their formulation (*i.e.*, synthetic vitamin E contains a mixture of stereoisomers while natural vitamin E contains only one stereoisomer) and in the relative concentrations of related molecules. The difference between the many different forms of vitamin E which occur in natural food substances and those that were used in failed clinical trials is striking (47).

When antioxidants are given in pharmacological doses, much higher than doses that can be attained by dietary intake, antioxidants may attenuate both deleterious and beneficial oxidative processes. This may be the reason for clinical trials that use pharmacological doses of antioxidants not expressing a beneficial effect when given arbitrarily to all individuals independent of their levels of oxidative stress. Moreover, the *in vitro* and animal model studies do not represent the biological process in human biological system (19,25,47).

In pharmaceutical research of antioxidants major factors to be accounted are dose of antioxidant administered, duration of the trail undertaken and synergism of the antioxidants. The knowledge regarding these effects has to lead to different strategies for development of antioxidative drugs that prevent cellular damage by ROS (25,33,47).

Mitochondrial targeting antioxidants would be potential therapy for diseases caused due to mitochondrial damage. Target specific antioxidant development would result in effective positive outcome to combat the damage due to ROS in biological system. The broader knowledge will enable to define the role of novel ROS-sources in different disease processes, which can arise from either under- or overproduction of ROS.

CONCLUSION

The dynamic balance between oxidation and antioxidation (redox balance) is significant for the homeostasis of biological system influencing not only ROS but antioxidant as well (2,19,25,47). Physiologic doses of exogenous antioxidants are essential for the maintenance of cellular homeostasis however; higher doses may disrupt the balance. Analysis of clinical studies involving antioxidants suggest that the health benefits of exogenous antioxidant supplements were observed primarily when taken in the natural form rather than high dose of isolated compounds as present in supplements. An approach that would be of interest for future clinical studies is inclusion of a compound that targets mitochondria besides mimicking the natural food matrix and available at near physiological dose (19,25,47).

The large amount of effort expended in testing antioxidants emphasizes the need for improved and more consistent methods and their standardization. Because of a wide divergence of results, more valid and rigorous guidelines and assay protocols are required to bring some order and agreement to this important field. Our understanding of the effects of antioxidant can only be improved if more specific methodology is used and is capable of defining what products are formed and inhibited by antioxidants depending on conditions, systems, and targets of protection (22,24).

Strategy to reinforce the antioxidant defense system and target the mitochondria need more studies, in particular, we need to

- decrease the production of ROS from the mitochondrial electron transport chain that occurs in response to high glucose and fatty acid levels;
- decrease ROS production without significantly affecting ATP production (development of mitochondrial uncoupling agents);
- differentiate the physiologic ROS level from pathologic ROS, which necessitate and motivate the development of better evaluation methods;
- increase the bioavailability of antioxidants, studying their passage through the barriers must be taken in consideration;
- standardize protocols and methods of antioxidants and ROS evaluation.

In terms of best designed antioxidant for tomorrow, we need to develop antioxidants that catalytically remove excessive oxygen radicals and ROS, rather than the current compounds in which each antioxidant molecule defeats one ROS. That is, what we need is something that will go in (to target the oxidative stress and mitochondria) and clean out all of the damaging radicals and excessive ROS. However, to set up good strategies to use antioxidants in human, we need to differentiate the physiologic level of ROS from the pathologic one, which necessitate urgently the development of better evaluation methods (53).

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