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Review

Mitochondrial Dysfunction and Redox Imbalance as a Diagnostic Marker of "Free Radical Diseases"

EKATERINA GEORGIEVA[1], DONIKA IVANOVA[1], ZHIVKO ZHELEV[1,2], RUMIANA BAKALOVA[3,4,5], MAYA GULUBOVA[1] and ICHIO AOKI[3]

[1]*Medical Faculty, Trakia University, Stara Zagora, Bulgaria;*
[2]*Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences, Sofia, Bulgaria;*
[3]*Department of Molecular Imaging and Theranostics, and*
[4]*Group of Quantum-state Controlled MRI, National Institute of Radiological Sciences (NIRS), QST, Chiba, Japan;*
[5]*Medical Faculty, Sofia University "St. Kliment Ohridski", Sofia, Bulgaria*

Abstract. *The intracellular redox balance (redox status) is a dynamic system that may change via many factors. Mitochondria are one of the most important among them. These organelles are the main intracellular source of energy. They are essential for maintaining cellular homeostasis due to regulation of many biochemical processes. The mitochondrial dynamics change during cellular activities and in some cases, can cause an overproduction of reactive oxygen species (ROS), which encourages the induction of oxidative DNA damage and up- or down-regulation of phosphatases, proliferative/anti-proliferative factors, apoptotic/anti-apoptotic factors, etc. Moreover, mitochondrial dysfunction and redox imbalance can continuously support and contribute to a wide range of pathologies, termed as "free radical diseases" (e.g., cancer, neurodegeneration, atherosclerosis, inflammation, etc.). This review article is focused on the mitochondrial dysfunction and cellular redox status as a hallmark of cell homeostasis and diagnostic marker of cancer. It is intended to broad readership – from students to specialists in the field.*

Correspondence to: Dr. Rumiana Bakalova, Department of Molecular Imaging and Theranostics, National Institute of Radiological Sciences (NIRS), QST, 4-9-1 Anagawa, Inage-ku, Chiba 263-8555, Japan. Tel: +81 8034307755, e-mail: bakalova.rumiana@qst.go.jp

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Reactive Oxygen Species, Redox Imbalance and Oxidative Stress

Maintaining the level of reactive oxygen species (ROS) in a balanced state and proper functioning of redox systems are crucial for redox status in the living cells. In the organism, the redox balance is based on the generation and elimination of ROS by endogenous and exogenous sources (1). Normal cells of healthy mammals are characterized by low steady-state levels of ROS and constant levels of intracellular reducing equivalents (2). ROS are universal products of aerobic metabolism, which can be generated during cellular respiration or as a result of specific enzymes that seem to be centrally involved in redox signalling (3, 4). The balance between generation of ROS and their neutralization by endogenous cellular defense mechanisms is crucial for maintaining normal cell homeostasis, because some types of ROS (*e.g.*, superoxide, hydrogen peroxide, nitric oxide) serve as signaling molecules (5, 6). Low/moderate levels of ROS are involved in normal biochemical pathways: (i) cellular response against infections; (ii) intercellular recognition and signal transduction; and (iii) induction of mitogenic response (7-9). Abnormal generation of ROS induces oxidative stress and "free radical pathologies" *via* damages of biological macromolecules and genotoxicity (10). The carcinogenesis is a classical example. As a result of intracellular redox imbalance, the abnormal levels of ROS may lead to cell dysfunction – inhibition of protein phosphatases and activation of protein kinases, malignant transformation and tumor development and progression (11, 12) (Figure 1). These events are accompanied by activation of transcription and translation factors, accumulation of

defective proteins, and adaptation to high levels of ROS and resistance to ROS-dependent apoptosis – a common behaviour of all cancer cells (13). Many authors indicate that the oxidative stress is a direct or indirect result of ROS-mediated damage on biological macromolecules and a major factor not only for carcinogenesis, but also for neurodegeneration, cardiovascular disease, inflammation, atherosclerosis, diabetes, aging, *etc*. (14-20).

The disruption of cellular homeostasis by activation of oncogenes, mitochondrial dysfunction and/or oxidative stress leads to an increased genomic instability and the target cells are able to adapt to the changing environment (21). As a result, we observe several events: enhancement of cell proliferation, angiogenesis, and metastatic potential, which induces cancer development (22). In turn, chronic inflammatory processes, caused by biological, chemical, and physical factors, are also associated with an increased risk of developing a variety of malignancies (Figure 2) (23).

Historical origins of the concept of redox status used to determine the ratio between the mutually convertible oxidized and reduced forms of a specific endogenous redox-pairs relative by the Nernst equation (24). In addition to determination of the redox potential of different redox-pairs, the Nernst equation can be an instrument for providing a quantitative assessment of various intracellular redox systems and to evaluate the cellular redox status (25). At a later stage, the "redox status" as a term in redox biology and medicine is widely used to describe oxidation-reduction changes, caused by free radicals and oxidative stress.

Cancer and non-cancer cells are characterized by an entirely different redox status, which is the basis of diagnostics and development of new therapeutic strategies. It is widely accepted that a moderate increase in ROS can promote cell proliferation and differentiation, but extremely excessive amounts of ROS can cause irreversible oxidative damages of biomacromolecules, apoptosis and cell death (26). Therefore, maintaining ROS homeostasis at low levels is crucial for normal cell survival, while the moderate enhancement of ROS is associated with adaptation, abnormal cancer cell growth and conservation of redox imbalance. Prolonged operation of cells at abnormal steady-state levels of ROS provokes genetic mutations, which makes them well-adapted to oxidative stress. This process is in the basis of malignant transformation. The cancer cells develop an enhanced endogenous antioxidant capacity. The cells that survive intrinsic oxidative stress mobilize a set of adaptive mechanisms, which not only activate ROS-scavenging systems to fight with the oxidative stress, but also to inhibit apoptosis. Recent evidences suggest that such adaptation contributes to malignant transformation, metastasis and resistance to anticancer drugs (26-30).

Which is the main endogenous source of ROS and how the substantial biochemical difference between normal and cancer cells and tissues could be used as a diagnostic marker and therapeutic target?

Role of Mitochondria in "Free Radical Pathologies"

Mitochondria play a central role in the regulation of cellular bioenergetics, which is responsive to changes in the environment, caused by hormones, nutrients, partial oxygen pressure, oxygen amendments and others (31, 32). They are essential for cell viability. However, mitochondrial gene mutations, which are often found in cancer cells, can impair mitochondrial energy metabolism or mitochondrial bioenergetics and biosynthesis, change the status and serve as a trigger of mitochondrial "retrograde signaling" of the nucleus (33, 34). The mitochondrial redox control is important not only for oxidative phosphorylation, ATP synthesis, calcium homeostasis, thermogenesis, apoptosis and ROS production, but it affects the redox balance of the entire cell (35). Mitochondria not only provide energy for the cell, but also participate in many other cellular functions, including calcium signalling, membrane potential regulation, heme and steroid synthesis, cell proliferation, apoptosis, *etc*. (36). Thus, mitochondria are the main source of intracellular ROS (*e.g*., superoxide and hydrogen peroxide) (Figure 3) (26, 37-41). The production of ROS in mitochondria is tightly regulated by the mitochondrial superoxide dismutase (SOD2) and glutathione-peroxidase (GPx), as well as by catalase and non-enzymatic antioxidants.

Overproduction of ROS in mitochondria and alteration of mitochondrial dynamics can promote carcinogenesis through suppression of complex I, induction of oxidative mitochondrial DNA damage, increased excessive calcium ion influx, inhibition of key phosphatases, induction of key kinases and transcription factors (42, 43). Data indicate also a key role of mitochondrial dysfunction in cardiovascular pathology and aging. Mitochondrial damage could trigger ROS overproduction, leading to detrimental structural and functional effects on the cardiovascular system (44). An increasing number of studies have demonstrated that mitochondrial oxidation of thiol-containing proteins is a major event in myocardial infarction and stroke. A basic characteristic of heart diseases, accompanied by oxidative stress, is mitochondrial dysfunction due to formation of ROS during reperfusion (45). Similar picture has been observed in aging, diabetes, atherosclerosis and neurodegeneration (46-49).

Substantial efforts were made in the understanding of the role of mitochondrial dysfunction and oxidative stress in these "free radical diseases". Different theories of aging have been proposed by many researchers, including free-radical and mitochondrial theories of aging. Currently, one of the most widely-accepted explanations for the cause of aging is the gradual accumulation of dysfunctional mitochondria and oxidative damage with age (29). This mechanism is also one of the most widely discussed in carcinogenesis.

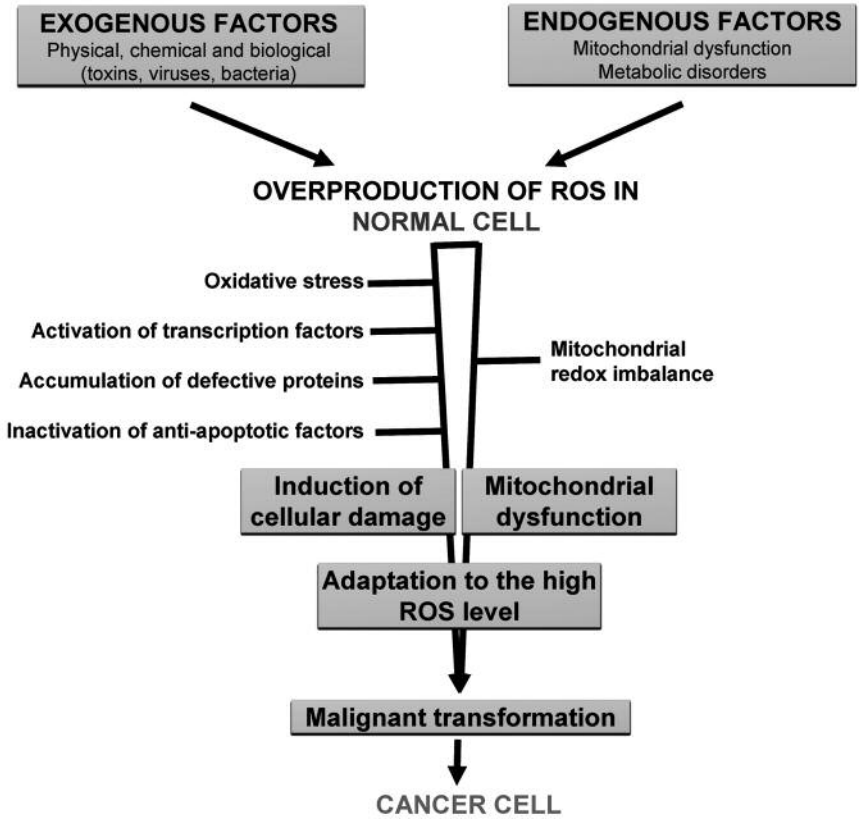

Figure 1. *ROS-dependent malignant transformation in cells – a potential mechanism.*

Models of Oxidative Stress and Mitochondrial Dysfunction in Living Cells

Many studies suggest that superoxide has been implicated in the pathogenesis, while hydro-gen peroxide has been implicated in the apoptosis (50). Because the mitochondrial ROS are essential for both processes, cell signaling regulation and ROS-induced damage, we need a more detailed understanding and developing of methods for detection of redox dynamics in living organisms.

The most popular approach is to use a specific selective mitochondrial inhibitor to block electron leakage, especially from complex-I or complex-III of the electron-transport chain, *e.g.*, antimycin A, cyanide, rotenone, myxothiazol, and oligomycin (51-59). Many researchers have used such experimental models of mitochondrial dysfunction to enhance drug-induced apoptosis in isolated mitochondria or intact cells. These studies were conducted to investigate the mitochondrial pathology and related oxidative damages (55, 60, 61). Mitochondrial in-hibitors of complex-I and complex-III cause a rapid increase in intracellular Ca^{2+}, disruption of mitochondrial potential and depolarization of the mitochondrial membrane (59). For ex-ample, cyanide as an inhibitor of complex IV binds to the cytochrome *c* oxidase heme a3-CuB binuclear center to inhibit oxygen utilization in cells and compromises the oxidative phosphorylation and ATP synthesis (62-64). As a result of cytochrome *c* oxidase inhibition, a cascade of reactions is initiated, which leads to mitochondrial electron transport inhibition and overproduction of ROS at complexes I and III (65). Rotenone, cyanide, myxothiazol and oligomycin significantly inhibit resting background K^+ by simulating the effects of hypoxia, in which leads to membrane depolarization (59).

In 2003, Pelicano *et al*. have described a basic strategy for exogenous induction of mitochondrial dysfunction in living cells, accompanied by overproduction of superoxide (Figure 4) (55). The authors have used simultaneously two specific compounds – rotenone and 2-methoxyestradiol (2-ME). Rotenone is an inhibitor of the electron flow through complex I and causes generation of superoxide. 2-ME is an inhibitor of mitochondrial SOD (Mn-SOD) and causes a further accumulation of superoxide. The combination of both leads to abnormal generation of superoxide radicals in the cells (55).

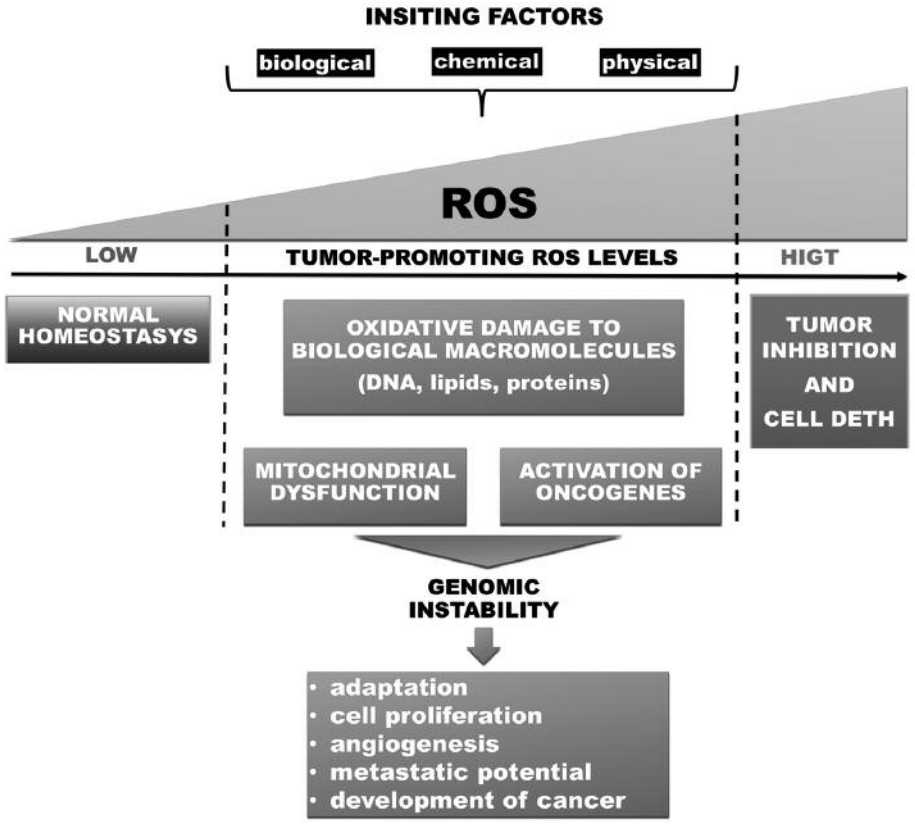

Figure 2. *Role of low and high levels of ROS on cell response – survival or cell death.*

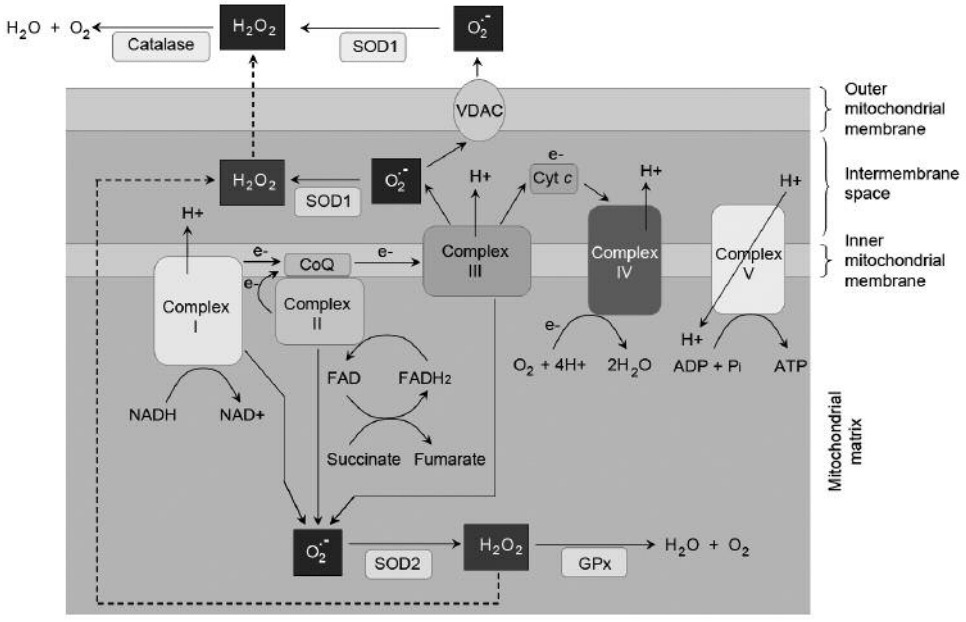

Figure 3. *Generation of ROS in the mitochondria (according to refs. 26 and 41). SOD: Superoxide dismutase; GPx: glutathione peroxidase; CoQ: coenzyme Q; Cyt c: cytochrome c; VDAC: voltage-dependent anion channel.*

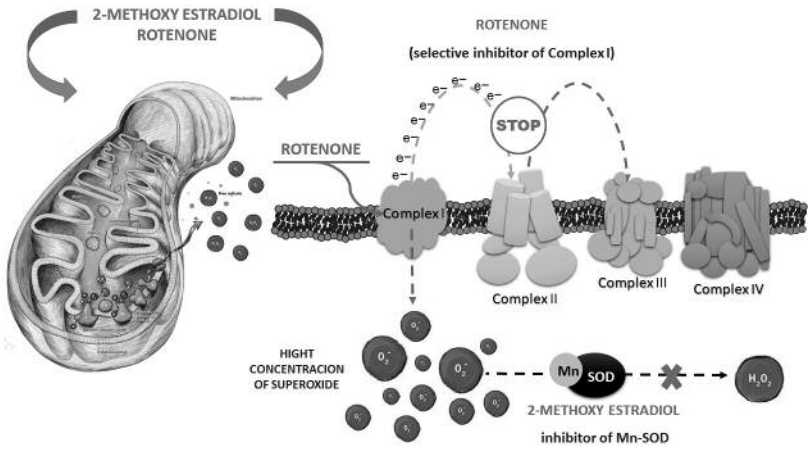

Figure 4. *Experimental model for induction of mitochondrial dysfunction, overproduction of superoxide and oxidative stress by treating cells with rotenone and 2-methoxyestradiol.*

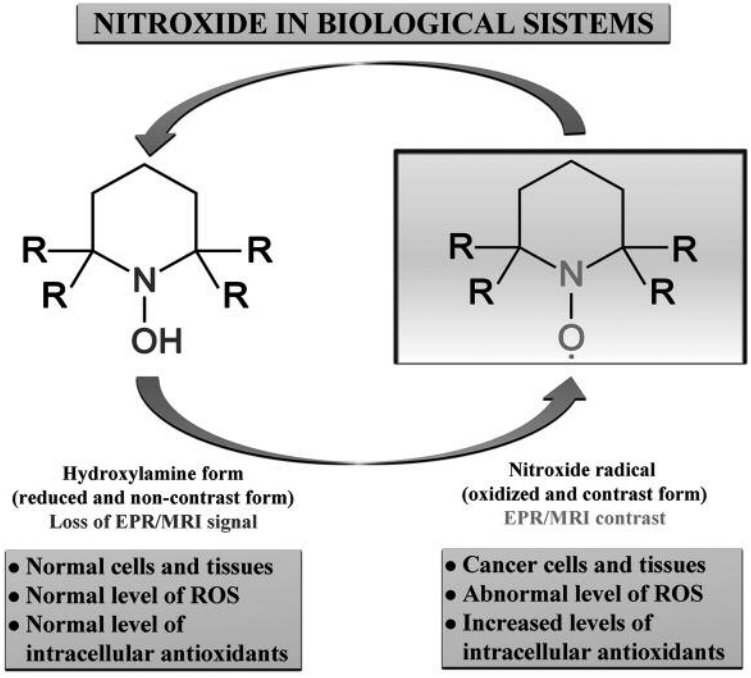

Figure 5. *Schematic presentation of the redox transformations of nitroxide radical in bio-logical systems.*

Rotenone is a naturally-derived plant compound (66). Its main effect is to increase the pro-duction of mitochondrial ROS and to decrease the mitochondrial membrane potential and cellular ATP levels (67, 68). It can prevent a transfer of electrons from the Fe-S center of the mitochondrial NADH-dehydrogenase (complex I) to ubiquinone. As a result, rotenone de-creases overall ATP production and at the same time leads to production of abnormal levels of superoxide. The low levels of ATP and increased superoxide cause oxidative stress in the cells and consequently result in cell death (69). A number of commentaries have shown that rotenone is able to induce apoptosis *via* abnormal mitochondrial ROS generation in a variety of cells. The rotenone-dependent mitochondrial ROS induce apoptosis by DNA fragmenta-tion, cytochrome *c* release, and caspase 3 activation (70). The inhibition of neuronal mito-chondrial

complex I with rotenone leads to an enhanced extracellular production of superox-ide and was primarily mediated by microglial NADPH-dependent oxidases in mice (71).

2-ME is a natural endogenous metabolite of 17β-estradiol that exerts anti-proliferative, anti-angiogenic, pro-apoptotic and transcriptional activity in various cells, including induction of cell-cycle arrest (72-76). Experiments have demonstrated that 2-ME is involved in the inhibi-tion of the polymerization of tubulin *in vitro*, thus disrupting normal microtubule function (77). Also, 2-ME leads to inhibition of mitosis at the metaphase and as a consequence – to the inhibition of cell proliferation and induction of apoptosis (78). 2-ME has been reported to have unique properties including cytotoxic, anti-proliferative and apoptotic effects in a vari-ety of malignancies (79). 2-ME can also stimulate pro-apoptotic factors and production of intracellular ROS. Another mechanism of action involves the inhibition of hypoxia-inducible factor (HIF) and interference with mitochondrial function by related compounds, which are inhibitors of complex I of the mitochondrial electron-transport chain (77). Specifically, 2-ME has been reported to inhibit the Mn-SOD, resulting in the release of cytochrome *c* from mitochondria and finally, in stimulation of caspases and generation of abnormal superoxide production (55, 80, 81). Thus, the treatment of cells with combination of rotenone and 2-ME is one of the best models for induction of mitochondrial dysfunction and oxidative stress in living cells.

Nitroxides as Redox Sensors for Detection of Mitochondrial Dysfunction and Oxidative Stress in Cells and Tissues

The cyclic nitroxides, also known as aminoxyls, are stable free radicals that have unique chemical and biochemical properties. They are widely used as probes for measurement of oxygen, glutathione, pH change and redox status in living cells and tissues (82, 83). Nitroxide radicals are organic compounds containing an aminoxyl group (N-O$^{\bullet}$) and have been used for many years as biophysical tools, due to the ability to interact with free radicals (Figure 5). They are stabilized by methyl-groups at the α-position in five-membered pyrrolidine and six-membered piperidine ring structures. Methyl-groups could be substituted with other groups (-R) on the ring. This produces a diverse range of compounds allowing modulation of specific properties (*e.g.*, hydrophobicity, intracellular delivery, delivery across blood-brain barrier, *etc.*) and stability to reduction. For example, TEMPO derivatives with different substitutes at 4-position in the ring could influence the ROS scavenging activity and time for interaction with superoxide (84).

Many researchers have reported that stable nitroxide radicals are appropriate redox sensors for ROS imaging and undergo bioreduction to hydroxylamine derivatives, which can react with superoxide (85-87). For example, nitroxides as mito-TEMPO and mito-carboxy-PROXYL are mitochondria-targeted derivatives of TEMPOL and carboxy-PROXYL, which currently are widely used as redox-sensors for detection of abnormal superoxide generation in living cells and tissues (2, 28, 37, 88-91).

During the past 15-20 years, most studies have focused towards the biochemical interactions, biologically-relevant effects, and diagnostic and therapeutic applications of nitroxides. These studies generally describe their ability to degrade superoxide and hydroperoxides, to inhibit Fenton reactions and to undergo radical-radical recombination. Furthermore, they can alter the tissue redox status and to change the metabolic processes (92).

In vitro studies have demonstrated that various reducers or oxidizers may convert the contrast form of nitroxide radical to non-contrast hydroxylamine, which depends on physiological conditions (2, 28, 91, 93). Figure 5 shows the transformations between the oxidation radical state of nitroxide, hydroxylamine, and oxoammonium states. The radical and oxoammonium forms act as an efficient redox-pair *via* reversible one-electron redox reactions, while hydroxylamine and nitroxide radical forms do not constitute an effective redox-pair. This feature determines nitroxide radicals as perfect compounds for imaging of intracellular redox balance (92, 94). This redox cycle can be detected, using magnetic resonance imaging techniques as electron-paramagnetic resonance (EPR) spectroscopy and imaging (EPRI), as well as magnetic resonance imaging (MRI).

In the living systems, under physiological conditions, nitroxide radical (which possesses EPR and MRI contrast) undergos one-electron reduction to hydroxylamine (which is non-contrast form). Those reactions are reversible (93). The balance between oxidized and re-duced forms depends on the environment and especially from the oxygen availability, super-oxide production and status of the endogenous redox-pairs (*e.g.*, NADH/NAD^{+}, NADPH/NADP^{+}, ascorbate/dehydroascorbate, *etc.*), which leads to reduction of nitroxide radical or oxidation of hydroxylamine. Thus, the ratio between radical and hydroxylamine forms directly depends on the redox status of the cells and tissues. Since only the radical form is characterized by EPR/MRI contrast, this could be used as a quantitative marker for the as-sessment of the redox status in biological objects *in vitro* and *in vivo* (91). Many excellent review articles have described this possibility (92, 95-98).

In conclusion, many researchers are focused on the differences between the redox status of normal and cancer cells and a variety of other pathologies, accompanied by high levels of ROS and disturbance of the intracellular redox homeostasis. Mitochondria are considered the main sources of intracellular ROS and as such they are essential for the functioning of normal and cancer cells. Recently, increasing studies are directed to detection of redox status of cells, tissues

and body fluids. Development of various methodologies, including EPR spectroscopy and MRI in combination with nitroxide radicals as redox-responsive contrast substances, can improve the diagnosis of many diseases, characterized by mitochondrial dysfunction and re-dox imbalance.

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