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Biological importance of reactive oxygen species in relation to difficulties of treating pathologies involving oxidative stress by exogenous antioxidants

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ABSTRACT

Findings about involvement of reactive oxygen species (ROS) not only in defense processes, but also in a number of pathologies, stimulated discussion about their role in etiopathogenesis of various diseases. Yet questions regarding the role of ROS in tissue injury, whether ROS may serve as a common cause of different disorders or whether their uncontrolled production is just a manifestation of the processes involved, remain unexplained. Dogmatically, increased ROS formation is considered to be responsible for development of the so-called free-radical diseases. The present review discusses importance of ROS in various biological processes, including origin of life, evolution, genome plasticity, maintaining homeostasis and organism protection. This may be a reason why no significant benefit was found when exogenous antioxidants were used to treat free-radical diseases, even though their causality was primarily attributed to ROS. Here, we postulate that ROS unlikely play a causal role in tissue damage, but may readily be involved in signaling processes and as such in mediating tissue healing rather than injuring. This concept is thus in a contradiction to traditional understanding of ROS as deleterious agents. Nonetheless, under conditions of failing autoregulation, ROS may attack integral cellular components, cause cell death and deteriorate the evolving injury.

1. Introduction

Evidence from animal- and clinical-based studies about involvement of free oxygen radicals and reactive oxygen species (ROS) in a variety of pathologies prompted investigations on ROS in their etiology and pathogenesis. For several decades it was widely accepted that excessive ROS production is an underlying mechanism of particular tissue injuries (Fig. 1, black columns). Although mostly demonstrated under *in vitro* conditions, this concept was mainly substantiated by findings that ROS readily react with most biological macromolecules, causing their oxidative modification, which ultimately leads to the loss of their primary functions (Bartosz, 2009; Lenaz, 2012). Therefore, ROS started to be considered as one of the key players in tissue injury, which if occurring in a massive extent may result in organ dysfunction. Pathologies where ROS were identified as causal factors were then termed free-radical diseases. Disease states of this category comprise a heterogeneous group including adult respiratory distress syndrome, atherosclerosis, inflammation, rheumatoid arthritis and other autoimmune diseases, degenerative disorders associated with aging, diabetic complications, stress related injury, processes of mutagenesis and cancerogenesis, ischaemia-reperfusion injury, organ transplantation complications, etc. (Becker, 2004; Halliwell, 2007; Harman, 2003; Jaeschke, 2011; Reed, 2011; Venardos et al., 2007). Along these lines, in anticipation of beneficial effects, the use of exogenous antioxidants has been proposed as a treatment of choice for free radical diseases (Aboul-Enein et al., 2013; Apostolou et al., 2013; Jaeschke and Woolbright, 2012; Rodrigo et al., 2013; Stagos et al., 2012). However, antioxidants exerting protection of essential macromolecules *in vitro* often fail to do so *in vivo*.

Almost simultaneously questions started to be raised as to whether excess of ROS may indeed serve as a general mechanism underlying such a variety of diseases. More specifically, do oxidative modifications of essential macromolecules by ROS always lead to cell death and tissue injury? In fact, there were reports suggesting that ROS overproduction may be a consequence rather than a

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cause of tissue injury (Feinendegen, 2002; Halliwell, 1994, 2009). If the former holds, ROS could actually modulate processes in the affected tissue and/or serve as markers of its actual state. Indeed, an increasing number of reports highlight physiological functions of ROS, particularly their messenger and mediatory role in intracellular signaling and intercellular communication (Fig. 1, cross-hatched columns), i.e. mechanisms underlying homeostasis maintenance (Armogida et al., 2012; Halliwell et al., 2000; Nikitovic et al., 2013; Nair et al., 2007; Nisticò et al., 2008; Sarsour et al., 2009; Upham and Trosko, 2009; Zweier et al., 2011). To contribute to the understanding of the role of ROS in health and disease, the present review focuses on apparent paradoxes of ROS, and discusses controversial findings regarding effects of ROS. Special attention is paid to the difficulties encountered in treating oxidative stress-related disease by exogenous antioxidants.

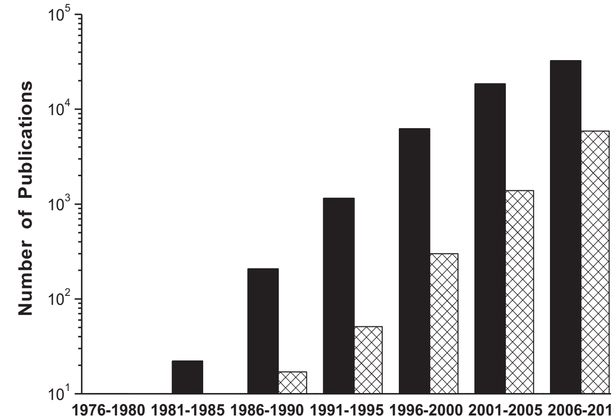

Fig. 1. Number of publications dealing with ROS in tissue injury (black columns) and with the signaling role of ROS (cross-hatched columns) published in 1976–2010. Source: PubMed.

2. Biological importance of ROS: role in emerging of life, evolution and biodiversity

Intriguing paradoxes regarding the involvement of free (oxygen) radicals, and ROS in processes of life emergence, evolution of higher forms of life and species diversity as well as in protecting vital functions of current aerobic organisms have been noted (Fig. 2).

2.1. Free radical reactions were crucial for the creation of life on Earth

Life emerged about 3.5–4 billion years ago from amino acids and nucleotides, which were synthesized utilizing free radical-mediated reactions from simple reduced inorganic compounds (Line, 2002). Necessary energy was provided by cosmic and sun radiation and geo-volcanic processes (Cody et al., 2000; Huber and Wächtershäuser, 2006).

2.2. Oxygen and ROS as important factors of evolution

About 2.6 billion years ago, cyanobacteria started to utilize water. As a result of their metabolism, molecular oxygen as a by-product began to be released into both the hydrosphere and the atmosphere (Mulkidjanian and Galperin, 2009). Increasing concentration of oxygen, which is basically toxic due to its high oxidative strength for most of anaerobes living then, represented a significant evolution pressure resulting in evolvement of effective oxygen-detoxifying mechanisms (Jenney et al., 1999). Gradually, unicellular organisms *via* symbiosis with cyanobacteria gave rise to the first plant cells (Lang et al., 1999). Intracellular symbiosis of eukaryotes with prokaryotes, on the other hand, led to emergence of the first simple animal unicellular organisms, which were nonetheless capable of "detoxifying" molecular oxygen by its reduction to water. Survival under highly oxidative environment led also to implementation of other specific strategies. To minimize the presence of oxygen in its free unbound form in higher organisms, systems of tightly working oxygen carriers, storage and reduction *via* hemoglobin, myoglobin and cytochrome c oxidase, respectively, have evolved (Collman et al., 2004). In this manner the giant step of eukaryotic energy metabolism was established (Emelyanov, 2003; Gray et al., 1999; Taylor and Pouyssegur, 2007). Oxygen "toxicity" hence played a momentous role in the evolution process, paving the way for the emergence of aerobic organisms (Lindahl, 2008; Raymond and Segrè, 2006).

2.3. Utilization of oxygen and ROS involvement in the evolution of multicellular organisms

Molecular oxygen is abundant in biological systems, and its high electronegativity, i.e. significant oxidation potential, determined its usage as a final electron acceptor in aerobes. Utilizing

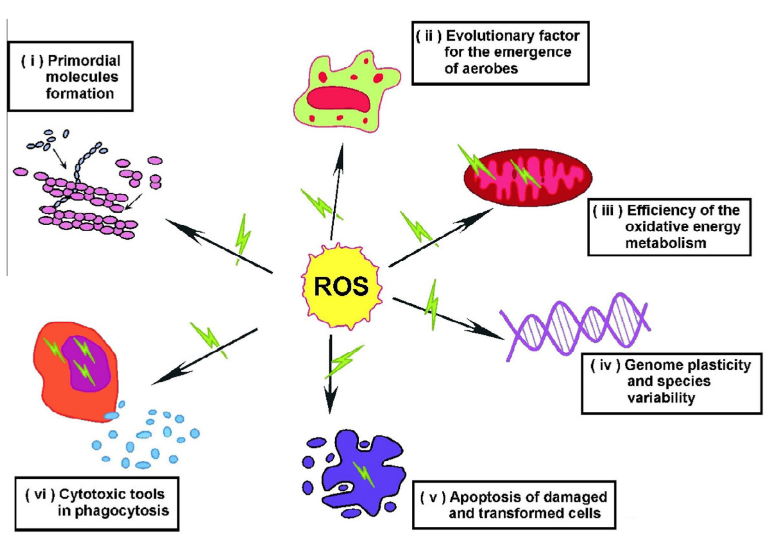

Fig. 2. Biological paradoxes demonstrating beneficial roles of ROS during life emergence and evolution as well as in tissue homeostasis: (i) synthesis of primordial life building blocks; (ii) evolution of life forms; (iii) facilitation of efficient oxidative energy metabolism; (iv) affecting genome plasticity and species variability; (v) participation in the apoptotic mechanisms of damaged and transformed cells and (vi) main cytotoxic tools in phagocytosis.

oxygen resulted in yielding such amount of energy from ingested organic compounds that enabled emergence of multicellular organisms (Koch and Britton, 2008; Jiang et al., 2010). Up to 100% of utilized oxygen enters mitochondria where it undergoes four-electron reduction to water while producing energy in the form of adenosine triphosphate (ATP). Yet, about 1–2% of the transported electrons may leak from the respiratory chain. This allows formation of partly reduced oxygen intermediates, like superoxide anion radical, hydrogen peroxide and hydroxyl radical, i.e. ROS (Kovacic et al., 2005; Lenaz, 2012). And ROS exhibited evolutionary pressure for developing effective antioxidative mechanisms (Min and Jian-xing, 2007). That substantially limited occurrence of partial oxygen reduction eventually yielding ROS. Concluding, single-electron chemistry of mitochondrial oxidative phosphorylation generates ROS by default. Recently, an intriguing relation between the efficiency of oxidative phosphorylation and mitochondrial ROS production has been reported (Brookes, 2005; Venditti et al., 2013). Going a step further, the indicated feedback mechanism in the regulation of both processes could conceivably be involved in the evolutionary processes guiding the development of the aerobic metabolism.

2.4. ROS involvement in transition of life in water into terrestrial forms

Organisms living under conditions of rarefied atmosphere were permanently exposed to high-energy ionizing radiation, a condition extremely deleterious as it facilitates the homolytic cleavage of water molecules which yields highly-reactive hydroxyl radical and hydrogen. Moreover, radiation energy can cause the excitation of molecular oxygen from its triplet to singlet state, yielding thus another ROS type (Bartosz, 2009). In order to survive, organisms found ways of protecting themselves from ionizing radiation. Initially, they inhabited relatively deep sea layers where they could get enough light to cover their energy needs yet the intensity of radiation was not as high as to be prohibitive. During millennia, life forms gradually developed protective mechanisms and ways for utilization and effective neutralization of ROS. These gained benefits enabled them move vertically upwards and start to inhabit layers closer to sea surface (McCord, 2000; Min and Jian-xing, 2007). The decisive event in aerobic evolution occurred, some 500 million years ago, when due to effects of ionizing radiation the increased amounts of atmospheric oxygen led to formation of ozone, which started to accumulate in higher layers of the atmosphere (Lindahl, 2008; Massabuau, 2003). The ozone layer significantly reduced the amount of the high-energy radiation reaching the Earth surface, transforming gradually the inhospitable environment into a permissive one. These environmental changes allowed organisms with already developed antioxidative mechanisms to gradually migrate from the water habitat onto terra firma.

2.5. ROS involvement in genome plasticity, species variability and biodiversity

As byproducts of biochemical reactions, ROS are involved in oxidative modification of essential macromolecules including nucleic acids. Oxidative alterations of DNA occur quite often. In fact, up to ten thousand DNA lesions, including those occurring during the process of DNA replication, are daily events in a single cell (Burrows, 2009). The resulting DNA lesions are mutagenic and, unless repaired, lead to a variety of mutations and consequently to genetic instability. Thus, modifications and mutations of DNA may accumulate in the cell and ultimately lead to dysfunction of the affected part of genome. Damage in genes coding for oncological suppressors, may result in up-regulation of oncogenes expression and concomitant uncontrolled cell proliferation. In order to protect themselves, organisms have evolved mechanisms to discriminate and repair critical DNA lesions and mutations (Cairns, 1998). Oxidatively induced DNA damage is repaired in living cells by different pathways that involve a large number of proteins (Dizdaroglu, 2012). On the other hand, repair of DNA is not always fully effective, and hence not every mutation is removed. Some mutations are harmless, i.e. they do not cause any particular defect of important genes, and others may remain unrecognized, e.g. when not crucially affecting normal cellular function. However, if damaging DNA alterations are extensive and are not repaired, the cell is usually directed to apoptosis (Baltmr et al., 2010). Indeed, failure in recognition of critical DNA damage and its repair as well as the absence of apoptosis may have severe consequences for the affected tissue/organ. Unrepaired and accumulated DNA lesions may lead to different disease processes including carcinogenesis (Sedelnikova et al., 2010).

Hence, DNA, which is under permanent attack by a variety of agents, including ROS, undergoes many oxidative and non-oxidative modifications every day. Therefore, due to perpetual modifications along with effective repairing mechanisms, DNA represents an exceptionally dynamic structure. Critical mutations are usually eliminated, whereas many alterations possess neutral character without having any immediate negative influence on the cell. Consecutively, some of the modifications will not be removed and will subsequently be inherited by daughter cells. Indeed, these processes are considered as the basis of genome plasticity, which during evolution resulted in a great number of species and high biodiversity (Burrows, 2009).

2.6. ROS involvement in defense of living organisms

Defense of the organism from harmful influence of both endogenous and exogenous factors involves, but is not limited to, inflammatory reaction, including phagocytosis. Phagocytes including neutrophils, monocytes, macrophages, dendritic cells and mast cells are specialized cells of the immune system, designed to engulf and destroy harmful microorganisms inside the newly formed phagosome. The latter is an intracellular organelle that is transformed into a toxic environment within minutes and disappears once the pathogen is destroyed. The process of phagocytosis removes foreign cells or particles and even the organism's own cells when damaged (Nüsse, 2011). The activation of phagocytes is associated with increased consumption of oxygen, with the resulting *oxidative burst* being characterized by increased ROS production. ROS are thus one of the main cytotoxic tools for removing damaged cells and destroying pathogens and particles in phagosomes (Halliwell, 2006; Jaeschke, 2011). Neutrophils can be cytotoxic to tumor cells through the release of large amounts of neutrophil elastase and ROS. However, neutrophils recruited directly and indirectly by cancer cells, can also contribute to tumor progression by preparing the microenvironment for tumor invasion (Wu et al., 2000; Houghton, 2010). Thus, neutrophils bind to tumor-secreted chemoattractants, become activated, adhere to the vascular endothelium, and transmigrate through the endothelial cell layer and into the tumor extracellular matrix (ECM). This process is accompanied by a strong neutrophil release of ROS, and NO as well as ECM remodeling, which eventually facilitates tumor cell-transmigration (de Larco et al., 2004). The effects of ROS on tumorigenesis seem to be strictly concentration (Valko et al., 2006) and duration of exposure-dependent (Brieger et al., 2012). Thus, while prolonged exposure to high ROS concentrations may lead to non-specific damage to proteins, lipids, and nucleic acids, low to intermediate ROS concentrations exert their effects rather through regulation of cell signaling cascades. Biological specificity is achieved through the amount, duration, and localization of ROS production (Nikitovic et al., 2013). Hence, ROS in a concentration/time dependent manner may protect the organism from infection and, may affect tumor development and progression (Halliwell, 2007). Importantly the ROS-producing activity of phagocytes can actually be modulated by antioxidative agents (Puertollano et al., 2011). Targeted modulation of ROS release from activated neutrophils, e.g. by treatment with antioxidant flavonoids (Ciz et al., 2012), may thus offer a basis of targeted therapy in inflammatory disease and cancer.

Summarizing, molecular oxygen is an obligatory component for the functioning of aerobic organisms. ROS are formed under physiological conditions as by-products of key cellular functions, particularly during the processes of energy metabolism (Venditti et al., 2013). On the other hand, the high oxidative potential of oxygen represents a constant risk for undesired oxidation defining thus aerobic metabolism as a condition of permanent oxidative attack (Beckman and Ames, 1998). Nonetheless, the majority of life forms have emerged, evolved and gained their complexity due to the presence and action of ROS. Indeed, selective force of ROS led to the evolvement of effective detoxifying systems and antioxidative mechanisms (Jenney et al., 1999). The selective pressure perpetrated by ROS has also stimulated living organisms to acquire specific enzymes for controlled ROS production, enabling them to utilize ROS in a manner beneficial for their own needs (McCord, 2000; Thannickal and Fanburg, 2000).

3. ROS production, elimination and oxidative stress

Under physiological conditions, ROS are formed as by-products of basal cellular metabolism. It has been proposed that cellular respiration is regulated by ADP, O_2 and NO preserving thus the notion that energy demands drive respiration but places the kinetic control of both respiration and energy supply in the availability of ADP to F_1-ATPase and of O_2 and NO to cytochrome oxidase. Indeed, for the regulation of the intramitochondrial steady state the concentration of NO itself and other reactive species such as superoxide anion radical (O_2^-) and peroxynitrite ($ONOO^-$) was found to have a key role (Boveris et al., 2000). Importantly, the amount of ROS formed within mitochondria has been reported to be proportional to the partial oxygen pressure (D'Agostino et al., 2007). Besides mitochondrial electron chain transfer, there are also other sources of ROS production including the system of cytochrome P-450, oxidative enzymes, such as endothelial xanthine oxidase, phagocyte NAD(P)H oxidases and myeloperoxidases, arachidonate oxygenases (Altenhöfer et al., 2012; Bedard and Krause, 2007; Kleikers et al., 2012). Furthermore, auto-oxidative reactions of endogenous substances, such as catecholamines, or exogenous substrates, such as xenobiotics, as well as the oxidation of reduced metabolites, accumulated e.g. in the processes of anaerobic metabolism, have also been indicated as a significant source of ROS (Kovacic et al., 2005; Lenaz, 2012).

To be effectively protected from potentially deleterious ROS, aerobes have evolved a battery of specific defense mechanisms to detoxify ROS. Briefly, the defense mechanisms include specialized antioxidative enzymes, e.g., superoxide dismutase, catalase, glutathione peroxidase, and non-enzymatic low molecular weight antioxidants, like glutathione, tocopherols, ascorbic acid, etc. (Yu, 1994). In addition, to cellular defense, a system of repair mechanisms has emerged. It comprises of specific degradative and synthetic enzymes, like proteases, peptidases, phospholipases, acyl transferases, endonucleases, exonucleases, polymerases, ligases, i.e. enzymes that cleave irreversibly damaged macromolecules and synthesize new molecules in their stead (Elliott et al., 2000). Significantly, all three systems are integrated creating a network working in concert under physiological conditions (Bartosz, 2009; Berry and Kohen, 1999; Venardos et al., 2007).

Uncontrolled ROS production occurring as a result of an imbalance between ROS formation and ROS elimination while favoring prooxidant processes and oxidative damage has been termed *oxidative stress* (Sies, 1985). Generally accepted dogma states that under physiological conditions, antioxidative system does not permit detrimental action of ROS. Indeed, an increasing number of publication reports that ROS and/or their products, such as lipid and protein oxidation metabolites, may play important signaling roles (Armogida et al., 2012; Forman and Dickinson, 2004; Nakashima et al., 2003). These mediators have been indicated to be involved in maintaining cell homeostasis, defending tissue against further insults by promoting its preconditioning, mediating physical endurance by exercising, regulating gene transcription, removing abnormally proliferating cells by inducing apoptosis, thus protecting against cancer, etc. (Becker, 2004; Fleury et al., 2002; González-Flecha and Demple, 2000; Maslov et al., 2012; Nisticò et al., 2008; Obrenovitch, 2008; Ravati et al., 2000; Veskoukis et al., 2012). The present review attempts to contribute to better understanding of the role of ROS in tissue injury. Using an example of ischaemia–reperfusion, ROS are introduced as potential mediators of the evolving injury and not a primary cause of cellular damage. This understanding may help explaining some discrepancies and certain disillusions on often missing beneficial effects of exogenous antioxidants intended to combat pathologies involving oxidative stress.

4. ROS and cytosolic calcium in tissue injury

Involvement of ROS in tissue injuries developing during ischaemia–hypoxia events and subsequent reperfusion–reoxygenation has been widely established for many organs and systems (Jaeschke and Woolbright, 2012; Obrenovitch, 2008; Rodrigo et al., 2013). Mechanism of ROS generation by the xanthine oxidase-mediated process has been described in detail (Han et al., 2012). Briefly, mitochondrial respiration and oxidative phosphorylation and thus ATP synthesis are inhibited by a lack of oxygen. Indeed, dysfunction of the electron transport chain itself leads to increased ROS formation. To cover cellular energy needs, synthesized ATP is rapidly hydrolyzed, yielding adenosine di- and monophosphate and adenosine, which are finally catabolized to inosine and hypoxanthine. Accumulated hypoxanthine, which is normally metabolized by xanthine dehydrogenase, is under hypoxia degraded by xanthine oxidase isoform, a potential producer of the superoxide anion radical. Importantly, superoxide derived from endothelial xanthine oxidase was found to be involved in attraction and activation of neutrophils, and antioxidants were demonstrated to reduce accumulation of neutrophils in postischemic tissue, and the resulting neutrophil depletion reduced reperfusion injury (for review see e.g. Juránek and Bezek, 2005). Superoxide was also demonstrated to release iron from ferritin, what may result in generation of hydroxyl radical *via* the Haber–Weiss reaction (Valko et al., 2007). Hence, by involving several other pathways, xanthine oxidase-derived superoxide can lead to further generation of other ROS in reoxygenation. And, it is of particular significance that the hypoxic conversion of xanthine dehydrogenase into oxidase isoform is mediated by calcium-dependent protease calpain.

In fact, cytosolic calcium itself plays an important role in ischemic-hypoxic insult as hypoxia-induced increase of cytosolic calcium concentration activates calcium-dependent degradative enzymes, such as proteases, phospholipases, nucleases. Their upregulation leads to degradation of essential macromolecules and may eventually result in cell death – necrosis and/or apoptosis. Indeed, intracellular calcium overload was observed to occur just before cell death (Takuma et al., 2004). Thus, a variety of calcium-dependent processes is getting activated under hypoxia-ischaemia. Besides the calpain-mediated activation of xanthine oxidase, calcium-dependent activation of NO synthase, NADPH oxidase and phospholipases A_2, with subsequent increase of arachidonic acid metabolism, also occur. And, importantly, all these processes are capable to generate ROS during oxygen reintroduction into the hypoxic tissue (Kleikers et al., 2012; Maslov et al., 2012). In support of the above concept, calcium entry blockers and/or chelators of cytosolic calcium were observed to both protect tissues from postischaemic injury and inhibit ROS production. On the other hand, it has been suggested that, in order of importance, energy metabolism failure – due to lack of oxygen, calcium overload and ROS overproduction, are crucial in developing reperfusion injury (Takuma et al., 2004). In addition, a number of studies indicate that the involvement of ROS is crucial in the induction of ischemic preconditioning (Armogida et al., 2012; Nisticò et al., 2008; Ravati et al., 2000; Upham and Trosko, 2009).

5. Controversies in treating oxidative stress-related pathologies by antioxidants

5.1. Findings from experimental models

According to traditional free radical hypothesis, tissue injury evoked by ischaemia–reperfusion develops due to overproduction of ROS and their subsequent deleterious action towards essential cellular constituents (Cuzzocrea et al., 2001; Valko et al., 2007). Briefly, readily oxidizable fatty acids of phospholipid membranes are good targets for peroxidative attack of ROS, resulting in alterations of membrane permeability and fluidity. That in turn may cause dysfunction of membrane proteins, including ionic pumps, whose activity depends on the membrane lipid milieu. Causal role of ROS was reported for ischaemia–reperfusion injury in the brain, myocardium, liver, kidney, intestine, etc. (Jaeschke and Woolbright, 2012; Maslov et al., 2012; Kleikers et al., 2012; Rodrigo et al., 2013).

On the other hand, ROS may play a role as mediators in postischaemic stage and they need not to be involved in the etiology of ischemic injury. In fact, there is a number of evidence that massive generation of ROS occurs mostly as a consequence of ischaemia/hypoxia, i.e. at reperfusion phase when reintroduction of oxygen into the postischemic/posthypoxic tissue takes place (for review see Juránek and Bezek, 2005). This assumption is of considerable relevance as it may help in understanding of some apparent discrepancies as regarding effects of exogenous antioxidants in treating the tissue injury. Indeed, reduction of ischaemia–reperfusion injury by antioxidants was often found to be only partial irregardless whether significant inhibition of ROS production was observed or not (Georgakouli et al., 2013; Kahles and Brandes, 2012; Veskoukis et al., 2012). Some of the negative findings may be attributed to improper dosage, timing or low bioavailability of exogenous antioxidants applied, though there is also evidence that ROS production did not coincide with occurrence of tissue injury.

Overall, the findings indicate that ROS are unlikely a causal factor of postischaemic injury and this understanding represents a remarkable shift in the perception of ROS playing a pivotal role in the etiology of tissue damage. An exception comprises tissue injury caused by hydroxyl radicals, formed in the process of water radiolysis upon exposure to ionizing radiation (Lehnert and Iyer, 2002; Mitchell et al., 2000). In agreement, this type of tissue injury was beneficially affected by antioxidants acting as radioprotectors (Kilciksiz et al., 2008; Singh, 2010; Weiss and Landauer, 2000). Based on the evidence provided, we feel confident that ROS overproduction should be generally considered as the consequence rather than the cause of tissue injury.

5.2. Disillusion of clinical trials

From a clinical point of view, the most important are findings of large epidemiological studies. They indicate that cancer and ischaemic heart disease, in particular, might be inversely related to the endogenous antioxidant status. The Physician Health Study (PHS) I (1982–1995) – focused on a long-term effect of β-carotene and aspirin on the occurrence of cardiovascular diseases and skin and lung cancer, and PHS II (1999–2001) – studying effect of β-carotene and vitamins C and E, and their combination, both comprising a large cohort of volunteers, failed to reveal any positive effects on the cardiovascular system and cancer occurrence (Christen et al., 2000; Goodman et al., 2004). In addition, the study CARET (Carotene and Retinol Efficacy Trial, 1988–1996) ended untimely because of unexpected negative results of the increased incidence of lung cancer (by 28%) and mortality due to lung cancer (by 17%), in heavy smokers (while having no effect in light smokers) after taking β-carotene and vitamin E in comparison to the placebo group (Goodman et al., 2004).

The Finland ATBC (α-tocopherol-β-carotene) study, involving 59–69-year old smokers taking α-tocopherol and/or β-carotene for 5 to 8 years, reported an increased rate of lung cancer (18%), increase in total mortality (8%) and decreased occurrence of prostate cancer (32%) (ATBC Cancer Prevention Study Group: New Eng. J. Med, 1994; Virtamo et al., 2003). In another study focused on head and neck cancer, patients were given α-tocopherol and β-carotene during radiotherapy for up to 3 years. A tendency of having less severe acute adverse effects due to radiotherapy has been observed in the test group in comparison to the placebo group. However, the test group had a higher trend of cancer recurrence (Bairati et al., 2005).

Summarizing, depending on conditions, antioxidants may both prevent or promote cancer progression. Though the role of ROS involvement in mutagenesis favoring cancer has been reported, one has to keep in mind that in the process of elimination of cancer cells, ROS often serve as executors (Feinendegen, 2002). Treatment with antioxidants may thus block the anti-cancer effect of ROS favoring survival and proliferation of neoplastic cells, and consecutively promote cancer rather than protect against it (Aboul-Enein et al., 2013; Halliwell, 2007; Nair et al., 2007).

6. Concluding remarks

Although the reaction of ROS with important cellular constituents may result in their degradation *in vitro*, mechanisms of ROS-mediated cytotoxicity *in vivo* are as yet not well understood. Free radical-mediated lipid peroxidation, protein oxidation and oxidative damage to nucleic acids are considered to be crucial events of the cytotoxic actions of ROS. Nonetheless, the most convincing evidence, i.e. unambiguous inhibition of free-radical diseases by pretreatment with antioxidants, is still missing. In fact, there are quite a number of papers reporting failure of antioxidant therapeutic or preventive effect in conditions where the primary causal role of ROS was believed to be involved (Christen et al., 2000; Heart Protection Study Collaborative Group, 2002). Moreover, contrary to anticipating effects, there are findings on serious complications arising from treatment by exogenous antioxidants (Goodman et al., 2004; Stocker and Keaney, 2004). The equivocal findings may result from inappropriate timing of administration or suboptimal drug availability at target tissue.

The above presented data indicate that postischaemic tissue injury may actually occur as an inevitable consequence of hypoxia-induced increase of cytosolic calcium resulting in the up-regulation of enzyme-mediated breakdown of essential intracellular constituents. Moreover, calcium overload seems to play a crucial role

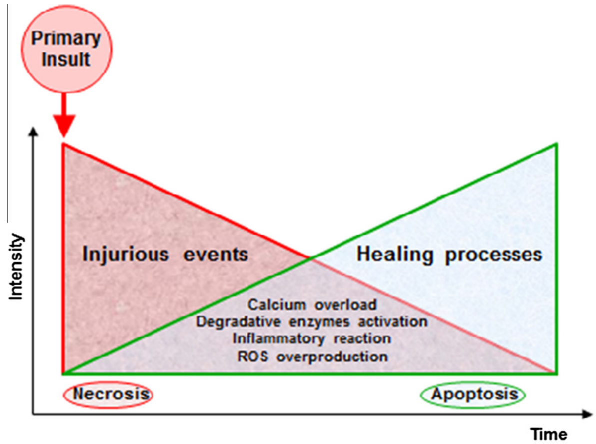

Fig. 3. Schematic depiction of tissue injury evolvement. After primary insult, injurious events immediately activate repair/healing processes (the former depicted in red, the latter in green). Eliminating injurious factors aims at maintaining or restoring functional and structural of the tissue (for details see text). To be resolved successfully, injurious processes should decline in time and those of healing have to prevail gradually. Injuring and healing involves complexity of mechanisms, including calcium overload, degradative enzyme activation, inflammatory reaction, and reactive oxygen species (ROS) production. Importantly, as both processes occur simultaneously the underlying mechanisms get overlapped, and hence it may be difficult to distinguish unequivocally which of them are primarily injurious and which are critical for effective healing. Regarding the cell death involved, necrosis occurs predominantly at the beginning while apoptosis, removing defected cells also *via* ROS mechanism, prevails later.

in cell death. In fact, calcium overload may be readily responsible for both activation of degradative processes and ROS overproduction, occurring upon reoxygenation of the hypoxic tissue. Hence, without injuring, ROS may then be excessively produced as a consequence of calcium overload.

When discussing the tissue injury, one has to realize that starting from the same beginning, a number of processes are initiated, aiming to eliminate deleterious insult and to restore tissue integrity and maintain its function (Fig. 2). During early stage, it is actually quite difficult to differentiate clearly injurious and healing events. Tissue responses are mediated by various messengers. Intriguingly, ROS have been reported to be released as by-products of both cyclooxygenase and lipoxygenase pathways depending on the partial oxygen pressure (Juránek et al., 1999, 2002). Interesting possibility is that similarly to a function of eicosanoids produced by these reactions, ROS may eventually play a role of mediator and as such to be involved in tissue effort to maintain homeostasis. Hereby, we described postischaemic injury developing as a consequence of intracellular calcium overload due to hypoxia with subsequent ROS formation as illustrative example. In fact, calcium-dependent processes themselves may trigger degradation of important cellular components leading to cell death, tissue injury and organ dysfunction. Indeed, cytosolic calcium overload (i) activates degradative enzymes, and (ii) mediates conversion of xanthine dehydrogenase to xanthine oxidase. The former may result in irreversible injury just as a consequence of hypoxia only. The latter is essential for the production of xanthine oxidase-derived ROS serving as a signal for other processes, e.g. neutrophil activation, to get involved. Activated neutrophils may then produce another portion of ROS. Nonetheless, produced ROS may not be involved in triggering injury at all. On the other hand, once generated, ROS may participate in evolving or resolving the injury, either directly or indirectly (e.g. by neutrophil activation), depending on the overall conditions (see Fig. 3).

There are authors suggesting that excessive production of ROS may be rather a *smoke after the fire*, i.e. as an inevitable consequence of some primary injury, than the injurious insult itself (Halliwell, 1994; Juránek and Bezek, 2005). In fact, ROS play an important role in both physiological and pathological signaling (Armogida et al., 2012; Bartosz, 2009; Halliwell, 2009; Corsini et al., 2013; Nikitovic et al., 2013). Moreover, it has been indicated that ROS are involved in limiting tissue lesion, preventing infection, cellular adaptation to stressful stimuli, i.e. preconditioning, increasing skeletal muscle endurance after physical exercise, etc. (Kerasioti et al., 2013; Kerksick et al., 2013; Varamenti et al., 2012).

In conclusion, rather than targeting ROS directly, current studies are designed to target specifically the concrete source involved in the particular disease (Juránek et al., 2010; Han et al., 2012). One of the promising inhibitory strategies aims at targeting specific isoforms of NADPH oxidases, particularly under ischaemia-reperfusion conditions (Bedard and Krause, 2007; Kahles and Brandes, 2012; Tang et al., 2012). The present review summarizes certain difficulties of using exogenous antioxidants to treat free-radical diseases. A major concern is the strategy of attenuating *toxic* effects of ROS. Yet, as antioxidant remedies are capable to affect redox homeostasis and alter particularly important physiological functions of ROS, their application should be cautious. Excess of antioxidants may in fact promote cell proliferation, inhibit immune response, alter developmental processes, affect mitochondrial function, prevent preconditioning and increasing of physical endurance due to exercising, etc. (Covarrubias et al., 2008; Gomes et al., 2012; Pallauf et al., 2013; Ristow and Zarse, 2010). Finally, if ROS are involved in cell signaling and likely in healing of tissue injury, treatment of free-radical diseases with antioxidant remedies may not be always desirable as the net result in injury healing may be deteriorated due to antioxidant action. Therefore, having in mind beneficial pharmacological intervention, any antioxidant supplementation directed to cope with free radical diseases should be carefully considered.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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