

Review Article

The Neglected Significance of “Antioxidative Stress”

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Oxidative stress arises when there is a marked imbalance between the production and removal of reactive oxygen species (ROS) in favor of the prooxidant balance, leading to potential oxidative damage. ROSs were considered traditionally to be only a toxic byproduct of aerobic metabolism. However, recently, it has become apparent that ROS might control many different physiological processes such as induction of stress response, pathogen defense, and systemic signaling. Thus, the imbalance of the increased antioxidant potential, the so-called antioxidative stress, should be as dangerous as well. Here, we synthesize increasing evidence on “antioxidative stress-induced” beneficial versus harmful roles on health, disease, and aging processes. Oxidative stress is not necessarily an un-wanted situation, since its consequences may be beneficial for many physiological reactions in cells. On the other hand, there are potentially harmful effects of “antioxidative stress,” especially in the cases of overconsumption of synthetic antioxidants. Antioxidants can neutralize ROS and decrease oxidative stress; however, this is not always beneficial in regard to disease formation or progression (of, e.g., cancer) or for delaying aging.

1. Introduction

The process of aging or senescence is complex; it may derive from a variety of different mechanisms and is caused by a variety of different factors. In recent years, oxidative stress has been implicated in a wide variety of degenerative processes, diseases, and syndromes, including the mutagenesis, cell transformation, and cancer; atherosclerosis/arteriosclerosis, heart attacks, strokes, and ischemia/reperfusion injury; chronic inflammatory diseases, such as rheumatoid arthritis, lupus erythematosus, and psoriatic arthritis; acute inflammatory problems, such as wound healing; photooxidative stresses to the eye, such as cataract; central nervous system disorders, such as certain forms of familial amyotrophic lateral sclerosis, certain glutathione peroxidase-linked adolescent seizures, Parkinson’s disease and Alzheimer’s dementia; a wide variety of age-related disorders, perhaps even including factors underlying the aging process itself [1]. There are many theories trying to explain the aging process, each from a different angle. The most recent studies support the idea that oxidative stress is a significant marker of senescence;

this was established in different species [2]. Harman first proposed the free radical theory of aging in the 1950s and extended this idea to implicate mitochondrial production of reactive oxygen species in the 1970s [3]. According to the free radical theory of aging [4–7], enhanced and unopposed metabolism-driven oxidative stress plays a major role in diverse chronic age-related disorders. The free-radical theory of aging states that organisms age because their cells accumulate free radical damage over time. Halliwell and Gutteridge later suggested to rename this free radical theory of aging as the “oxidative damage theory of aging” [8], since aging and diseases are caused not only by free radicals but also by other reactive oxygen and nitrogen species. Theory links oxygen consumption, metabolism, ATP, and ROS formation and holds that increases in ROS accompany aging and lead to functional alterations, pathological conditions, and even death [9]. Furthermore, impairment of mitochondrial activity is assumed to be one of the main causes of the aging process [10–12]. Mitochondria are the main site of intracellular oxygen consumption and the main source of ROS formation [10, 13, 14]. Mitochondrial ROSs originate

from the electron transport chain and the nitric oxide synthase reactions. Nonmitochondrial sources of ROS include environmental pollutants, pollutants in food, radiation, or they are the by-products of other metabolic processes within organisms. Majority of free radicals are generated inside the cell rather than coming from the environment [10, 15, 16]. The mitochondrial damage theory has been recently reviewed by Wilken [17]. Age-related functional deficits have been observed in some, but not all, studies of aging mitochondria, adding support to the mitochondrial damage theory. The age-related increases in the levels of both oxidative damage and mutational load of mtDNA predicted by the mitochondrial theory of aging have been confirmed in multiple species and organ systems [18]. However, whether this damage affects mitochondrial function or significantly modulates, the physiology of aging has remained controversial [19, 20]. On the other hand, the “vicious cycle” theory, which states that free radical damage to mitochondrial DNA leads to mitochondria that produce more superoxide, has been questioned by some scientists since the most damaged mitochondria are degraded by autophagy (mitophagy), whereas some defective mitochondria (which produce less ATP as well as less superoxide) remain to reproduce themselves [21]. Several lines of direct and indirect evidence generated over the past two decades have demonstrated a positive relationship between the increased oxidative stress *in vivo* and biological aging. In reality, the oxidative damage potential is greater than antioxidant defense, and thus there is a constant free radicals formation in low amounts, which escapes the cell defenses. Estimates of how much oxygen is turned into free radicals vary; however, typically cited values are around 1.5–5% of the total consumed oxygen [7, 22]. These estimates have been questioned by Hansford et al. [23] and Staniek and Nohl [24], who suggested that H₂O₂ production rates were lower than 1% of consumed O₂. Yet, even if we accept a conservative value of 0.15%, it still represents a substantial amount of free radicals formation [25]. Thus, high levels of reactive oxygen species (ROS) compared to antioxidant defenses are considered to play a major role in diverse chronic age-related diseases and aging. Reduction of oxidative stress was reported to be associated with prolongation of life expectancy, and ROS-lowering interventions were proposed as antiaging strategies [26–40].

Since a biological antioxidant has been defined as any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate [8], the questions arise whether reduction of oxidative stress in cell environment with antioxidant treatment would be beneficial; how it would influence the health outcome and what adverse effects could this trigger. At this point, it should be stressed that the antioxidants cannot distinguish among the radicals that play a beneficial physiological role and those that cause oxidative damage to biomolecules.

Oxidative stress was first defined by Sies as “a disturbance in the pro-oxidant/antioxidant balance in favor of the former, leading to potential damage.” The term “antioxidative stress” was used for the first time by Dundar and Aslan [41] for the negative effects of antioxidants. Here, we present and discuss

the evidence on how “antioxidative” and oxidative stresses or antioxidative imbalance can be damaging for the organism on the examples of cancerogenesis and aging process.

2. Antioxidants and Human Trials

The results of many clinical trials in which individuals received one or more synthetic antioxidants failed to obtain beneficial results. Recent evidence suggests that antioxidant supplements (although highly recommended by the pharmaceutical industry and taken by many individuals) do not offer sufficient protection against oxidative stress, oxidative damage or increase the lifespan. Some recent studies showed that antioxidant therapy has no effect and can even increase mortality [42–53]. Schulz and coworkers showed that nutritive antioxidants completely abolish the extension of lifespan by inhibiting an adoptive reaction to ROS called mitohormesis [54]. It was demonstrated by *in vivo* and *in vitro* studies that vitamin C, vitamin E, SOD, glutathione and beta carotene have a potential to cause also “antioxidative stress” in addition to prooxidative stress under certain conditions [55–59]. There are evidently homeostatic mechanisms in cells that govern the amount of allowable antioxidant activity. This indicates that other substances in fruits and vegetables, or a complex mix of substances (e.g., inhibitors of P450 from grapefruit, garlic; inhibitors of cell proliferation, e.g., resveratrol, green tea polyphenols, curcumin; antagonists of estrogen, e.g., some flavonoids; inhibitors of metastases, like some flavonoids; inhibitors of angiogenesis, like genistein, epigallocatechin gallate), contribute to better cardiovascular health and decreased cancer incidence observed in individuals who consume more of fruit and vegetable [60, 61].

Dosing the cells with exogenous antioxidants might decrease the rate of synthesis or uptake of endogenous antioxidants, so that the total “cell antioxidant potential” remains unaltered. Cutler described “the oxidative stress compensation model” which explains why dietary supplements of antioxidants have minimum effect on longevity [62, 63]. Most humans are able to maintain their set point of oxidative stress and so no matter how much additional antioxidant supplement they consumed in their diet further decrease in oxidative stress does not occur. Antioxidant supplements thus do not appear to significantly decrease oxidative stress or/and increase life expectancy in humans [64]. Besides, to exert beneficial physiological effects, antioxidants should be well absorbed in the body and reach the site of ROS formation in cells, where they should be present and in appropriate amounts for a sufficient time.

3. ROS as Signaling Molecules, Regulators of Transcription Factor Activities, and Other Determinants of Gene Expression

Human cells also generate some hydrogen peroxide and other ROS molecules deliberately to use them as chemical signals to regulate everything from glucose metabolism to cellular growth and proliferation [65]. The most commonly synthesized ROSs are superoxide radical and NO, which

are produced by NADPH oxidases and NO synthases, respectively, in different places of the organism [66]. These enzymes are highly active in most of the reproductive tissues, indicating that some ROSs are required for reproduction.

ROSs induce various biological processes that include a transient elevation of intracellular Ca^{2+} concentration, phosphorylation of specific proteins, activation of specific transcription factors, modulation of eicosanoid metabolism, and stimulation of cell growth [67]. Nitric oxide was identified as a signaling molecule as early as 1987 [68] and is now a well-known regulator of some transcription factor activities and other determinants of gene expression. Hydrogen peroxide and superoxide have similar intracellular effects [69]. ROS can affect directly conformation and/or activities of all sulfhydryl-containing molecules, such as proteins or glutathione (GSH), by oxidation of their thiol moiety. Among many other enzymes and membrane receptors, this type of redox regulation affects many proteins important in signal transduction and carcinogenesis, such as protein kinase C, Ca^{2+} -ATPase, collagenase, and tyrosine kinase [70]. For several transcription factors, ROSs are physiological mediators of transcription control [71]. The well-known examples of redox-sensitive transcription factors are nuclear factor- κB (NF- κB) and activator Protein-1 (AP-1). Thus, increased oxidative stress is not beneficial for the cell; however, an increase in cellular antioxidants might impact redox regulation and signal transduction. The complete elimination of free radicals would thus disrupt, rather than extend, the normal functioning of the body.

In addition to ROS, regulators of transcription factor activities and other determinants of gene expression also act as signaling molecules. Even the products oxidized by ROS (e.g., lipids, proteins, sugars, and nucleic acids) may act in the similar way. For example, lipid peroxidation products may modulate signal transduction pathways and mediate biological processes through receptors or receptor-independent pathways. At the same time, it has been shown that the lipid peroxidation products induce adaptive response and increase tolerance against forthcoming oxidative stress by upregulating defense capacity [72].

4. “Antioxidative Stress” and Cancer

Increasing cellular viability by antioxidants prior or after the toxic compound-induced toxicity (e.g., Cr(VI), UV-radiation, ionizing radiation) might not always be beneficial. Carcinogen-induced growth arrest and apoptosis are at the molecular decision point between carcinogen toxicity and carcinogen carcinogenesis [73]. When normally growing cells are in contact with carcinogens, they may respond by undergoing growth arrest, apoptosis, or necrosis. A population of genetically modified cells may also emerge, which exhibits either intrinsic or induced resistance to apoptosis [73]. Control of intracellular concentration of ROS is critical for the survival of cancer cells. It was demonstrated recently in human lung cancer cells that acute increases in intracellular concentrations of ROS caused the inhibition of glycolysis through glycolytic enzyme pyruvate kinase M2

thus diverted the glucose flux into the pentose phosphate pathway [74]. This generated sufficient reducing potential (antioxidant response) for detoxification of ROS. This promoted the metabolic changes required for proliferation and enabled the cancer cells to withstand oxidative stress. Therefore, precancer cells may lead to neoplasia as a result of their altered growth/death ratio, due to disrupted cell cycle control, genomic instability, or altered metabolism. This, however, raises the question whether decreasing carcinogen toxicity by antioxidants might actually increase the incidence of cancer by allowing the inappropriate survival of altered cells. This hypothesis was confirmed by human intervention studies in which smoking male volunteers were exposed for 5–8 years to daily supplements of vitamin E and beta carotene. The overall mortality of male smokers increased in those taking supplements of beta carotene and was most probably due to its effect on cell proliferation [8, 75]. Supplementation by alpha-tocopherol or beta-carotene did not prevent lung cancer in older men who smoke [75]. Beta-carotene supplementation at pharmacologic levels may modestly increase lung cancer incidence in cigarette smokers, and this effect may be associated with heavier smoking and higher alcohol intake. Randomized human trials have demonstrated the antineoplastic and neoplastic effects of antioxidants, with neoplastic effects in patients from higher risk groups due to smoking and alcohol consumption, or patients undergoing chemo- or radiation therapy. The influence of “antioxidative” stress and cancer was confirmed also by the study of Schafer and coworkers [76], who revealed an unanticipated mechanism for survival of epithelial cells detached from the extracellular matrix. Normally such cells lose the ability to take up glucose, which results in ATP deficiency. Such cells do not survive normally in these conditions but can be rescued through two different pathways. The expression of cancer-promoting oncogene ErbB2 in detached endothelial cells restores the cell’s ability to take up glucose and reduces the levels of ROS through the antioxidant-generating pentose phosphate pathway. Alternatively, the ATP deficiency could be rescued by antioxidant treatment, which stimulates fatty acid oxidation otherwise inhibited by detachment-induced ROS. The latter cell rescue occurs without the rescue of glucose uptake. Therefore, the addition of antioxidants promotes the survival of preinitiated tumor cells even in unnatural matrix environments by altering metabolic regulation, which results in enhanced malignancy [76].

Antioxidants may thus have dichotomous activities with respect to tumorigenesis, namely, suppressing tumorigenesis by preventing oxidative damage to DNA [77, 78] and promoting tumorigenesis by allowing survival of cells that are metabolically impaired (e.g., in altered matrix environments).

Also, iron metabolism, iron balance, and iron stores in relations with antioxidants (Fenton’s chemistry) are important in ROS-induced damage (see Section 4.1). Given that the cells require iron, restricting its supply could also limit the growth of cells, including tumor cells [79–90]. Conversely, the iron carrier neutrophil gelatinase-associated lipocalin (NGAL, Siderocalin) is overexpressed in tumours [91–93].

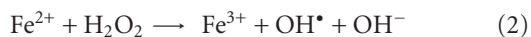
The role of iron-promoting oncogenesis is documented well [94–110].

4.1. Is Iron Overload the Reason Why Human Trials with Synthetic Antioxidative Supplements Failed to Obtain Beneficial Results? Potential strategies to decrease oxidative stress may involve not only overall reduction of oxidative stress but also the use of iron and other metal chelators hampering Fenton-type chemistry [111]. Harmful reactions, in which partially reduced oxygen species such as superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) may be formed, are, in part, mediated by redox-active metals, the most physiologically relevant of which are iron and copper. These transition metal ions can play an important role in ROS production in the cell. Reduced forms of redox active metal ions participate in the Fenton reaction where the hydroxyl radical (HO^\bullet) is generated from hydrogen peroxide.

Fenton Reaction



For example,

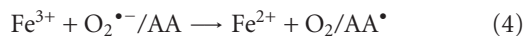


Furthermore, the Haber-Weiss reaction, which involves the oxidized forms of redox active metal ions and superoxide anion, generates the reduced form of metal ion, which can be coupled to Fenton chemistry to generate hydroxyl radical (see also below). Superoxide and H_2O_2 themselves are not excessively reactive; thus, they are not especially damaging at physiological concentrations. However, their reactions with poorly liganded iron species can lead to the production of hydroxyl radical, which is extremely damaging, and a major cause of chronic inflammation [112].

Haber-Weiss Reaction



For example,



AA ascorbate

AA^\bullet ascorbate $^\bullet$.

In the net reaction molecule of hydrogen peroxide is converted into hydroxyl radical and water in the presence of iron as a catalyst. Because the iron concentration in biological systems is often very low, the very important factor for Fenton chemistry activity in biological systems is the presence of functional metal redox-cycling mechanism [113]. Superoxide anion radical ($O_2^{\bullet-}$) or certain antioxidants (e.g., ascorbate) play the role of such reducing agent in biological systems. Antioxidants reduce Fe(III) to Fe(II) which reenters the Fenton reaction. Thus, although ascorbate (or some other antioxidant) is “reducing” and an “antioxidant” agent,

its reaction with O_2 , especially when catalyzed by Fe(II), produces superoxide and thence HO^\bullet radicals that have prooxidant properties. It is clear that any increase in the levels of superoxide anion, hydrogen peroxide or the redox active metal ions are likely to lead to the formation of high levels of hydroxyl radical by the chemical mechanisms listed above.

Some compounds contribute to antioxidant defense by chelating transition metals and preventing them from catalyzing the production of free radicals in the cell. Metal-chelating antioxidants such as transferrin, albumin, and ceruloplasmin avoid radical production by inhibiting the Fenton reaction catalyzed by copper and iron. Particularly important is the ability to sequester iron, which is the function of iron-binding proteins such as transferrin and ferritin [114]. The cellular pools of low-molecular-weight iron are not characterized well [115, 116]. If these are in contact with ascorbate (or other antioxidants), prooxidant effects may occur. As already mentioned, ascorbic acid reduces Fe(III) to Fe(II) which reduces oxygen to hydroxyl radical [117]. According to Herbert [118], every advertisement and label for iron supplements and/or vitamin C should warn the consumers: “Do not take this product until your blood iron status has been determined.” Six percent of Americans have the negative iron balance, and such multivitamin/mineral product may help them. Twelve percent of Americans have positive iron balance, and such product may hurt them. If the inappropriate iron balance is a confounding factor in trials with antioxidants, then the ranges of negative effects from these studies should overlap with those of iron balance. Indeed, there was approximately 5–15% rise when different antioxidants were assessed for increased mortality. For example, when the different antioxidants were assessed separately in the study of Bjelakovic et al. [49], there was a significantly increased mortality by vitamin A (RR 1.16, 95% CI 1.10 to 1.24), beta-carotene (RR 1.07, 95% CI 1.02 to 1.11), and vitamin E (RR 1.04, 95% CI 1.01 to 1.07); however, there was no significant detrimental effect of vitamin C (RR 1.06, 95% CI 0.94 to 1.20). The effects of a combination of beta carotene and of retinol (vitamin A) on the incidence of lung cancer were compared by Omenn et al. [42]. The relative risk of death from any cause was 1.17 (95 percent confidence interval, 1.03 to 1.33) in the treated group: 1.46 (95 percent confidence interval, 1.07 to 2.00) of death from lung cancer and 1.26 (95 percent confidence interval, 0.99 to 1.61) of death from cardiovascular diseases. As there is no direct evidence to connect the inappropriate iron balance with antioxidants treatment, the only conclusion could be that iron status should be considered in further clinical trials with antioxidants.

Dietary antioxidants can therefore act in complex and synergistic ways depending on iron status and promote the production of OH^\bullet radicals in the presence of inappropriately or inadequately liganded Fe(II) [119–122]. In this regard, the use of elemental iron plus ascorbate in food supplements [123] does not seem appropriate. The problem of iron overload increases with aging since iron stores tend to increase with age [124–128], partly due to dietary reasons [129–131]. Several commonly used dietary vitamin and

mineral supplements may be associated with increased total mortality risk in elderly women; this association is strongest when supplemented iron [47]. On the other hand, anemia was reported in older population [132, 133]. Killilea et al. [134] reported additionally that the iron content of cells increases also as the cells age normally. Some excessive iron can be removed from the body by regular blood transfusions, like in the case of haemochromatosis. Additionally, certain polyphenols inhibit the absorption of iron. Flavonoids act as antioxidants, through, both, free radical scavenging and metal chelation [135]. Many other iron chelators have been reviewed elsewhere (e.g., [85, 86, 112]). The reduction of redox stress thus requires appropriate amounts of both antioxidants and effective iron chelators.

Additionally, calorie restriction (CR) is thought to be the only experimental manipulation that considerably increases both mean and maximum lifespan in a phylogenetically wide variety of animals [136]. CR provokes a mild stress response, causing enhanced cell defences and metabolic changes, coordinated by the endocrine system. CR may improve iron status, since age-related iron accumulation was found to be markedly suppressed by dietary restriction (DR) in all tissues of 344 male Fischer rats [137]. This improved iron status could in part promote longevity [138]. While we have comparatively little influence on the production of superoxide and peroxide in mitochondria, we can try and improve the speciation of iron ions by pharmacological or dietary means [112].

5. ROS and Adaptive Responses

The key to the future success of regulating oxidative stress induced damage should be the suppression of oxidative damage without disruption of the well-integrated antioxidant defense network. Approach to neutralize free radicals with antioxidants should be changed into prevention of free radical formation in the first place. Evidence that such approaches are possible was summarized in the recent article by Poljsak [2].

The term “hormesis” describes beneficial actions resulting from the response of an organism to a low-intensity stressor. The basic concept behind the idea of hormesis is to provoke the intrinsic capability of a body rather than to supply exogenous natural or synthetic antioxidants to try to compensate for age-related decline of physiological activities in the overall maintenance mechanisms of life [139].

Hormetic pathways activated by phytochemicals may involve kinases and transcription factors that induce the expression of genes that encode antioxidant enzymes, protein chaperones, phase-2 enzymes, neurotrophic factors, and other cytoprotective proteins. Specific examples of such pathways include the sirtuin/FOXO pathways, the NF- κ B pathway, and the Nrf-2/ARE pathway [140]. Examples of moderate (usually intermittent) stress inducers include ischemic preconditioning, exercise, and dietary energy restriction [141]. The work of Lee and Yu [142], Koizumi et al. [143], and Chen and Lowry [144] strongly suggest that food restriction (energetic stress) enhances the overall

antioxidant capacity to maintain the optimal status of intracellular environments through the concerted interactions of cellular components to regulate ROS and membrane stability against peroxidative stress [137]. However, recent studies do not provide compelling evidence that the CR-associated longevity is mediated by antioxidants [145]. Attention is thus directed to other targets of nutrient signaling that result in the increased production of cytoprotective and restorative proteins in cells.

Finkel and Holbrook [146] stated that the best strategy to enhance endogenous antioxidant levels may actually be oxidative stress itself, based on the classical physiological concept of hormesis. Studies have reported the antiaging and life-prolonging effects of a wide variety of the so-called stressors, such as prooxidants, aldehydes, H₂O₂ [147], CR [148–151], shear stress [152, 153], irradiation [154], radiation stress [155], UV-radiation [156], physical exercise [157], heat shock, hyperbaric conditions [158], and hypergravity. ROS production is increased by several environmental factors of stress, such as exposition to high levels of light, drought, heavy metals, salt concentrations, temperature extremes, air pollution, UV radiation, herbicides, and pathogen attacks. Whether ROS will act as damaging, protective, or signaling factors depends on the delicate equilibrium between ROS production and scavenging at the proper site and time [159]. Heat and cold can also work as hormesis agents [160–164]. Thermal stress induces a heat-shock response involving increased expression of heat-shock proteins (HSP). Overexpression of certain HSPs in the mitochondria can significantly extend the longevity of normal-lived animals [165, 166]. The so-called adaptive response processes may explain how ROS formation could culminate in promotion of health and life span. For example, moderate physical activity enhanced mitochondrial activity and subsequently increased ROSs formation that ultimately induces an adaptive response, which culminates in metabolic health and extended longevity. On the other hand, health-promoting effects were demonstrated to be reduced, if the subjects exposed to physical activity were treated also with antioxidant supplements [167, 168].

According to Ristow and Schmeisser [145], endogenously produced ROS presumably not only induce ROSs defense enzymes, but also increase activities of phase II response enzymes that protect from damage beyond ROS. Increased oxidative stress over extended period of time is harmful as well as is the extended period of antioxidative stress. Increased ROSs induce endogenous defence mechanisms culminating in increased stress resistance. Interestingly, low doses of ROS with intermittent duration seem to exert the hormesis effects, whereas higher doses of ROS are unquestionably detrimental [145]. The question arises whether Le Chatelier’s principle can be used also in situation of antioxidative stress to predict the effect of a change in conditions on a chemical equilibrium. The principle states

“If a system in equilibrium is subjected to a stress the equilibrium will shift in the direction which tends to relieve that stress.”

6. The Importance of Determination of the Oxidative/Antioxidative Status *In Vivo*

To establish the mechanism of toxicity as ROS mediated, there are direct and indirect methods. Direct methods relate to ROS measurement such as superoxide, H_2O_2 , OH^\bullet . These species are very reactive, and their quantitation can be difficult. The only technique that can detect free radicals directly is the spectroscopic technique of electron spin resonance (ESR), sometimes called electron paramagnetic resonance (EPR). The indirect methods are used in order to overcome these problems. Indirect methods usually measure changes in endogenous antioxidant defense systems or measure the products of damage by ROS among the cellular components [169]. Therefore, changes in endogenous antioxidant defense systems and damages on cellular components, caused by ROS, are measured by indirect methods. The principle behind fingerprinting methods is to measure products of damage by ROS, that is, to measure not the species themselves but the damage that they cause. Measuring the damage caused by ROS instead of direct measuring of ROS seems logical, since it is the damage caused by ROS that is important rather than the total amount of ROS generated. A good marker of oxidative damage must be increased in the presence of oxidative stress, and it must remain unchanged in its absence [170]. The marker must measure a product that is endogenously present, not produced during the isolation procedure. Multiple methods of measurement are available today, each has their own benefits and limitations. Ascertaining the true importance of ROS has been hard, as these evanescent species are difficult to measure *in vivo*. It is generally accepted that two or more assays should be used whenever possible to enhance their validity, since different parameters are measured by different assays. There are inherent limitations in all methods, and no method can be said to measure accurately the amounts of ROS by itself [171]. For example, in order to determine oxidative stress, both, the ROS potential as well as the antioxidative defense potential should be measured. Besides, it is not possible for a reactive free radical produced in an extravascular space of living tissue, with a lifetime of microseconds, to diffuse into the blood to be detected at the distant site. Numerous *in vitro* methods are described for the antioxidation potential determination that are easy to perform and largely used in screening. However, the results of such tests are relevant only partially for humans as certain active compounds (e.g., those with large molecular masses) are poorly absorbed from the gastrointestinal tract and/or may undergo metabolic degradation. Therefore, new experimental models are required to provide information if protective effects take place in humans under realistic conditions.

There are many challenges regarding the methodology for oxidative state detection on cellular level, for example, which method(s) to use, which antioxidant should be used for a standard, on the most appropriate positive control (e.g., an oxidising agent or catalysts of Fenton reaction), and so forth. There are no specific recommendations on methods which evaluate best the oxidative stress in biological

systems although there are several test systems with different end-points [169]. Additionally, there are no reference values of what is the optimal antioxidative potential in urine, blood, or even intracellularly. It is not known how much antioxidants and in which combinations they are needed for a beneficial antioxidant effect *in vivo*. Besides, typical oxidative stress status of an individual is not established yet because it is difficult to measure [172]. Determination of antioxidative potential per se is thus not enough, since it is difficult to establish how the individual antioxidants work: by preventing the formation of ROS, by scavenging free radicals, by inducing the signaling pathways, or by repairing the oxidative damage. The improvement in methodology will likely overcome at least some of these drawbacks.

7. Antioxidant Supplements and the Consequences of Oxidative Imbalance

Mitochondria are the main site of intracellular oxygen consumption and the main source of ROS formation [10–14]. Cellular and tissue defenses against ROS include the enzymes superoxide dismutase (Mn-SOD, Cu/Zn-SOD, extracellular (EC)-SOD), catalase, glutathione peroxidase, peroxiredoxins, and the nonenzymatic antioxidants, like glutathione (GSH), thioredoxin, ascorbate, α -tocopherol, and uric acid [173]. In reality, the oxidative damage potential is grater than antioxidant defence, and thus there is a constant small amount of toxic free radical formation, which escapes the cellular defenses. These may lead to a conclusion that endogenous levels of antioxidants are not high enough to fight the stress insults and that the addition of exogenous antioxidants could enhance the cellular antioxidant capacity.

On the other hand, low levels of ROS function as signalling molecules to induce adaptive responses. Overconsumption of exogenous antioxidants could thus lead to the state of “antioxidative” stress, where antioxidants might attenuate or block the adaptive stress responses. Additionally, reactive oxygen species in moderate concentrations are essential mediators of defence against microorganisms and unwanted cells. Thus, if administration of antioxidant supplements decreases the levels of free radicals, this may interfere with essential defence mechanisms for removal of damaged cells, including those that are precancerous and cancerous [174]. Therefore, antioxidant supplements may actually cause some harm [44, 175–177]. Our diets typically contain safe levels of vitamins; however, supplementing them with high levels of antioxidant supplements can potentially upset an important physiological balance [44, 175–177]. A systematic review and meta-analysis done by Bjelakovic et al. [176] concluded that the long-term treatment with beta carotene, vitamin A, and vitamin E may increase mortality. It is not known whether the increased mortality is due to iron overload, adaptive stress response prevention, repression of immune system, or any other mechanism. Presented findings provide indirect evidences for the hypothesis that “anti-oxidative” stress influences ROS production and subsequently inappropriate induction of ROS defenses. On the other hand, also excessive oxidative stress for an extended

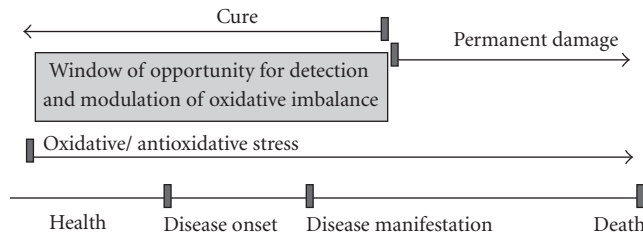


FIGURE 1: Early detection of “antioxidative” or oxidative stress enables successful prevention and treatment. The window of opportunity for detection and modulation of oxidative imbalance should be explored in order to timely recognise and ameliorate the oxidative imbalance to prevent or postpone its consequences, namely, the free radical-related disease development and premature aging.

period of time increases oxidative damage with negative influences on health and longevity.

Oxidative or “antioxidative” stresses occur early in the process that may lead to pathology; therefore, they are not associated with any specific clinical symptoms or signs at an early stage. Often they are not recognized until the development of irreversible damage to the patient that manifest itself as a symptom of a chronic disease. It is therefore important to recognise oxidative imbalance at an early stage in order to prevent the long-term oxidative and antioxidative stresses (Figure 1). These point to the need for determination of individual’s oxidative status before starting or ending the supplement therapy with antioxidants. This may prevent or reverse the development of diseases which arise because of chronic oxidative imbalance.

The question arises whether determination of individual’s oxidative balance/imbalance status would lead to a better preventive or therapeutic interventions that may slow down the aging process. If so, who would benefit from it most? We believe that individuals with oxidative stress because of their diets, lifestyle, metabolic errors, exposure to environmental pollution or iron overload would benefit from regular monitoring of their oxidative status. The individuals who overdose antioxidant supplements and enter the status of “antioxidative” stress may benefit even more.

8. Conclusion

The current level of awareness of safety and quality of supplements with antioxidative properties is inadequate, as there are still many gaps in our knowledge regarding the mechanisms of bioavailability, biotransformation, and action of these supplements. There are several studies in which the addition of antioxidants affected and even increased the mortality.

Exogenous antioxidants tend to depress the endogenous antioxidant levels. Changing the level of one antioxidant causes a compensatory change in the levels of others, while the overall antioxidant capacity remains unaffected. Dosing cells with exogenous antioxidants might decrease the rate of synthesis or uptake of endogenous antioxidants, so that the total “cell antioxidant potential” remains unaltered. Thus,

the key to future success of dietary antioxidant supplementation should be in the suppression of oxidative damage without the disruption of the well-integrated antioxidant defense network.

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