REVIEW ARTICLE

"Mild" mitochondrial uncoupling as potentially effective intervention to slow aging

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Abstract
The main objective of this review is to elucidate the role of endogenous reactive oxygen species (primarily mitochondrial origin) in the aging process. We have attempted to highlight the findings from several investigations about the relationship between reduction in mitochondrial production of free radicals (using specific antioxidants or "mild" mitochondrial uncoupling) and life span. Several studies on animal models have shown that aging rates and life expectancy could be modified using mitochondria-targeted antioxidants and uncouplers. In particular, different uncoupling strategies were able to extend life span in models ranging from yeast to mammals. These findings are in line with the "uncoupling to survive" hypothesis, suggesting that uncoupling could be an approach to promote life span extension due to its ability to prevent the formation of reactive oxygen species. Obviously, because of the high toxicity, 2,4-dinitrophenol and other uncouplers themselves can not be applied in practical geriatrics, but their low toxic analogues having controlled and "soft" action either agonists affecting the natural way of uncoupling (uncoupling proteins, UCPs) are promising for the development of means for control of tissue redox state and animal life span.

INTRODUCTION

Great current interest in the aging process, both in scientific and public settings, has been stimulated by a number of factors. First of all, advances in medicine and public health have essentially increased average life expectancy over the past 200 years. According to the data from the National Institute of Aging (NIA), United Nations and Statistical Office of the European Communities [1-3] there are several trends in global aging:

-a considerable aging of the population is now occurring in economically developed countries, *i.e.*, the fraction of elderly persons has increased especially during the last quarter of the XX century;

-the number of oldest old is rising. People aged 85 and over are now the fastest growing portion of many national populations. Average human life expectancy has progressively increased largely due to the improvements in nutrition, vaccination, antimicrobial agents and effective treatment/prevention of cardiovascular disease, cancer, *etc*;

-all these trends have serious economic consequences, since population aging will have dramatic effects on social entitlement programs, labor supply, trade and savings around the globe.

Altogether, this global demographic change represents one of the main social, scientific and economic challenges of our time. On the one hand this indicates essential achievements of mankind in health care and solution of social problems, but on the other hand it is associated with many problems which require fundamental understanding of aging mechanisms and novel therapeutic developments. An enormous effort has been expended to understand how the aging process is regulated at the molecular and cellular levels, but unfortunately the final aging mechanisms are not established.

MECHANISMS OF AGING

As known, modern understanding of the mechanisms of aging are based on two most important aspects of the aging process: ***(1)*** aging is characterized as a progressive decline in biological functions with time, and ***(2)*** aging results in a decreased resistance to multiple forms of stress, as well as an increased susceptibility to numerous diseases [2].

But despite well-described definition, aging remains one of the most poorly understood phenomena, due in large part to its inherent complex and integrative nature, as well as the difficulty in dissociating the effects of normal aging from those manifested as a consequence of age-associated disease conditions [2]. As a result, it is proposed a large number of theories that attempt to explain why we age, and no single theory has been completely successful in explaining the aging process [4, 5].

For example, more than a century ago, Max Rubner coupled the observation that maximal life span of mammalia increases with body size and the mass-specific rate of metabolism of mammalia decreases with body size. Later, this perspective was elaborated and extended by Raymond Pearl, who proposed that life span differences of animals maintained at different temperatures could be explained by differences in metabolic rate and gave the "rate-of-living theory of aging" its name [6].

But one of the prevalent theories in the current literature revolves around free radicals, as causal agents in the process of aging. "Free radical theory", formulated by Harman [7] in 1950s is one of the leading hypotheses because it rather satisfactorily explains many observations of modern gerontology. It is established that generation of reactive oxygen species (ROS), which are molecules containing unpaired, highly reactive electrons, contributes to the accumulation of oxidative damage to cellular constituents (nucleic acids, proteins and lipids, among others), resulting in the process of "aging".

Thus, a more modern version of this tenet is the "oxidative stress theory" of aging. The term "oxidative stress", popularized by Helmut Sies [8], was defined as "a disturbance in the pro-oxidant/ antioxidant balance in favor of the former, leading to potential damage". In the context of the aging process, oxidative stress was interpreted to connote a cellular state in which the antioxidant defenses are insufficient for the complete eradication of various ROS, thereby resulting in the age-dependent accrual of macromolecular structural damage [9].

Nevertheless, there are relatively few substantive differences between the basic propositions of the original "free radical" and the later "oxidative stress" hypotheses. Both postulate that the rate of aging, i.e., the progression of age related deleterious alterations, is a function of the imbalance between ROS fluxes and antioxidant defenses and both predict that the narrowing of this gap should reduce the amount of structural damage and prolong the life span. Therefore, from this historical perspective, the postulated mechanism implicating ROS in the aging process according to Sohal and Orr [9] can be characterized as the "structural damage-based oxidative stress hypothesis".

So, today the variants of "oxidative stress theory" are among the most widely accepted mechanistic explanations of aging and life span, and there is much evidence to support it.

OXIDATIVE STRESS IN NORMAL AGING AND EFFECTS OF ANTIOXIDANT MANIPULATION OR CALORIE RESTRICTION ON LONGEVITY

One of the central themes of the oxidative stress hypothesis is that ROS are the primary causal underlying aging associated declines in physiological functions. Several sets of direct and indirect evidences generated over the past two decades have demonstrated a strong correlation between aging and an increase in oxidative damage to tissues in species ranging from *Caenorhabditis elegans* to humans [10-12]. But although numerous studies have demonstrated a correlation between *in vivo* oxidative damage and aging, a more insightful test of the oxidative stress theory would be to assess the direct effects of antioxidants on aging processes.

Antioxidants

Over the past few decades, research in natural dietary compounds with use of various animal models provides a new strategy for anti-aging. Natural dietary compounds act through a variety of mechanisms to extend life span and prevent age-related diseases [13].

For example, a polyphenolic antioxidant resveratrol can reduce oxidative damage and cognitive impairment in senescence-accelerated mouse [14]. Chondrogianni *et al* [15] have identified quercetin and its derivative, namely quercetin caprylate, as a proteasome activator with antioxidant properties that consequently influence cellular life span, survival and viability of primary human fibroblasts (HFL-1, human fetal lung fibroblasts). Grape intakes, especially grape pomace with the highest content of flavonoids, beta-carotene, tocopherols and dietary fiber, showed the effective capacity of inhibiting age-related increase of lipid peroxidation and DNA damage [16].

These and many other results suggest that the pharmacological action of natural antioxidants may offer a perspective therapeutic strategy for the treatment of age-related conditions. And now many people consume antioxidants (synthetic or natural) in order to decrease oxidative stress, to modulate the aging process, and to extend the health-span. For example, the data from the Food and Drug Administration (FDA) of the United States suggest that more than 29,000 different nutritional supplements (many of which are antioxidants) are available to consumers. It also appears that in the USA more than 12 billion US$ per year may be spent on supplements, and supplements represent a market of over 30 billion $ worldwide [17, 18].

Calorie restricted diets

Probably one of the most important arguments in favor of the free radical concept of aging are the

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results obtained from keeping animals on a calorie restricted diets (CR). CR is the only non-genetic treatment that has been shown clearly to increase maximal life span in most, if not all, species where it has been applied [19]. A growing body of evidence supports the hypothesis that CR, with no malnutrition and an adequate mineral intake, also works by decreasing oxidative stress [10, 20].

In particular, Zheng *et al*'s investigation [21] and our own results [22], obtained in the study with *Drosophila*, support the universal character of the CR geroprotective action on organisms of different levels of organization and show that CR in flies, as in mammalia, slows the accumulation of oxidatively damaged macromolecules.

Thus, a large body of evidences supports the notion that ROS are produced in cells and can manifest damage, but a causal link between ROS and aging has still not been clearly established [6]. Furthermore, in addition to being potentially toxic, ROS were considered to be physiologically important regulatory molecules. Accordingly, oxygen utilization by aerobes was regarded as both vital for their survival, by virtue of its capacity to accept electrons during respiration, and potentially deleterious: a phenomenon that was referred to as the "oxygen paradox" [9].

Cellular sources of reactive oxygen species: mitochondrial theory of aging

As generally known, ROS include a number of molecular species derived from oxygen that are relatively reactive, but biologically most of them are derived from either superoxide ($O_2^{\bullet -}$) and/or hydrogen peroxide (H_2O_2).

In mammalian cells, a number of endogenous sources of ROS are known including mitochondria (mainly complex I & III, but also monoamino oxidase and α-ketoglutarate dehydrogenase), endoplasmic reticulum (mainly cytochrome P-450 and b_5 enzymes), peroxisomes (mainly fatty acid oxidation and D-amino acid oxidase), cytosol (NO synthases and lipoxygenases), plasma membrane (NADPH oxidases, lipoxygenases), and extracellular space (xanthine oxidase) [23].

Mitochondrial energy metabolism is recognized today as one of the most quantitatively important source of ROS in the eukaryotes. Hydrogen peroxide production from mitochondria has been first recorded more than 40 years ago [24]. In these pioneering works it was recognized that H_2O_2 production in mitochondria accounts for up to 2% of the total oxygen consumption and the rate of mitochondrial H_2O_2 production is strongly dependent on the metabolic state: it is high in state 4, when the NADH/NAD^+ and ubiquinol/ubiquinone pools are largely reduced; and it is low in state 3, when the steady-state concentrations of the potential oxygen reductants are decreased [24, 25].

However, the other point of view, according to which the frequent assertion that 1-4% of mitochondrial O_2 consumption is diverted to ROS *in vivo* is wrong. It is based on the maximum rate of superoxide production from complex III in mitochondria in the presence of antimycin, at saturating substrate and O_2. Less unrealistic conditions predict lower values, only 0.15%, and which of the specific sites of mitochondrial ROS production is the most active in cells in the absence of inhibitors of the electron transport chain is probably unclear [26].

Generally, little is known about the regulation of mitochondrial function *in vivo*; thus it is unknown how much superoxide is produced by mitochondria *in vivo*. But it would still be considered, that under certain physiologically relevant conditions, mitochondria are thought to contribute significantly to the steady-state level of H_2O_2 in cells [27].

As it is well known, the mitochondrial electron transport chain (ETC) links the transfer of electrons from reduced cofactors to the ultimate electron acceptor of the ETC (*i.e.*, oxygen) with the simultaneous transport of protons from the mitochondrial matrix across the inner mitochondrial membrane and into the intermembrane space. Movement of protons out of the matrix and into the intermembrane space results in an electrochemical potential (ΔΨ) which can then be used to drive protons back into the mitochondrial matrix through the ATP synthase (complex V), facilitating the synthesis of adenosine-5′-triphosphate [12, 28]. However, at various sites within the ETC, electrons may occasionally leak to oxygen, forming $O_2^{\bullet -}$ via single electron reduction [29].

About half of the total ROS ($O_2^{\bullet -}$ and H_2O_2) produced by heart mitochondria at high NADH/NAD^+ ratio can be accounted for as being generated by the respiratory chain components (complexes I and III) [27]. Inhibitory analysis suggests that the major part of superoxide produced by the respiratory chain (up to 70%) is generated by complex I [30].

The reductant of oxygen to produce $O_2^{\bullet -}$ in complex I is not known and published reports are highly conflicting [31]. However, recent studies of Russian scientists [32] have shown that complex I bears at least two NADH (NAD^+) specific sites. One site (F) has high affinity to NADH and low affinity to NAD^+, serves as the entry point for NADH oxidation by ubiquinone and coupled translocation of 4 H^+ per molecule of NADH oxidized, and the other site (R) is specifically involved in univalent reduction of oxygen. The presence of the specific site R

participating in the $\Delta\mu_{H+}$-dependent ubiquinol:NAD^+ reductase reaction (reverse electron transfer) and in $O_2\bullet^-$ generation by complex I raises the question of the physiological significance of these reactions. According to Vinogradov and Grivennikova [32] of the above mentioned study, succinate, the most widely used substrate, is not the only and, most likely, not the major electron donor at ubiquinone level. Each first dehydrogenation in the fatty acid β-oxidation cycle, oxidation of α-glycerophosphate, choline, sarcosine, and dihydroorotic acid (in eukaryotes) is coupled with direct ubiquinone reduction, and site R may participate in "universalization" of the reducing equivalents in the form of NADH via reverse electron transfer.

But in general, despite the large amount of scientific results, there is still much uncertainty as to the mechanisms and physiological role of generation of ROS in the mitochondrial membranes. As previously stated in this review the proportion of oxygen consumed that emerges as ROS is highest under non-phosphorylating conditions rather than phosphorylating ones, most determinations of mitochondrial ROS production are made under state 4 conditions. This is combined with the fact that in intact cells, mitochondria normally respire in metabolic states that are intermediate between state 3 and state 4. Consequently, more studies of ROS generation are needed to elucidate the sites and rates of ROS production *in vivo* [31].

In addition, according to the report of Vinogradov and Grivennikova [32] physiological significance of $O_2\bullet^-$ generation by the respiratory chain (mainly by complex I) seems to be of minor importance, at least under normal mitochondrial functioning. The rate of $O_2\bullet^-$ formation is quite low and combined operation of antioxidant enzymes in matrix is expected to diminish or neglect completely the potential ability of the respiratory chain to create a significant amount of $O_2\bullet^-$. This, by no mean, makes $O_2\bullet^-$ production by mitochondria unimportant [32]. But this process may become of great importance under some extreme conditions (such as reoxygenation after anoxia), and the relative "leakage" of the respiratory chain for univalent oxygen reduction may be tissue specific; therefore comparative quantitative analysis of mitochondria from different tissues and species would be the most interesting [32].

At present, the analysis of relative contribution of different enzymes into overall superoxide and hydrogen peroxide production by mitochondria remains strongly dependent on reliability of the methods for quantitative determination of these analytically "difficult" compounds [32]. According to Brand [26], there are two major problems hindering identification of the specific mitochondrial sites of ROS production in cells. The first one is the methods to quantify ROS production; fluorescent probes such as dichlorodihydrofluorescein or dihydrorhodamine have interpretational problems: their specificity is often unclear and they may themselves cause ROS production. The second problem is the use of electron transport inhibitors to identify sites of ROS production, which poses the same problems as with isolated mitochondria.

Overall, despite the lack of consensus in the interpretation of results obtained in different laboratories, probably it can be considered that mitochondria are still attracting the attention of researchers as an important intracellular source of ROS, which are known to be important determinants in cell function, participating in many signaling networks and also in a variety of degenerative processes [33].

For example, mitochondrial ROS are considered to be totally or partially responsible for several diseases including Alzheimer's disease, Parkinson's disease and several human cancers [34]. Furthermore the "mitochondrial free radical theory of aging" (MFRTA) hypothesizes that mitochondria are the critical component in control of aging. It is proposed that electrons leaking from the ETC produce ROS and that these molecules can then damage ETC components and mitochondrial DNA, leading to further increases in intracellular ROS levels and a decline in mitochondrial function [2].

In support of MFRTA, there are comparative data showing that the production of ROS is low in mitochondria isolated from large long-lived mammals compared to small short-lived ones, with mitochondria from mammals of intermediate life span and body mass having intermediate rates of ROS production, and ROS production rates of mitochondria from long-lived birds are lower than those from similar-sized short-lived mammals (rats and mice) [35].

Lambert *et al* [35] critically tested the hypothesis that longevity correlates with mitochondria radical production. Authors investigated this correlation by comparing rates of H_2O_2 production by heart mitochondria isolated from groups or pairs of species selected to have very different maximum life span but similar body masses (small mammals, medium-sized mammals, birds). It was shown that during succinate oxidation, H_2O_2 production rates were generally lower in the longer-lived species. Additional data were obtained from large species and the final dataset comprised mouse, rat, white-footed mouse, naked mole-rat, Damara mole-rat, guinea pig, baboon, little brown bat, Brazilian free-tailed bat, ox, pigeon and

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quail. In this dataset, maximum life span was negatively correlated with H_2O_2 production at complex I during reverse electron transport [35]. These findings indicate that enhanced longevity may be causally associated with low free radical production by mitochondria across species over two classes of vertebrate homeotherms [35]. However, further studies with greater numbers of species are still required to test the generality of the hypothesis, particularly since one exception was found: heart mitochondria from long-lived naked mole-rats produced more ROS than expected (or the naked mole-rats lived longer than expected) compared to the other species that were examined [35].

Thus, the present data from different laboratories suggest that complex I can probably play a central role in the regulation of longevity. Stefanatos and Sanz [36] propose that complex I regulates aging through at least two mechanisms: ***(1)*** ROS-dependent mechanism that leads to mitochondrial DNA damage, and ***(2)*** ROS-independent mechanism through the control of the NAD^+ to NADH ratio.

In summary, "mitochondrial free radical theory of aging" is currently mainly supported by indirect data which show a negative correlation between free radical production in isolated mitochondria and life span in several different model organisms. However, correlations can suggest but not demonstrate causality. In fact, the only definitive way to test MFRTA is to specifically decrease (or increase) mitochondrial ROS production and to study the effect of such modification on life span.

CONDITIONS MODIFYING MITOCHONDRIAL FUNCTION AND ANIMALS' SURVIVAL

It does not cause a big surprise that the selected life conditions that are able to improve survival in animals are similar to the recommendations that are commonly followed by many aging humans, *i.e.*, moderate physical exercise, antioxidant supplementation, CR and so on [12]. It is known that CR rats showed structural and functional liver mitochondrial properties (fatty acid pattern, respiratory chain activities, antioxidant level, and hydroperoxide content) similar to those of younger rats [37]. It was reported that long-term CR led to an essential decreasing in the rate of mitochondrial H_2O_2 generation and in oxidative damage to mitochondrial DNA in the rat skeletal muscle [38]. These and other results support the possibility that CR increases maximum life span at least in part through decreases in mitochondrial oxidative stress.

At the same time although mitochondria can produce ROS at complexes I and III, it was shown that CR decreases ROS production exclusively at complex I, because the decrease in oxygen radical generation occurs with pyruvate plus malate, but not with succinate plus rotenone, as substrate [20]. Furthermore, according to Barja [39], the mechanism allowing the decrease in ROS production during CR probably is not a simple decline in mitochondrial oxygen consumption because it stays unchanged. Instead, the percentage of total electron flow directed to ROS generation (the free radical leakage) is decreased with CR.

And recent data indicate that the decrease in mitochondrial ROS generation may be also due to protein restriction rather than to calorie restriction, and more specifically to dietary methionine restriction. According to Pamplona and Barja [40], lowering of methionine levels is involved in the control of mitochondrial oxidative stress and vertebrate longevity by at least two different mechanisms: ***(1)*** decreasing the sensitivity of proteins to oxidative damage, and ***(2)*** lowering of the rate of ROS generation at mitochondria level. So, at present the MFRTA probably should be considered as one of the most widely believed and supported theories of aging. But in spite of its attractiveness, MFRTA has received some recent criticism [41]. For example, there is a standing criticism by Nohl and colleagues that mitochondria *in vivo* are not an effective source of $O_2\bullet^-$ and H_2O_2 and that the determined rates are artifactual [42]. According to Brown and Borutaite [23], mitochondria are a significant source of ROS, but not the main source at least in mammalian liver.

Long-lived animals were shown to produce fewer free radicals and to have lower oxidative damage levels in their tissues. However, it does not prove that free radical generation determines life span. In fact, the longest-living rodent, naked mole rat *Heterocephalus glaber*, produces high levels of free radicals and has significant oxidative damage levels in proteins, lipids and DNA [35, 43]. So in summary, available data concerning the role of free radicals in longevity control are sometimes contradictory and do not always prove MFRTA. In fact, the only way to test this theory is by specifically decreasing mitochondrial free radical production without altering other physiological parameters. If MFRTA is true animals producing fewer mitochondrial ROS must have the ability to live much longer than their experimental controls.

Some alternatives will be discussed which might be effective in decreasing mitochondrial oxidative stress. It seems more promising to reduce the free radical formation in mitochondrion than trying to neutralize free radicals after they have been produced. Recent evidences discussed below show that such possibilities exist.

PROBABLE WAYS TO TEST THE MITOCHONDRIAL FREE RADICAL THEORY OF AGING BY SPECIFICALLY DECREASING FREE RADICAL PRODUCTION

Over-expression of mitochondrial antioxidant enzymes

One approach to protect mitochondria from oxidative damage is targeting antioxidant compounds, including low molecular weight antioxidants and antioxidant enzymes, specifically to mitochondria. In support of this strategy, mitochondrially-targeted catalase mitigates multiple age-related pathologies, including cardiac tissue pathology, hearing loss, and comorbidity factors including tumor burden [44].

In contrast to these mitochondria-targeted catalase mice, over-expression of the same enzyme in either the nucleus or peroxisomes did not result in significant life span effects, and in other experiments, over-expression of the mitochondrial matrix MnSOD decreased ROS without a corresponding life span increase [28, 45]. In accordance with the study of Jang *et al* [45], Perez *et al* [46] collected life span data from multiple studies using mice either under- or over-expressing different antioxidant system components. Of these, only SOD1-/- mice had a significantly shorter life span. However, these mice also displayed levels of oxidative damage 4-5 fold higher than in aged wild type mice, and had a high incidence of hepatocellular carcinoma, suggesting that their life span deficit may not represent normal mechanisms of aging [44].

According to Mookerjee *et al* [44] two major considerations emerge from these studies: in addition to the amount of ROS, both the species of ROS and the subcellular location of antioxidant activity, and not simply ROS levels, can strongly affect whether increased ROS production correlates with decreased life span. At the same time the mitochondria-targeted catalase mice probably may be considered proof-of-principle for the potentially significant, multi-factorial efficacy of targeted antioxidant interventions in the context of age-dependent diseases and aging [28].

Mitochondria-targeted antioxidants

Penetrating cations

It is known that a well-established approach to targeting small molecules to mitochondria utilizes the negative charge of the mitochondrial matrix against to the cytoplasm to facilitate accumulation of positively charged molecules (cations) in the mitochondrial matrix [47]. However, small charged molecules are typically hydrophilic in nature and moving them from the aqueous extracellular or cytosolic compartment into the hydrophobic interior of cell or mitochondrial membranes is energetically unfavorable. Only if a cation is combined with suitably hydrophobic groups the overall molecule become sufficiently hydrophobic to allow entry into the hydrophobic interior of biological membranes. Such lipophilic or "penetrating" cations are then able to cross through the hydrophobic interior of the phospholipid bilayer of biological membranes. As a result, lipophilic cations will accumulate inside mitochondria [47, 48].

While a wide range of lipophilic cations could in principle be utilized for drug targeting purposes (for example commonly used fluorescent dyes such as rhodamine 123), to date only one such compound has been extensively characterized in this context. Triphenylphosphonium (TPP) consists of a positively charged phosphorus atom surrounded by three hydrophobic phenyl groups, giving it an extended hydrophobic surface despite the positive charge of the phosphorus atom. TPP and TPP-conjugated compounds have been reported to firstly accumulate 5-10 fold inside cells relative to the extracellular space, and then to further accumulate up to 1,000 fold inside energized mitochondria both *in vitro* and *in vivo* [48, 49].

A range of antioxidant moieties has been conjugated to TPP for targeting to mitochondria. Using aliphatic linkers of various lengths between TPP and the antioxidant moiety, the degree of hydrophobicity of such compounds can be modified, and their efficacies have been explored both *in vitro* and *in vivo* [48, 49].

Mito-compounds

As it is known**,** one of the first proof-of-principle compounds for the feasibility of utilizing the TPP moiety to target antioxidants to mitochondria was mitochondria-targeted α-tocopherol, called MitoVit E: [2-(3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-yl)ethyl]triphenylphosphoniumbromide [43]. While *in vivo* data show that MitoVit E can be chronically administered to animals in drinking water, has good bioavailability and limited toxicity [50], it has not been investigated for its therapeutic potential in humans.

To date, most intervention studies have focused on TPP conjugated to ubiquinone via a 10-carbon aliphatic carbon chain, namely ubiquinonyl-decyl-triphenylphosphonium or MitoQ. Ubiquinol was chosen as the antioxidant moiety because of its ability to inhibit lipid peroxidation and because it can be regenerated by the mitochondrial ETC following oxidation [50].

Multiple lines of evidences indicate that most MitoQ and related TPP compounds found in tissue indeed localize to mitochondria [48, 50]. But were reported *in vitro* evidence that MitoQ, at high enough concentration, can undergo redox cycling and act as pro-oxidant [48, 51]. At the same time, some authors

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have also reported no evidence that such pro-oxidant effects were observed *in vivo*, possibly because MitoQ under physiological conditions was not able to interact sufficiently with the flavin site of complex I to cause such pro-oxidant effects [28].

Nevertheless, MitoQ's potential as a modulator of aging has been examined in *Drosophila melanogaster*. In life span experiments using wild type *Drosophila* maintained under normal culture conditions, MitoQ was reported to have no [52] or at best a marginal positive effect [48] on life span. When administered to a short-lived mutant strain, deficient in the mitochondrial form of the antioxidant enzyme superoxide dismutase, MitoQ significantly extended life span in adult female but not male flies [52].

While MitoQ does not seem to modulate aging rate, at least in those model organisms that have been investigated (flies, nematodes) [52, 53], it might be suitable as an intervention into age-dependent conditions that are associated with increased oxidative stress and elevated mitochondrial damage, but an obstacle to the widespread use of this drug may be a "slight difference" between anti- and pro-oxidant doses of the MitoQ.

Mitochondria-targeted plastoquinone (SkQ)

Probably, the most active research groups in mitochondria-targeted antioxidants investigation are from Russia and some other countries. They are headed by Vladimir Skulachev, who developed an alternative series of mitochondria-targeted antioxidant compounds based on the hydrophobic cation targeting approach. Skulachev *et al* [48] used in their research plastoquinone, a quinone in the electron transfer chain of chloroplasts, in place of the ubiquinone antioxidant moiety of MitoQ, reasoning that plastoquinone might provide better antioxidant activity.

Several related "Sk" compounds were synthesized, exploring a range of plastoquinone derivatives, as well as ubiquinol as antioxidant moieties, and also including some alternative lipophilic targeting cations [48]. Using planar bilayer phospholipid membrane, autors [54, 55] selected SkQ derivatives with the highest permeability, namely plastoquinonyl-decyl-triphenylphosphonium (SkQ1), plastoquinonyl-decyl-rhodamine 19 (SkQR1), and methylplastoquinonyl-decyl-triphenylphosphonium (SkQ3).

Anti- and pro-oxidant properties of these substances and also of MitoQ were tested in aqueous solution, detergent micelles, liposomes, isolated mitochondria, and cell cultures [54]. In mitochondria, cationic quinone derivatives in micromolar concentrations were found to be pro-oxidant, but at lower (sub-micromolar) concentrations they displayed antioxidant activity decreasing in the series SkQ1 = SkQR1 > SkQ3 >MitoQ. SkQ1 was reduced by mitochondrial respiratory chain, *i.e.* it is a rechargeable antioxidant. Essentially than in cell cultures, SkQR1, a fluorescent SkQ derivative, stained only one type of organelles, namely mitochondria [54].

So, the most important feature of the cationic derivatives of antioxidant plastoquinones is the extremely low concentrations required for their effects to be seen: in experiments on cells this is the range of 10^{-12} to 10^{-9} M; in the case of treatment of senile ophthalmic diseases, one drop of $2.5 \cdot 10^{-7}$ M solution daily; in therapy of heart arrhythmia, $1 \cdot 10^{-10}$ mol/kg per day; in experiments on life span elongation, $5 \cdot 10^{-10}$ to $5 \cdot 10^{-9}$ mol/kg per day [56].

Another factor providing the high efficiency of SkQ1 is its ability to recover from its oxidized form to the initial reduced (working) form. All the above-said allows authors [56] to conclude that SkQ1 is an artificial rechargeable antioxidant addressed to the inner mitochondrial membrane.

The greatest interest for the purposes of this review is the evidence that in the fungus *Podospora anserina*, the crustacean *Ceriodaphnia affinis*, fly *Drosophila* and mice, SkQ1 prolonged life span, being especially effective at early and middle stages of aging [54]. In particular, addition of SkQ1 to *Drosophila melanogaster* growing medium has been reported to extend life span in virgin [53] but not in mated flies [57]. SkQ1 exerted its protective effect in virgin flies predominantly by protecting flies from early death and the observed life span benefit was small (~15%) yet significant [53].

Using long-term monitoring of SkQ1 effects on the *Drosophila* life span, Krementsova *et al* [58] analyzed different integral parameters of *Drosophila* survival and mortality under SkQ1 treatment. Adding SkQ1 to fly food was associated with a reduction in early mortality and a decrease in random variation in life span. Analysis of the Gompertz function parametric plane demonstrated significant differences between points corresponding to experimental and control cohorts. These findings indicated that the SkQ1 effect on life span was associated with both elevation of life quality and slowing of aging. Furthermore, according to Krementsova *et al* [58], the results presented in their work show that the nature of the SkQ1 effect on *Drosophila* longevity was constant for six years, regardless of fluctuations in the control life span, differences in preparation and administration of SkQ1 solution, or the year and season when the experiments were conducted.

What is more, SkQ1 affected the life span of not only inbred and outbred laboratory mice but also wild rodents housed in outdoor cages or kept under conditions that simulated natural seasonal changes in

temperature and illumination, the dwarf hamster *Phodopus campbelli* and the mole vole *Ellobius talpinus* [59].

Taking the data together, the authors concluded that SkQ1 increased the survival of young flies and primarily improved the quality of life. The same tendency of predominant early-in-life SkQ1 effect was observed for other animals, for example, mice. SkQ1 prolonged the median life span of dwarf hamsters and mole-voles, and the effect being especially great in the case of mole-voles treated with SkQ1 from an early age [59].

There are some reports suggesting that SkQ1 may reduce baseline levels of oxidative damage in young, healthy animals. For instance, protein carbonyls in muscle tissue have been reported to be reduced by SkQ1 treatment in healthy wild type rats [60].

It was shown that SkQ1 not only prevented age-associated hormonal alterations but partially reversed them [61]. In mammals, the effect of SkQs on aging was accompanied by inhibition of development of such age-related diseases and traits as cataract, retinopathy, glaucoma, balding, canities, osteoporosis, involution of the thymus, hypothermia, torpor, peroxidation of lipids and proteins, *etc* [54]. Thus, the involvement of mitochondrial ROS in senescence was confirmed in Skulachev group's experiments showing that cationic derivatives of antioxidant SkQs selectively accumulated in mitochondria, lengthen the life span of a wide circle of eukaryotes (from fungi to mammals), and retard the development of typical symptoms of senescence and senile diseases [48, 56]. So, SkQs look promising as potential tools for treatment of senescence and age-related diseases [48].

In this context, it is worth remembering the fact that TPP compounds can facilitate mitochondrial uncoupling [55], an intervention that has itself been explored as a possible life span modulator.

"Mild" mitochondrial uncoupling modulators

In practice, the two approaches are possible to reduce the generation of ROS in the mitochondria. One of these is the use of highly specific antioxidants which would work directly in the mitochondria (*e.g.*, penetrating SkQ1 cation or antioxidants such MitoQ [48]) as mentioned above. However, an alternative approach would be to modulate mitochondrial function with the aim of producing less ROS while maintaining adequate ATP production.

As noted earlier in this review, mitochondria are believed to be one of the major sources of ROS, although 98% of oxygen consumed by mitochondria is converted into water and only 2% produce ROS during side chemical reactions in chain of respiratory enzymes [24, 25].

According to Skulachev [62] this "parasitic" chemical reaction appears to be inevitable since the initial and middle steps of the respiratory chain contain very reactive electron carriers of negative redox potential that are chemically competent in the one electron reduction of oxygen. The carriers in question are short-lived under conditions of active respiration but seem to become long-lived in the resting state when the respiration rate is limited by ADP availability. As a result of these relationships, the rate of the $O_2\bullet^-$ production by mitochondria increases when ADP is exhausted. And in 1994 Skulachev suggested that mitochondria possess a special mechanism called "mild" uncoupling, which prevents a strong increase in $\Delta\mu_{H+}$, and hence, in $O_2\bullet^-$ formation under resting conditions [62, 63].

Later Skulachev's group showed that 15% decrease in membrane potential $\Delta\Psi$ under resting conditions caused by an uncoupler, a respiratory inhibitor or the oxidative phosphorylation substrates ($ADP + P_i$) results in 10 fold decrease in the H_2O_2 production by heart mitochondria [64]. In addition, for undefined reasons linked to the proton-pumping mechanism of complex I, ROS generation is more dependent on changes in the transmembrane pH gradient (ΔpH) than on the membrane potential ($\Delta\Psi$) [65]. As mild uncoupling decreases proton motive force (Δp) by lowering both ΔpH and $\Delta\Psi$, it is an effective means to lower mitochondrial superoxide production at the cost of efficient ATP synthesis [29].

Thus, "mild" mitochondrial uncoupling probably can be regarded as the reduction of the efficiency of energy conversion without compromising intracellular high energy phosphate levels [66]. Uncoupling also increases respiration, which decreases the local concentration of O_2 and therefore decreases the rate of ROS production [67]. Based on these observations, it has been suggested that a "soft" uncoupling should be a mechanism preventing excessive production of ROS [68] and as a result (in the frames of the free radical aging theory) should prevent shortening of the life span.

This theoretical postulate argues in favor of the fact that mild uncoupling will decrease ROS production and thereby extend life span even if it increases the "rate of living". To test this, Speakman *et al* [69] separated mice into quartiles of metabolic intensity and then investigated their longevity. They found that mice in the highest quartile lived longer than those in the lowest. In another study, a tightly-coupled human first dorsal interosseus muscle showed greater deterioration with age than a relatively uncoupled tibialis anterior one [70].

Also, in a comparison of strains of *Caenorhabditis elegans* carrying life span extending mutations, a

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lower mitochondrial membrane potential was associated with increased worm life span and the effect could be replicated using a chemical uncoupler carbonylcyanide-3-chlorophenylhydrazone (CCCP) [71]. These results are in line with a series of findings on an increase of longevity by mild uncoupling as well they are contradicting to others [72].

For example, although some experiments nicely illustrate the ΔΨ dependence of mitochondrial superoxide production [31], there are authors who argue that the conditions used to demonstrate this effect may not reflect conditions occurring *in vivo*. A recent study [73] has challenged the hypothesis that mild uncoupling could be neuroprotective by decreasing oxidative stress and found no significant change in the level of matrix superoxide in cultured rat cerebellar granuler neurons on addition of low protonophore (FCCP) concentrations.

According to Shabalina and Nedergaard [74] it is only ROS production from succinate under reverse electron-flow conditions that is sensitive to membrane potential fluctuations, and so, only this type of ROS production could be affected in uncoupling investigation; however, the conditions under which succinate-supported ROS production is observed include succinate concentrations that are supraphysiological. Any decrease in membrane potential, even 'mild uncoupling', must necessarily lead to large increases in respiration, i.e. it must be markedly thermogenic. Mitochondria within cells are normally ATP-producing and thus already have a diminished membrane potential, and treatment of cells, organs or animals with small amounts of artificial uncoupler does not seem to have beneficial effects that are explainable via reduced ROS production. Although it has been suggested that members of the uncoupling protein family (UCPs) may mediate a mild uncoupling, authors evidence does not unequivocally support such an effect, *e.g.* the absence of the truly uncoupling protein UCP1 is not associated with increased oxidative damage. Generally, the authors conclude that present evidence does not support mild uncoupling as a physiologically relevant alleviator of oxidative damage [74]. So, further experimental data are required to verify the hypothesis that a physiological uncoupling acts as a protective mechanism against oxidative stress.

In general, it should be noted that although there is no single point of view on the problem, the possibility that "mild" uncoupling could be protective against oxidative damage by diminishing ROS production has attracted much interest during the last decade. And according to Cunha *et al* [66] mild mitochondrial uncoupling by activation of mitochondrial uncoupling pathways may therefore represent a plausible mitochondrial-targeted strategy to modulate ROS production. Perhaps these ideas are the most clearly articulated in the "uncoupling to survive" hypothesis: the attenuation of ROS production by partial or mild mitochondrial uncoupling while maintaining sufficient ATP production is a potential mechanism for delaying cellular senescence (since reduce oxidative damage to DNA, proteins and lipids) and may extend life span [75].

So given results of experiments from several laboratories demonstrated that reduction of ROS generation in the mitochondria could be achieved by dissipation of the mitochondrial membrane potential using mild mitochondrial uncoupling. Uncoupling of mitochondria occurs naturally but may be induced by exogenous chemical protonophores such as "classical" protonophore 2,4-dinitrophenol (DNP) and by activating endogenous innate mitochondrial uncoupling pathways involving, for instance, UCPs [28].

Uncoupling proteins (UCPs)

As noted, ROS, natural by-products of aerobic respiration, are important cell signaling molecules, which can also severely impair cellular functions and induce cell death. Hence, cells have developed a series of systems to keep ROS in the nontoxic range. It is advisable in this connection to draw attention to the existence of endogenous regulators of respiration and oxidative phosphorylation, a family of uncoupling proteins, which are the likely natural modulators of mitochondrial functions. The "mild" uncoupling that they catalyze has been proposed to function as an evolutionarily conserved mechanism to attenuate ROS production [44].

The UCPs represent a family of transporters belonging to the mitochondrial carrier protein superfamily, which is found in all eukaryotic organisms. They transport substrates across the mitochondrial inner membrane and can dissipate the proton gradient [76]. This transporters family also includes the adenine nucleotide translocase (ANT), an ATP/ADP antiporter, and multiple metabolite and ion transporters [44].

UCP1 was the first identified uncoupling protein able to mediate non-shivering thermogenesis by brown adipose tissue [77]. But according to Ricquier [78], the ancient function of the UCPs may rather be associated with adaptation to oxygen and control of free radicals than to thermogenesis. UCP2 and UCP3 are the closest in amino acid identity to UCP1 (57% and 59%, respectively). But due to their relatively low abundance, the degree of uncoupling by UCP2 and UCP3 in cells is much lower than UCP1 [44].

A negative feedback mechanism has been suggested in which increased level of ROS induce UCPs

uncoupling to decrease the formation of oxygen toxic species inside mitochondria [79]. For example the lipid peroxidation product 4-hydroxy-2-nonenal can stimulate inhibitor-sensitive proton conductance through the UCPs and ANT [80].

Mailloux and Harper [79] not only confirmed that ROS activate UCP2 and UCP3, but also demonstrated that UCP2 and UCP3 are controlled by covalent modification by glutathione. In more recent studies, Mailloux *et al* [81] have identified glutaredoxin-2 (Grx2) as the enzyme responsible for regulating proton leak through UCP3 by glutathionylation. Specifically, by conjugating glutathione to UCP3, Grx2 inhibits proton leak in skeletal muscle mitochondria.

Moreover, despite the identification of UCP2 and UCP3 in 1997, the physiological functions of the mitochondrial uncoupling proteins are still under debate. A wide variety of roles for these proteins including fatty acid transport and metabolism, efflux of mitochondrial ROS by-products, glucose metabolism, and calcium homeostasis has been identified [31]. However, for the purposes of this paper the most important is the ability of UCP‘s to cause mild uncoupling and so diminish mitochondrial superoxide production, hence protecting against oxidative damage. The findings of Mailloux and Harper [79] are consistent with the conclusion that UCP2 and UCP3 function as the “first line of defense” against mitochondrial ROS production.

Thus, at the moment, multiple recent reviews discuss the putative biochemical and physiological functions of the UCPs [44]. For the purposes of this review, the most interesting is the application of these proteins to life span modulation. One approach to assess the importance of mitochondrial UCP function in aging and life span is to examine the correlation between the level of mitochondrial uncoupling and life span and if more uncoupled mitochondria may be favorable for long life span.

There are few studies that directly manipulate UCP levels and measure life span. For instance, fruit fly life span could be lengthened approximately 10-30% by over-expression of human UCP2 in the nervous system [82]. Closer inspection indicated that the production of ROS was reduced in human UCP2-expressing flies, and the transgenic flies were more resistant to exogenously applied oxidative stress than non-transgenic controls. While human UCP2 overexpression was associated with increased life span in *Drosophila*, the deletion of one of the endogenous *Drosophila* UCP homologs, DmUCP5, could also extend life span [82]. This evidence may support the hypothesis that high UCP activity may augment organism antioxidant defenses and increase life span.

But other studies found no change in life span of UCP1-/-, UCP2-/-, UCP3-/-, or transgenic UCP3-over-expressing mice relative to the wild type [83, 84]. However, Gates *et al* [85] observed reduced incidence of lymphoma and atherosclerosis in UCP1-overexpressing mice, suggesting that despite a lack of life span extension, an increase in “survival potential” could be attributed to UCP1-dependent uncoupling.

Chemical uncouplers

It is important to keep in mind that the mammalian uncoupling proteins are tissue specific and insufficiently active, and effective pharmacological agonists of these proteins in mammals are not known [86]. That's why unlike modulation of the UCPs’ abundance or function within naturally occurring mitochondrial uncoupling pathways, direct uncoupling of mitochondria using chemical uncouplers is may be more specific since they act directly to facilitate transport of protons across the inner mitochondrial membrane, from the intermembrane space into the mitochondrial matrix, thus dissipating the proton-motive force. Mild mitochondrial uncoupling using chemical uncouplers may therefore provide a stronger test for the “uncoupling to survive” hypothesis.

One of the chemical uncouplers is the “classic” DNP, which was extensively used as an obesity treatment in the 1930s, and recently has been increasingly utilized as a putative anti-aging substance. Known, that *Drosophila melanogaster* is an excellent model organism to study aging due to its short generation time and life span, the availability of the genome sequence and an enormous catalogue of genetic tools. In insects, as in mammals, there is a negative correlation between free radical production in isolated mitochondria and life span, and the extreme longevity of queen ants and bees is correlated with a resistance to oxidative stress [34].

That’s why in our laboratory the effect of a moderate (“soft”) DNP uncoupling of mitochondria on the life span and some parameters of biological age of *Drosophila melanogaster* Oregon-R strain was studied [87]. It was found that the insects treated with approximately 40 mM DNP during the larval stage had longer life span. For example, on the 30th day about 50% of the flies were dead in the control, whereas in the experiment not more than 35% of the flies had died. However, the maximal life spans of flies in both groups were not different [87]. In other available investigation [88], 0.1% DNP prolonged the average life span of *Drosophila* by 12.3%. In various series of our experiments, the increase in the average life span was not less than 20%.

This and other obtained results allowed us to assume

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that DNP can determine subsequent events: the soft uncoupling of mitochondria is accompanied by preventing the excess production of ROS by the mitochondrial respiratory chain, and this can be associated with a significant decrease in the amount of cell reserves of NADH- and $FADH_2$-providing substrates and ATP. This should decrease the total intensity of metabolic processes; moreover, just the increased consumption of O_2 without production of ATP lowers the level of free molecular oxygen capable for producing $O_2^{\bullet -}$ [68, 87]. This is likely to reduce oxidative damage of the cell and increase the life span.

In other series of experiments, we studied the effect of DNP and sodium nitroprusside (SNP; known donor of nitric oxide radicals) on protein oxidative damage and life span of *Drosophila melanogaster*, Oregon-R strain. It was shown that SNP had negative effect on flies viability connected, probably, with activation of processes of proteins oxidative damage. At the same time, DNP essentially corrected the SNP negative action on insects' survival rates and the "normalizing" action was revealed both at the level of sensitivity of flies to exogenic stresses and protein carbonyl levels, and at a level of insect life span as a whole. DNP was supposed to protect from SNP negative action on flies viability by reduction of intensity of free radicals production [89]. So, the results of our *Drosophila* experiments [87, 89] probably favor the point of view that "soft" DNP uncoupling of mitochondria has a positive effects on oxidative proteins damage degree and may increase the life span of flies.

Not so long ago, it was shown that in yeast *Saccharomyces cerevisiae*, treatment with 10 nM DNP was sufficient to increase both the chronological and replicative life span of yeast mother cells, the latter by approximately 15% [90]. At the same time mild uncoupling using low concentrations of DNP or FCCP (carbonyl cyanide-4-(trifluoromethoxy)phenyl-hydrazone) was revealed to strongly promote premature senescence in yeast according to the data of other authors [91].

Extension of life span by mild uncoupling was also described for mice. Animals treated with 5 μM DNP in drinking water had larger median (771 *vs* 722 days in controls) and mean (769.7 *vs* 718.8 days in controls) life spans than untreated mice [86], although the mice used in the study were of a short-lived strain. Nevertheless, this life span extension was accompanied by decreases in ROS production and biomarkers of oxidative damage for DNA (measured as 8-hydroxydeoxyguanosine levels) and protein carbonyl levels, in mouse brain, liver and heart tissues [86].

In other investigation, Cerqueira *et al* [92] demonstrated that murine life span can be extended by low doses of the DNP in a manner accompanied by weight loss, activation of eNOS and Akt-dependent pathways leading to mitochondrial biogenesis, lower serological levels of glucose, insulin and triglycerides as well as a strong decrease in biomarkers of oxidative damage and tissue ROS release.

The effect of uncoupler DNP on the oxidative processes intensity in liver biomembranes of rats of different age during longitudinal experiment was also studied in our laboratory [93]. On the young, 3-month old males it was shown that long-term xenobiotic administration had been accompanied by intensification of the rate of oxygen consumption, decrease of the rate of ROS formation in microsomal redox-chain, decline in lipid hydroperoxides and protein carbonyl levels in blood serum and liver microsomes, and (at the end of the long-term experiment) also by increase of their mean life span. Indeed, the average life expectancy at 50% mortality of animals was increased by 19.6% (677 ± 23 days *vs* 566 ± 55 days in control) [93]. An interesting fact is that the increase in the median life expectancy in the group treated with DNP doesn't depend on the level of model organization, as the fruit flies and rats exhibit the same increase of median life expectancy (20-25%) [87, 93].

It is important that other chemical uncouplers such as carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and FCCP were found to extend the life span of wild type *Caenorhabditis elegans* also [71].

Attempts to study the effect of mild uncoupling on cellular aging in culture have produced mixed results, with one study supporting the "uncoupling to survive" hypothesis [94], whereas others do not [91]. Overall, despite some contradictory research results it was established that uncouplers (in particular DNP) at certain doses could increase the life span of a wide range of model organisms including yeast [90], flies [87], mice [86] and rats [93].

Thus, the literature and our own results demonstrate that mild mitochondrial uncoupling is a highly effective *in vivo* antioxidant strategy, and show significant efficacy of DNP and other uncouplers in animal experiments as potential geroprotectors, and confirm the reasonability of further studies on the role of uncoupling in the regulation of development and aging of organisms.

Is it possible to use DNP in clinical practice?

As well known, dinitrophenols (DNPs) are a class of synthetic chemicals which do not occur in nature. All DNPs are highly toxic and the mechanism for toxicity common to all DNPs is uncoupling of mitochondrial oxidative phosphorylation. By mitochondrial uncoupling, the drug causes a marked increase in fat

metabolism. This shift in metabolism led to the use of DNPs as an ingredient in weight-loss pills in the early 1930s for the treatment of obesity [95, 96]. It should be mentioned that uncoupling agents could have severe health risks. As such, DNP is a metabolic poison that acts by uncoupling oxidative phosphorylation, leading to cataracts, renal failure, hyperthermia, tachycardia, diaphoresis and tachypnea, eventually leading to death [95, 97, 98] and as a result, it was banned for weight-loss purposes in 1938 [96].

In spite of this, fatalities related to exposure to DNP have been reported since the turn of the twentieth century. To date, there have been 62 published deaths in the medical literature attributed to DNP [95]. For example, the published data describe toxicological findings from two deaths, one in Tacoma, WA and a second in San Diego, CA, following the ingestion of DNP. In the Tacoma case, DNP was identified in capsules the decedent was taking for weight loss, while in the San Diego case, DNP was identified in a yellow powder found at the scene [97]. Another study reports the cases of two patients whose deaths were attributed to occupational and non-oral exposure of DNP. They were all poisoned through skin absorption and respiratory tract inhalation; common features were excessive sweating, hyperthermia, tachycardia, clouded consciousness and asystole [99].

Unfortunately, review of information available on the Internet [98] suggests that DNP is still illicitly promoted for weight loss, for example, among body building enthusiasts. Although, the drug is illegal in the United States, it can be purchased on the Internet under such names as “Sulfo Black”, “Nitro Klenup”, or “Caswell No.392” from commercial web sites which sell and promote the use of anabolic steroids [98]. Some of the websites promoting use of the DNP give no information about its dangers, though others include a disclaimer that it is not safe for human consumption.

So, DNP and other nitrophenols have long been known to be toxic at high concentrations (the ‘bad’ face of DNP), an effect that appears essentially related to interference with cellular energy metabolism due to uncoupling of mitochondrial oxidative phosphorylation. But at the same time other studies have provided evidence of beneficial actions of DNP (at low concentrations), including neuroprotection against different types of insult, blockade of amyloid aggregation, stimulation of neurite outgrowth and neuronal differentiation [100]. These results indicate that treatment with mitochondrial uncoupling agent DNP may provide a novel approach for the treatment of secondary injury following spinal cord contusion [101].

Moreover DNP uncoupling of the mitochondrial electron-transport chain may be the strategy to prevent intracellular oxidative stress during liver cold preservation/warm reperfusion injury. The results of Petrenko *et al* [102] suggest that reversible uncoupling may be one way to influence oxidative stress associated with hepatic cold preservation. In other investigation on rhesus monkeys with low cryoresistant ejaculates, reducing ROS through mild mitochondrial uncoupling had statistically significant beneficial effects on sperm cryopreservation [103]. Individuals or species that have higher sensitivity to cryodamage may derive the most benefit from these treatments.

Thus, despite a number of positive effects of mild uncoupling on the living organism, the use of DNP and other chemical uncouplers in clinical practice should be considered impossible due to the high toxicity. But the possibility of correction of oxidative processes intensity in tissues of mammals and their life span by means of modulation of ROS production in membrane electron transport chains does exist. In this connection, the search of non-toxic uncouplers (preferably of natural origin) is of great interest for the further studies on the role of uncoupling in the regulation of development and aging of organisms.

CONCLUDING REMARKS

Aging is a universal process commonly defined as the accumulation of diverse deleterious changes occurring in cells and tissues with advancing age which are responsible for the increased risk of disease and death. Understanding of the aging mechanisms is of major interest to scientists, physicians and general population as well. But attempts at understanding the causes of aging are limited by the complexity of the problem. More than 300 theories have been postulated and free radical theory of aging is one of the most prominent and well studied.

The free radical theory of aging hypothesizes that oxygen-derived free radicals are responsible for the age-related damage at the cellular and tissue levels. In a normal situation, equilibrium exists among oxidants, antioxidants and biomolecules. Excessive generation of free radicals may overwhelm natural cellular antioxidant defences leading to undesirable oxidation of biomolecules and further contributing to cellular functional impairment. The identification of free radical reactions as promoters of the aging process implies that interventions aimed at limiting or inhibiting them should be able to reduce the rate of formation of aging changes.

The main objective of this review is to elucidate the role of endogenous (primarily mitochondrial origin)

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ROS in the aging process. We have attempted to highlight the findings from several investigations about the connections between reduction in mitochondrial production of free radicals (using specific antioxidants or mild mitochondrial uncoupling) and life span.

It should be noted that the conclusions from this review do not provide entirely clear picture of the effect of mitochondria-targeted antioxidants and uncouplers on life span and it is presently unclear if mild mitochondrial uncoupling is useful as a therapeutic strategy for achieving extended human longevity. Further investigation of these interesting agents is necessary. Nevertheless, several studies on animal models have shown that aging rates and life expectancy could be modified using mitochondria-targeted antioxidants and uncouplers.

In particular, different uncoupling strategies were able to extend life span in models ranging from yeast to mammals [86, 87, 90, 93]. These findings are in line with Brand's "uncoupling to survive" hypothesis [29], suggesting that uncoupling could be an approach to promote life span extension due to its ability to prevent the formation of ROS. Indeed, mild mitochondrial uncoupling can be a highly effective intervention to prevent the formation of ROS.

Obviously, because of the high toxicity DNP and other uncouplers, themselves cannot be applied in practical geriatrics, but their low toxic analogues having controlled and "soft" action either agonists affecting the natural way of uncoupling (UCPs) are promising for the development of means for control of tissue redox state and animal life span. In this regard, we support Harman's point of view [7] regarding the fact that the extensive studies based on the free radical theory of aging hold promise that average life expectancy at birth and the maximum life span in principle can be extended, although we cannot yet answer the question of "When?".

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COMPETING INTERESTS

This work presents no conflict of interest.

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