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Forum Review Article

Dietary restriction and oxidative stress: friends or enemies?

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ABSTRACT

Significance: It is well established that lifestyle and dietary habits have a tremendous impact on lifespan, the rate of aging and the onset/progression of age-related diseases. Specifically, dietary restriction (DR) and other healthy dietary patterns are usually accompanied by physical activity and differ to Western diet which is rich in fat and sugars. Moreover, as the generation of reactive oxidative species is the major causative factor of aging, while DR could modify the level of oxidative stress, therefore it has been proposed that DR increases both survival and longevity.

Recent Advances: Despite the documented links between DR, aging and oxidative stress many issues remain to be addressed. For instance, the free radical theory of aging is under "re-evaluation", while, DR as a golden standard for prolonging lifespan and ameliorating the effects of aging is also under debate.

Critical Issues: This review article pays special attention to highlight the link between DR and oxidative stress in both aging and in age-related diseases. We discuss in particular DR's capability to counteract the consequences of oxidative stress and the molecular mechanisms involved in these processes.

Future Directions: Although DR is undoubtedly beneficial several considerations must be taken into account when designing the best dietary intervention. Use of intermittent fasting, daily food reduction or, DR mimetics? Future research should unravel the pros and cons of all these processes.

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INTRODUCTION

Aging, oxidative stress, and dietary restriction

Aging represents an inevitable stochastic process that involves the accumulation of diverse deleterious changes in the cells and leads to the increased risk of diseases and death at the end. Although aging cannot be avoided, the rate of aging is not fixed, but is plastic and subjected to modifications. Among environmental factors proven to be very potent in modulating aging is diet. For decades it is known that a reduced daily food intake significantly extends the life of various species (92).

Dietary restriction (DR) is one of the simplest, but most extensively investigated environmental procedures and with the widest anti-aging effects in a huge variety of species. Additional terms that are used are calorie restriction (CR) and food restriction (FR). Although they all refer to less energy intake, they do not always overlap fully. Dietary restriction is defined as a reduction in energy intake (typically 40% less than *ad libitum*) without malnutrition, typically by decreasing food consumption, while calorie-restricted subjects get the same amount of daily food intake as *ad libitum* subjects, but with reduced content of calories. In this review, we will use term DR to refer to all food-restricted protocols.

DR is the most widely known for its life-prolonging effects. However, DR has many other beneficial effects and it is associated with enhanced physiological functions in many species during aging. To date, it is the most successful intervention in delaying aging progression and age-related chronic diseases, like diabetes, incidence, and progression of various cancers, osteoporosis and sarcopenia, a decline in immune function, cardiovascular diseases and neurodegenerative disorders (123). Although in the focus of the research for more than 80 years now, the number of published reports about DR, longevity, and healthspan constantly increases, denoting that previous studies did not answer all the questions, but opened a lot of new ones.

According to David Sinclair's theory of aging and DR, DR is an active, highly conserved stress response that increases the organism’s likelihood of surviving harsh conditions (167). It has been considered that DR represents a stressor of mild intensity; therefore, by inducing a low level of stress, DR enables cells to counteract higher stress. This effect DR achieves by modifying key processes in cell protection, modulating

metabolism to permit a higher survival against adversity, and favoring maintenance and repair. This is in alignment with the '**Hormesis hypothesis"** of DR actions (77, 124, 127, 148).

Oxidants and antioxidants

"Stress" for sure is one of the most common but most terrifying words nowadays. Physiological stress is imperative for biological rhythms and the constancy of the homeostasis, but too strong stress can bring organisms above the reparative limit, causing damages (151). Herein, the stress concept was brought to the cellular level, with oxidative stress as one of the most notorious ones.

Oxygen is essential for aerobic organisms but also has a hazardous potential since it is a tool for the production of reactive oxygen species (ROS). ROS are produced by living organisms as a result of normal cellular metabolism. It is estimated that about up to 3 % of the oxygen consumed by aerobic cells is diverted to the generation of ROS (170). In this manner, mammalian cells are under constant barrage from oxidants, generated not only by normal aerobic respiration in mitochondria, but also from extrinsic sources, disease/pathology states (inflammation, obesity), exposure to UV light, ionizing radiation, or heavy metal ions (Figure 1). ROS has an important role in a broad array of physiological processes, but in high concentrations, they cause adverse modifications to biomolecules, including proteins, lipids, and DNA (186, 172).

ROS encompasses free radicals (molecules containing one or more unpaired electrons) and nonradicals (when two free radicals share their unpaired electrons), with the 3 most profound ones: superoxide anion ($O_2^{\bullet -}$), hydroxyl radical (OH•), and hydrogen peroxide (H_2O_2). Superoxide anion is directly generated from the reduction of oxygen and can be further converted to hydrogen peroxide that can penetrate the cell's membranes and in Fenton's reaction generates hydroxyl radical, which is the most reactive form of oxygen (12) (Figure 2).

The mitochondrial electron transport chain is considered to be the main source of ROS in the cells. It has been suggested that all the complexes of the mitochondrial electron transport chain are involved in ROS production (56) and that they can be assembled in supercomplexes whose progressive deterioration during aging causes an irreversible increase in ROS (50).

On the other hand, organisms possess a counterbalancing weapon to fight against oxidants - a variety of antioxidants, molecules that can be classified as exogenous (natural or synthetic) or endogenous, both responsible for removal of free radicals, scavenging ROS and preventing their production (52). Endogenous antioxidants can be further divided into two major classes: enzymatic and nonenzymatic. Enzymatic antioxidants embrace limited number of proteins, such as catalase (Cat), glutathione peroxidase (GPX), glutathione reductase (GR), and superoxide dismutase (SOD), with 3 very important forms: copper, zinc superoxide dismutase (CuZnSOD), manganese superoxide dismutase (MnSOD), and (EC-SOD), along with some auxiliary enzymes like heme oxygenase-1 and redox proteins: thioredoxins (TRXs), peroxiredoxins (PRXs) and glutaredoxins (12). Non-enzymatic component of antioxidant defense serves as the second defense system and includes antioxidants that are capable of direct protection against oxidative damage and for enhancing the function of endogenous enzymatic antioxidants, thus synergistically scavenging free radicals (143, 185). The following antioxidants belong to this group: vitamins (A, C, E), Coenzyme Q, glutathione (GSH), uric acid, glutathione, and polyphenols (11). Food, especially one of plant origin, is a great source of non-enzymatic antioxidants (Figure 3).

The antioxidant enzymes represent some sort of network aimed to detoxify excessive levels of ROS. They are generally ubiquitously expressed; however, subcellular localization is an important part of their protective capacity. A primary line of defense is provided by **catalase**, a peroxisomal enzyme that catalyzes the reduction of hydrogen peroxide to oxygen and water. **Glutathione peroxidase-1** is localized in both mitochondria and cytosol. It uses glutathione to catalyze the reduction of various oxidants, including hydrogen peroxide and fatty acid hydroperoxide. Oxidized glutathione is subsequently reduced by glutathione reductase with the reducing equivalent NADPH. **Glutathione S-transferase** takes part in detoxification and conjugates glutathione to several substrates and can be found in the nucleus, cytosol and in the cell membranes. **Superoxide dismutase** exists in three forms, depending on the protein fold and the metal cofactor. It is present in the cytosol (CuZnSOD), mitochondria (MnSOD) and extracellular space (EcSOD), and catalyzes the reduction of superoxide anion to oxygen and hydrogen peroxide (192).

When the antioxidant defense system in the cells fails to counterbalance the oxidants, a disturbance in the oxidants to antioxidants ratio happens, with the oxidants predominate. One of the oldest and long-established theories of aging - the redox stress theory of aging (68) explains aging exactly in this manner - as a failure of defensive mechanisms to respond to the oxidative damage induced by ROS (68).

In the elderly, antioxidant systems are not efficient enough to counteract intensified and elevated oxidative stress, thus with aging people become extremely vulnerable to oxidative damage and prone to many age-related diseases (26, 41,141,170). However, according to some new findings increased antioxidants expression could be ineffective, or could even have negative consequences on lifespan (reviewed in 9, 54), as aging can occur even if there is almost no ROS, in anaerobic conditions. Therefore, it is feasible ROS to have only a side role in the aging process. For instance, aging could be considered as a consequence of over-activation of growth-promoting signaling pathways such as mTOR (mammalian target of rapamycin) pathway that terminates life prior to ROS effects. According to this hypothesis, ROS are part of the mTOR theory of aging (13).

Dietary restriction, ROS production, antioxidant defense and repair mechanisms

The free radical theory of aging has been re-evaluated in recent years. Primary sources of ROS production and primary targets for ROS actions have become more important than the total ROS load and/or total oxidative damage. According to Harman's modified theory of aging arose 20 years after the first one, mitochondria got a fundamental role in the process of aging (68, 69). Mitochondria produce ROS, but ROS hit mitochondria in particular, damaging their structural and functional properties (26, 132). On the other hand, mitochondria are quoted as a target for DR to exert its protective potential during the process of aging (17, 112, 113, 114).

Since the beneficial role of a restricted food supplementation has been proposed, numerous studies described a positive impact of DR on mitochondria and their function. Protective actions of DR against ROS production, increased antioxidant enzymes level and/or activity, and increased turnover of oxidized macromolecules are anticipated. All of these DR beneficial pathways mutually intertwine (Figure 4).

Lower mitochondrial membrane potential and fluidity, lower mitochondrial free radical generation rate and lower oxidative damage of cellular macromolecules have been

reported in food-restricted animals and in various tissues (23, 24, 60) following DR. Numerous redox homeostasis parameters were significantly improved under the DR in 24 months old rats, and across various organ systems (31, 63, 115), even when DR started in the adulthood (166). In addition, DR influences mitochondria positively by increasing mitochondrial biogenesis and efficiency (114, 139). All mentioned processes could result in a decreased aging rate at cellular and organismic levels (34, 44, 104, 122).

Regarding the enzymatic component of anti-oxidative defense, no particular enzyme follows a consistent pattern during aging or is being consistently affected by DR, although animal studies implied a significant DR-induced increase in the activity of several endogenous antioxidant enzymes (2, 55, 100, 147, 169, 170).. Glutathione reductase activity has never been observed to decrease with DR, while catalase and glutathione S-transferase activities tended to increase following DR (192). SOD activity is the least likely to change due to DR, although in the same cases a greater gene expression and activity of SOD1/2 has been detected, while no effect has been noticed on catalase activity (143).

The tissue specificity has also been observed. For example, in the liver, the most prominent effect of DR observed was the increased catalase activity (192). In the heart of the male Wistar rats exposed to 50% DR for 35 days glutathione peroxidase and catalase activities were increased (34). On the other hand, the beneficial effects of DR in the brain may be due primarily to the glutathione redox system. Glutathione plays a critical role in the brain (38), and disruption of the glutathione system is associated with brain aging/neurodegeneration (144). Therefore, increased glutathione may be a primary mechanism through which DR protects the brain (192).

The third level of DR protection is DR-induced expression of proteins involved in protein turnover and DNA repair, and consequentially in the removal of damaged proteins and DNA that accumulate with age. DR has been shown to have a significant effect on major DNA repair pathways, such as base or nucleotide excision repair (BER/NER) (73, 74, 75, 146). Numerous studies have been conducted over the past few decades to detect the ability of DR to repair a variety of DNA damage in cells. Early studies showed that DR enhances considerably the cells' ability to repair the DNA damage induced by UV light or chemicals (73), while more recent investigations focused on specific DNA repair pathways and their key enzymes. DR led to a significant improvement in the repair mechanism in

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many cases (73) and resulted in a reversal of age-related DNA damage in multiple mouse tissues (16, 183). Surprisingly, it was shown that DR effects on the DNA repair capacity are heterogeneous, being gene- and strand- dependent. Furthermore, this protective action of DR was also evident in young animals exposed to DNA damaging agents (16) and during development (163). In this manner, DR would support life-long maintenance of genomic integrity and more importantly, it seems that even a short-term DR can provoke similar beneficial effects (18).

Molecular targets of DR action

There are numerous mechanisms and signaling pathways trough which DR exerts its anti-aging effects and impacts the capacity of the organisms to adapt to constant challenges of internal and environmental stimuli and to respond adequately to the stressful conditions (Figure 5).

Following nutrient deprivation cells experience a metabolic shift from glycolysis to oxidative phosphorylation. Although it is energetically more efficient, it causes increased mitochondrial activity and consequential increased ROS production. This metabolic switch is driven by nutrient sensors that also influence oxidative stress response (116). One of the strongest evidence for this link came from the studies of sirtuins (SIRT).

Among other factors, sirtuins gained great popularity due to their involvement in DR-mediated beneficial health and antiaging effects (98), including a role in regulating redox homeostasis (65, 131). Sirtuins represent a family of nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylases and include seven proteins, with six of them having a role in DR-related reduction of oxidative stress in mitochondria: SIRT1, SIRT3, and SIRT5 protect the cell from ROS, and SIRT2, SIRT6, and SIRT7 modulate key oxidative stress genes and mechanisms, while SIRT4 has both antioxidative roles and can induce ROS production (168).

From the initial studies on aging in yeast, SIR2 and its homologs in higher eukaryotic organisms – SIR1 were identified as possible molecules through which DR extends lifespan (84). In the absence of SIRT1 protein, DR cannot extend the lifespan of mice (109). On the other hand, an elevated SIRT3 level leads to the activation of SOD2, the major antioxidant enzyme in mitochondria (145).

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In addition to sirtuins, nutrient sensors involved in DR-mediated reduction of oxidative stress are AMPK (5' adenosine monophosphate-activated protein kinase), mTOR signaling network and general control nonderepressible 2 (GCN2), serine/threonine-protein kinase (145). AMPK is an energy sensor, while mTOR and GCN2 sense the availability of intracellular amino acids. AMPK has been proposed to reduce oxidative stress by inhibiting acetyl-CoA carboxylase 1 and 2 and maintaining the NADPH level, while the TOR and GCN2 activate autophagy and recycle damaged mitochondria. Also, nutrient sensors can respond directly to ROS (145).

It has also been shown that DR suppresses the age-related decrease in the activity of forkhead box (FOXO) transcription factors (48,51), known to increase resistance to oxidative stress, decrease ROS production, and slow down the accumulation of oxidative damage (6, 174).

Some of DR benefits are associated with the nuclear factor erythroid 2-related factor 2-antioxidant response element (Nrf2/ARE) pathway activation (76, 97,122, 189) and consequential increase in the enzymes with important detoxifying and antioxidant functions (189). For example, DR can promote the up-regulation of GST and NQO1, Nrf2/ARE-driven genes, in a variety of body tissues and organs (22, 82). Either by restricting calorie intake or meal frequency, DR-driven activation of Nrf2 has soothing effects on the oxidative stress, that could be especially important for the management of CNS disorders (189). Interestingly, it seems that DR/fasting could result in a transient, hormetic increase in ROS production that in turn may trigger activation of the Nrf2/ARE pathway and the up regulation of its target genes (76,97).

Longevity effects of DR can be also brought by heat shock transcription factor 1. Namely, a general protective effect against proteotoxicity and promotion of longevity, induced by specific form of dietary restriction called bacterial food deprivation, was showed in C. elegans models of various diseases (173).

The complex interplay between oxidative stress and DR in the cell includes also epigenetic modifications. While oxidative stress affects DNA, histones and histone modifiers, an age-related increase in oxidative stress could change the epigenetic landscape of the cell, leading to various diseases. ROS can influence DNA demethylation, mainly by affecting the function and the activity of the DNA methyltransferases (DNMTs),

histone methylase and histone deacetylase (HDAC). The family of sirtuins considered to be one of the key factors through which DR exerts its beneficial effect could be also considered as epigenetic enzymes (37). Also, several micro RNA (miRNA), termed as oxidative stress-responsive miRNAs, are both regulated by ROS and capable to modulate ROS production and scavenging, playing the role in age-related diseases like diabetes and atherosclerosis (45). It has been shown that several miRNAs may play a key role in linking the imbalanced redox state with dysregulated sirtuins. SIRT1 is highly sensitive to oxidative stress, while its activation can alleviate oxidative stress, promote mitochondrial biogenesis and prevent senescence. The interaction is even more complex as oxidative stress regulates a panel of miRNAs; some of those miRNAs directly regulate SIRT1, while the others influence SIRT1 regulators. Further, those regulators can be down- or up-regulated by oxidative stress and also influence SIRT expression, both positively and negatively. Finally, SIRT1 can regulate miRNAs through the modulation of their upstream transcription factors (reviewed in 25). All of these factors construct a complex network, leading to the different DR outcomes.

ROS and DR interplay in pathological conditions

All the above mentioned highlights oxidative stress as one of the major factors that pave the path to numerous pathological conditions, including cancer, atherosclerosis, hypertension, diabetes and neurological disorders (Figure 6). At the same time, in all of these pathologies a protective role of reduced food intake has been suggested (3, 21, 33, 62, 66, 109, 175, 185,186,192).

Neurodegenerative disorders

The brain can be highly susceptible to oxidative damage, due, in part, to its elevated oxygen demand, the presence of high amounts of polyunsaturated fatty acids that are easily targeted by free radicals, and also due to the lower levels of antioxidant enzymes in comparison to other organs (189). In recent years, several studies have examined the potential associations between oxidative stress and neurodegenerative diseases such as multiple sclerosis (MS), PD (14) and AD (155, 158).

There is growing evidence that oxidative stress plays a significant role in AD and mild cognitive impairment. Increased oxidative stress markers, protein nitration and carbonylation, and reduced antioxidant defenses are present in these conditions (130,

189). Numerous studies indicated that oxidative stress is an important contributor to PD pathogenesis (29, 87,128). The chronic neuroinflammation and characteristic dopamine metabolism could be the source of cytotoxic ROS in this case (13).

While epidemiologic findings suggest that high-calorie diets increase the risk for AD/PD due to an elevation of the free radical production, studies of animal models of these disorders have shown that reduced energy intake can reduce neuronal damage and improve behavioral outcome (125, 128). It has been shown that DR lessens neuropathology in several mouse models of AD, increases mitochondrial metabolism and biogenesis, inhibits oxidative stress and promotes autophagy (109). Also, a recent study showed that 30% DR for only 12 weeks can prevent neurotoxicity, significantly attenuated oxidative damage and cognitive impairment induced by acrolein, an environmental pollutant involved in AD etiology, and a potent inducer of oxidative damage (80).

Furthermore, it was reported that the activation of neuronal SIRT1 represents a possible mechanism underlying the protective effects of DR against the pathology of AD. DR has been shown to significantly increase the transcriptional levels of SIRT1 and tended to increase SIRT3 expression levels (58). A growing body of evidence suggests that, as in the case of AD, modulation of sirtuins activity by DR regimen can protect against neuronal loss and impairment in models of PD. Moreover, SIRT2 is necessary for DR to achieve the protective effect on dopaminergic neurodegeneration (67,171).

Obesity and diabetes

Inadequate caloric intake leads to the higher activity/hypertrophic of adipose tissue, with macrophage infiltration, increased inflammation, increased level of oxidative stress and obesity, with the development of metabolic syndrome (MetS) at the end (47). Hyperglycemia and chronic low-grade inflammation present in metabolic syndrome promote increased production of ROS and increased oxidative stress. On the other hand, weight loss induced by DR reduces oxidative stress and improves the clinical outcome of MetS in obese individuals. Namely, caloric restriction-induced weight loss results in an improvement in glucose metabolism and may lead to decreased inflammation as a result of decreased adiposity and adipokine/cytokine secretion, thereby reducing the oxidative stress (181). A recent study also revealed that the improvement of subclinical atherosclerotic markers and reduced risk of cardiovascular disease after dietary weight

loss intervention in obese individuals is associated with a reduction of oxidative stress and inflammatory pathways (111).

Emerging data of experimental and clinical studies imply that oxidative stress also plays a pivotal role in the pathogenesis of both type 1 and type 2 diabetes mellitus (DM). By oxidation of elevated glucose levels present in diabetes, by nonenzymatic glycation of proteins, and the consequent oxidative degradation of glycated proteins, increased levels of free radicals in diabetes are formed. Together with a decline of antioxidant defence mechanisms, that are also present in diabetes, increased oxidative stress causes the damage of cellular organelles and enzymes, increased lipid peroxidation and development of insulin resistance (119, 121).

The study by Roberts et al. (150) demonstrated that 3 weeks of low-calorie diet/exercise program led to the amelioration of inflammatory cytokines and oxidative stress markers in obese type 2 diabetic patients (42, 150), while a calorie-restricted vegetarian diet led to an enhancement of enzymatic and non-enzymatic antioxidant defence (91).

The probable mechanism for oxidative stress markers modulation by calorie restriction-induced weight loss could be due to the reversal of the mechanism by which obesity produces oxidative stress. Some of those mechanisms are mitochondrial and peroxisomal oxidation of fatty acids, which can lead to the overproduction of ROS, as well as over-consumption of oxygen, and subsequent greater generation of free radicals in the mitochondrial respiratory chain (150).

Cardiovascular diseases

Growing evidence demonstrates the pleiotropic beneficial effects of life-long DR on the cardiovascular system. Protective effect on atherosclerosis, plaque deposition, hypertension, and other cardiovascular complications DR exerts via the modulation of the activity of both SIRT1 and AMPK (45, 64, 106) whose activity reduces ROS generation (28, 35) and improves mitochondrial biogenesis and function (156). By activation of the SIRT1 pathway, DR upregulates genes involved in mitochondrial biogenesis, such as nuclear respiratory factor 1 and peroxisome proliferator-activated receptor coactivator 1 and contributes to an elevation of mitochondrial biogenesis in the heart (165). DR attenuated ROS production in endothelial and smooth muscle cells thus creating a microenvironment

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that does not favor atherogenesis and preserves vascular health (184). It increases the bioavailability of NO, activates the Nrf2/ARE pathway inducing ROS detoxification systems, exerts anti-inflammatory effects, significantly decreases vascular $O_2^{\bullet -}$ and H_2O_2 production and thereby suppresses the initiation/progression of vascular disease that accompanies aging and has also been shown to substantially attenuates oxidative DNA damage (184).

Human studies

Reduced food intake in the human population stands in opposition to obesity and increased risk for many diseases and health complications. Until recently, the effects of DR in longer-lived species remained unknown, although results from non-human primate research indicated that the same outcome of DR intervention noticed in lower organisms could be expected here (27).

The effects of DR on oxidative stress in humans are scantly investigated so far and mostly limited to few short-term studies investigating DR effects in a combination with exercise in obese people (46, 71, 72).

First data about the role of DR in lifespan extension in humans came from the epidemiological study of Okinawa people (90, 149) where a significantly increased number of centenarians in comparison to the rest of the world has been noticed. At the same time, low caloric intake has been detected in this population, since the young stage and persistently through life. Another study, examining the effects of alternate-day feeding in non-obese people, showed reduced health problems and death rates (149). In addition to that, a group of 18 individuals (so-called CRONIES) by being imposed voluntary to DR for 3–15 years displayed significant health benefits (45). Still, the question is - are those beneficial effects linked to oxidative stress?

Regarding the data in the obese population, it has been shown that DR for 4 weeks in a combination with exercise-induced the activities of superoxide dismutase and glutathione peroxidase, counteracting oxidative stress present in this population (108). Few other studies showed that even a modest (20-25%) and short– term restriction could have sustainable effects on oxidative stress markers (15, 78, 180).

Recently one of the long-expected studies, called CALERIE (Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy) appeared, describing the effects of 25% DR in 150 non-obese healthy persons. After 6 months of such a regimen,

two out of three biomarkers of longevity were improved, while DNA damage was reduced (72). CALERIE also showed that moderate short-term DR modulates some, but not all the components of antioxidant defense and oxidative stress (133). Namely, while an increased plasma glutathione peroxidase activity was detected, as well as decreased plasma protein carbonyl levels, no significant changes in the plasma antioxidants were noticed. It has to be noted, however, that in this case age, body mass index (BMI) status and short-term nature of reduction may play a role. In another short-term study, 24 healthy individuals underwent two 3-week treatment periods of IF with antioxidant supplementation. Only a minor increase in SIRT3 expression was found, without changes in oxidative stress markers (194).

Intermittent fasting versus dietary restriction

The question stays, however, is there any difference between the effects of intermittent fasting (IF or EOD- every other day feeding) and limited daily feeding, is it an overall reduction in calories that is lying in the base of DR protective effect or these two alternative dietary approaches activate different sets of metabolic pathways?

Fasting is a dietary approach that has been practiced for a very long time. It has been shown that IF or EOD protocols produce many of the effects characteristic for DR, like increased life span and decreased incidence of age-associated diseases (70). Beneficial IF action could be driven by increased resistance to stress and enhanced protection against oxidative DNA damage (20,126). In lower eukaryotes, chronic fasting has been shown to extend longevity through reprogramming metabolic and stress resistance pathways. In rodent models intermittent fasting (IF) has been shown to be protective against diabetes, cancers, heart disease, and neurodegeneration (reviewed in 110).

Fasting could be especially interesting in humans, having in mind religious reasons for people tend to follow Ramadan protocol, but also as IF is easier to perform and it seems that is equally effective in decreasing body weight and fat mass as classical DR diets (187). Fasting helps in reducing obesity, hypertension, asthma, and rheumatoid arthritis (reviewed in 110).

Human data indicates that IF in overweight asthma patients leads to a decrease in body weight, improved clinical findings, increased antioxidant defense and significant reductions in several markers of oxidative stress. More importantly, these beneficial

effects of IF appeared very fast- within 2 weeks of diet initiation and persisted for the 2 months (88). IF reduces oxidative stress and affects neurogenesis in the adult hippocampus (reviewed in 110). IF also improves endogenous SOD1, SOD2, and catalase expressions and increases cell proliferation, and dendrites complexity and maturation in the adult dentate gyrus of gerbils (reviewed in 1). Postoperative IF suppresses neuroinflammation and oxidative stress induced by chronic cerebral ischemia, thereby preserving cognitive function in a vascular dementia rat model. It has been shown that postoperative IF preserved SOD activity, GSH level, and synthesis of their regulatory enzymes (79).

Every-other-day feeding in a combination with exercise regulates both Coenzyme-Q level and Coenzyme-Q-dependent activities. Coenzyme-Q is an important component of many antioxidant and cell survival pathways, as it has an essential function in the mitochondrial electron transport chain and in the regulation of oxidative stress-dependent cell signaling (153). In this manner, by modulating Coenzyme-Q level and activities, EOD is capable of preventing oxidative damage and cell death in aged muscle (153). Long-term, late-onset intermittent food restriction, another form of IF (IFR-two consecutive days a week fasting) in addition to extended mean and maximum lifespan, enhanced the activities of antioxidant enzymes: catalase, glutathione peroxidase, and superoxide dismutase (193).

Ketogenic and Mediterranean diet

Besides DR, several other types of dietary interventions have been proposed as anti-aging strategies, or for the prevention and treatment of several chronic-degenerative diseases, including cancer, cardiovascular diseases, and neurodegeneration and metabolic disorders.

The ***ketogenic diet (KD)*** represents a high-fat, low-carbohydrate diet that provokes ketone body production in the liver through fat metabolism. The ketogenic diet mimics a fasting or starvation state without calorie deprivation. Numerous clinical evidences support KD use in the management of adult epilepsy, malignant glioma, AD, PD, migraine headache, motor neuron disease, and various other neurologic disorders (reviewed in 129). Although the mechanisms underlying the neuroprotective effects of KD are still

elusive, recent studies provided lots of evidence that ketone bodies exert their neuroprotective effects by modulating oxidative status in the cell (reviewed in 198).

Ketone bodies decrease oxidative stress, increase antioxidants and scavenge free radicals after TBI, and improve cerebral metabolism by providing alternative substrates, leading to the prevention of oxidative stress-mediated mitochondrial dysfunction (59). Additional evidence implies that neural protection ketone bodies show may be directed to mitochondria. KB-induced mitochondrial biogenesis and enhanced respiratory function, as well as reduced mitochondrial production of ROS in response to glutamate, has been shown (reviewed in 30).

Interestingly, as oxidative phosphorylation in mitochondria produces free radicals and the ketone metabolism is distinctively oxidative, it might also be disputed that ketogenic supplements should even worsen mitochondrial dysfunction; however, this does not happen. The explanation could lie in the fact that moderate levels of mitochondrial ROS actually can protect against chronic disease through improving mitochondrial capacity and up-regulation and endogenous antioxidant defense. This phenomenon called mitohormesis is induced through increased dependence on mitochondrial respiration that could be achieved through ketogenic diet/nutritional ketosis or exercise (134).

The Mediterranean diet (MD) represents one of the most extensively described and assessed diet in the scientific literature. The Mediterranean diet includes high intakes of fruits and vegetables, legumes, various nuts, grains, fish, seafood, and extra virgin olive oil (EVOO), The conventional MD is rich in antioxidant vitamins (β-carotene and vitamins C and E), natural folate, phytochemicals (flavonoids), carotenoids, and selenium. EVOO represents a significant source of antioxidant phenolic compounds, whose synergistic effects may modulate the oxidative stress. There is growing evidence that polyphenols from olive oil (especially hydroxytyrosol) induce the expression of SIRT1. In addition to hydroxytyrosol, oleuropein was shown to activate SIRT1 as well and could thus contribute to the antioxidant effects of olive oil. Mediterranean diet also includes a temperate intake of red wine, containing resveratrol capable to enhance the resistance to oxidative stress through several signaling pathways, including SIRT1, ntf-2, and NF-κB (173).

Mimetics and senolytics

Regardless of all the beneficial effects mentioned above, it is hard to believe that a lifelong 40% food reduction might ever be practical for humans. Cellular pathways engaged in DR-mediated anti-aging effect, including those related to ROS signaling and antioxidant activity, have become promising molecular points for developing pharmacological treatments that would provide a pro-health phenotype. Therefore, research of molecules that have been found to "mimic" the phenotypes of DR (105) led us to different opportunity: instead of reducing food intake, to introduce mimetics into our daily food routine (85,191). Mimetics and DR have many common cellular pathways (Figure 5).

Fisetin, a plant polyphenol and one of the DR mimetics, protects rat brain against age-induced oxidative stress. After six weeks of fisetin treatment, several levels of action were detected: fisetin significantly decreased the level of pro-oxidants, ameliorated mitochondrial membrane depolarization, down-regulated the expression of inflammatory (IL-1β and TNF-α) and Sirt-2 genes, while up-regulated the expression of SIR1 and autophagy genes and increased antioxidants level in the aging brain (168). There are many other reports about the role of fisetin in fighting oxidative stress: fisetin protected human retinal pigment epithelial cells from oxidative stress-induced cell death (81). It acts on multiple pathways to reduce the impact of age and age-related disease, including a direct antioxidant and chelating activity, and maintenance of GSH activity (118). Fisetin suppresses oxidative stress by modulating sirtuins and FOXO3a expression in human monocytes (95) and also shows antioxidant effects dependent on Nrf2/ARE pathway (199).

Another promising DR mimetic- ***resveratrol*** (RSV) seems to be capable to protect from inflammation, metabolic and cardiovascular insults (8, 28, 36, 152, 190). A strong antioxidant effect of resveratrol has been noticed in mice; resveratrol increased mitochondrial biogenesis and mtDNA quantity (8, 83, 152). Resveratrol among other targets can induce FOXO transcription factors and it seems that in low doses may prime cells to deal with DNA-damage causing agents similarly to the DR mechanism. Moreover, it can mimic the DR effect by SIRT-mediated deacetylation of pro-inflammatory complexes such as NF-κB (53). Resveratrol partially prevents both maternal and offspring oxidative stress induced by maternal protein restriction during pregnancy (190). An interesting study

aiming to compare the effects of 30% DR and resveratrol on physiological and behavioral parameters during aging was conducted by Marchal et al., (120). They concluded that both DR and resveratrol induced heterogeneous effects, leading to a higher level of oxidative stress first and then to a decrease, due to 3 different mechanisms: decreased ROS production increased antioxidant defenses and an enhanced repair process of damaged molecules. This two-step pattern fits with the "hormesis" theory of DR action when a stressor of low-intensity preconditions subject to stress. In a review by Lam et al., (103) resveratrol vs. calorie restriction effects were summarized from rodents to humans. Resveratrol showed broad protective effects *in vitro* and *in vivo*, from rodents to primate, and at least partially by suppressing oxidative stress (103). Epidemiological studies showed that resveratrol for six weeks significantly reduced ROS production in mononuclear cells. Further, an improvement in mitochondrial efficiency was also observed (103). However, it seems that at higher levels resveratrol could become a stressor itself (53).

Rapamycin, a macrocyclic lactone-based compound and inhibitor of mTOR (40), increases autophagy (89) and ameliorates numerous age related-disease phenotypes. Similarly to DR, rapamycin acts on mTOR signaling and results in an increased expression of proteins protecting against oxidative damage. SOD1, glutathione reductase, δ-aminolevulinate dehydratase were increased in rapamycin-treated mice (99). In two different in vitro studies, rapamycin prevents oxidative injury-induced cell-death, by inhibition of ROS production (164, 176). Genes involved in oxidative stress and DNA damage were down-regulated by rapamycin (137). Although it is capable of increasing antioxidant defense, it seems that rapamycin, similarly to the effects driven by DR, acts predominantly at the level of suppressing the production of free radicals. Interestingly, it seems that the effects of rapamycin are not only time-specific but also species/cell-type specific (53).

Metformin is a commonly used treatment for type II diabetes. In addition to this well-known role, it has been shown that metformin possesses both direct and indirect antioxidant properties (102,140,160,177,188), with the activation of AMPK pathways being the main mechanisms for its effects. Metformin-activated AMPK leads to hyperphosphorylation, nuclear exclusion and up-regulation of mitochondrial gene expression (61). It inhibits mitochondrial respiratory-chain complex I (43, 140) and

modulates ATP production by mitochondria. Other, AMPK-independent actions of metformin were suggested (10), and several mechanisms that explain metformin's beneficial actions have been proposed, like NF-kB inhibition, increased in NO activity and/or production and SIRT1 activation and mTOR inhibition (53,138). By delaying p53 activation, metformin reduces oxidative stress, and it seems that in mice liver it could induce stress-response and antioxidant-linked proteins, including SOD (53, 122). Metformin inhibits the induction of the mitochondrial permeability transition in endothelial cells, preventing apoptosis (156). It seems that metformin shows tissue-specific action, a feature attributed to DR. Namely it inhibited ROS production in macrophages (53) while increasing ROS levels in white adipose tissue of mice (4).

Senolytics. One of the proposed mechanisms of action of oxidative stress is the induction of a cellular growth arrest state i.e. cellular senescence, characterized by the secretion of pre-inflammatory senescence-associated secretory phenotype (SASP) factors. Stress-induced senescence, often called stress-induced premature senescence (SIPS) or premature senescence (PS) is one of the forms of non-telomeric senescence, suggested to strongly contribute to the development of age-associated diseases, especially cancer, cardiovascular diseases, and neurodegenerative disorders. In that context, drugs that specifically target and induce apoptosis of senescent but not non-senescent cells named senolytics could be a promising route for antiaging interventions. The discovery of first senolytics, *Dasatinib* and *Quercetin* is closely related to the potential of the caloric restriction to delay the age-related increase in senescent cells (101) and senolytics and DR target the same signaling pathways in the cells (107). In humans, it has been shown that senolytics significantly decrease senescent cell burden (74). However, much remains to be optimized in this field before regular use of senolytics in humans, above all to show their effectiveness in a broad spectrum of age-related disorders and in reducing the multimorbidity commonly present in the elderly.

The effects of DR depend on the onset, duration and tissue type

Previous studies have shown that DR effects could vary significantly and that both duration and onset of DR could intervene with several physiological positive effects, including amelioration of age-associated increase in the level of oxidative stress in rhesus

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monkeys (94, 96) exposed to an adult-onset long-term (2.5 or 5 years) moderate DR (30% below control level). The same diet implemented in monkeys from early adulthood up to middle-age (10 years) resulted in a decrease of the expression of genes involved in ROS metabolism, detoxification, NO synthesis and oxidative deamination (93).

The age-dependent effect of DR has been also found in murine studies. Negative DR effects were noticed when DR is implemented in early life (19, 86, 197) or in aged subjects (49, 136). Moderate (60% of AL) restriction (19) or semi-starvation (50% of AL intake) (86) introduced for 2 months in adolescence rats (4 weeks of age), worsened performances in the MWM (19) and anxiety level (86). Dietary approach with a restricted amount of food available in order to keep the animal weight limited to the level of young Wistar rats showed that long-term DR causes negative effects on cognitive functions if started before the adulthood, at 2.5 months of age (197). In addition, 60% DR with a late-onset (after 18 months of age) and short duration (3 months) could also have a significant negative impact on motor abilities, cognitive performances and frailty level in 24 months old Wistar rats, as we have shown previously (179). Interestingly, in male Wistar rats late-onset EOD (18-21m) compensates an age-related increase in oxidative stress (162). In addition, a study in the male Sprague–Dawley rats indicated that 40% calorie restriction for 6 months improves the redox homeostasis in male rat heart during aging, even when it started in middle-adulthood (18 months) and when the bodyweight is stable (166). Others showed that 40% lifelong caloric restriction started early in life (196) shows its favorable effect and reverses the adverse effects of age-induced oxidative stress in the adrenal medulla and hypothalamus of aged Fischer 344 x Brown Norway rats.

Short-term DR could have positive effects both in the case of the early (161), or late onset (162) EOD lasted for 3 months and implemented early (3m) in Wistar strain young adult male albino rats induced beneficial effects on antioxidant system against kainate-induced excitotoxicity, and in different brain regions (161). The latter study showed broad beneficial outcomes of EOD started at 21 months of age in Wistar male rats on oxidative molecular damage to proteins (162). Authors suggested that even the short-term DR regimen has capability reversing an age-associated decline in oxidative stress scavengers and stress response proteins, at least partially and regardless of the age of the subjects.

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A 40% DR increased antioxidant capabilities in the brain and liver of old mice and rats, but not in young animals (32, 112). Rodriguez-Bies and colleagues demonstrated that 6 months of EOD (in a combination with exercise) increased Coenzyme Q level, prevented the age-related increase in lipid peroxidation and protein carbonylation (153), but only in aged and not in young male Swiss-OF1 mice. In C57BLY6 mice it was shown that specific age-related changes can be suppressed only by early onset DR (104), while other studies showed beneficial effects of DR even when light (10-20%) DR is implemented from adulthood, at 12 months of age (195). Protective effect of DR on the ROS production in mitochondria was found both after short term (1-month duration) and long-term (eight months) 60%DR in male C57BL/6 mice (23,24,25).

DR effects could also depend on a combination of onset and the tissue. For example, DR-induced reduction in protein oxidative damage was observed after 4 and 12 months of DR in the heart mitochondria, while in the liver beneficial effect of DR was evident only after life-long restriction (reviewed in 60). This indicates the existence of tissue specificity in a response to DR (192). The highest influence on oxidative damage DR exerts in the same tissues where the highest degree of oxidative damage was noticed and those tissues are composed predominantly of post-mitotic cells, such as the brain, heart and skeletal muscle (170). However, a strong impact on protein oxidation and antioxidant activity DR was shown also in spleen and plasma (39, 178).

An interesting region-specific action of a restricted diet was observed in the study by Whidden et al., (196) where an adult (18months) and very old (38months) male Fischer 344xBrown Norway rats were exposed to 40% CR regime. While in the adrenal medulla DR completely prevented the age-induced increase in lipid peroxidation, in the hypothalamus it exerted its effect on a different level, by restoring the age-related decline in antioxidant enzymes, CuZnSOD, and catalase. Although the adrenal glands and the hypothalamus are closely related structures playing a role in the sympathetic nervous system, DR exerted site-specific effects. This specificity in action is most probably due to the specific age-related changes present in these two organs. Namely, the majority of the antioxidant enzymes of the adrenal medulla remained unchanged with age, thus even a small enhancement of free radical production would exceed the ability of the organ to defend against oxidative damage, and therefore DR targeted the production of free radicals,

rather than directing the antioxidant system. On the other hand, in the aged hypothalamus, there was no statistically significant increase in oxidative stress and lipid peroxidation, but there was a significant reduction in both CuZnSOD and catalase level; therefore the logical target for DR action here is to increase antioxidant defense by raising the level of antioxidants. It should be also mentioned that in the same study lifelong DR failed to attenuate a marker for nitric oxide (NO)-dependent oxidative stress. Therefore, DR exerts a very specific cellular target-dependent protective action. On the other hand, as the aging brain is especially prone to oxidative damage, the maintenance of antioxidant capacity is crucial, and this action of DR is highly needed (196). Similarly, a heart and kidney-specific DR- induced increase in catalase activity was noticed; since both organs are very vulnerable to H_2O_2, and catalase modulation may be a primary mechanism by which DR acts (192). On the other hand, measurements of oxidative stress and antioxidant status in testis tissue showed that dietary restriction failed to improve the oxidative status, except for some individual antioxidant parameters (5). The organ specificity has been also found in the study by Parrado-Fernández and colleagues (142), where it has been shown that 60% average dietary intake implemented at 3 months old male C57BL/6 mice has organ-dependent effects: increasing Coenzyme Q levels in muscle, decreasing in heart and keeping it at a stable level in the brain (142). Resveratrol, a DR mimetic, also induced a tissue-specific response in antioxidant activity in the liver, heart, and muscle of mice (182).

Conclusion:

Since aging is an inevitable process, a great effort was invested during decades to understand it, and subsequently to find a way to slow it down. So far, dietary restriction is the only reproducible experimental intervention extending life expectancy in a broad array of species, from yeast to mammals (135) and a gold standard for the anti-aging interventions (192). With its property to target the production of free radicals in the cells, DR also targets the free radical theory of aging. It is very likely for reduced food amount to act as a mild stressor and trough preconditioning prepares cells to cope with the oxidative and metabolic stress associated with aging (124, 148).

Understanding the molecular mechanisms underlying beneficial effects of dietary restriction may also lead to the development of novel therapeutic agents –DR mimetics, which may reproduce the effects of DR, and reverse aging and diseases.

Nevertheless, an interesting question stands at the end-can we talk about the anti-aging effect of reduced food/ calorie intake on cellular oxidative stress if the free radical theory of aging is incorrect? Recently, this famous theory starts "getting old". Studies appear critically evaluating the premise that ROS-induced oxidative damage is responsible for aging (7, 57, 117, 154, 159) and proving that majority of the antioxidant defense system components are not linked with longevity (200), but on contrary- antioxidants correlate inversely with maximum longevity in vertebrates, and increase in their concentration does not increase maximum lifespan (7). At the same time, long-lived organisms have low rates of mitochondrial ROS production and low levels of oxidative damage of mtDNA. At the same time it is extensively and undoubtedly proven that DR extends life span in many species including mammals, and among other beneficial effects on the cells, decreases ROS production and therefore lowers oxidative stress and oxidative damage to biomolecules. However, although the effects of DR on general health and well-being are striking, they are not straightforward, but depend on various factors and have their limitations regarding the protective capacity. The ideal outcome of DR depends on the applied protocol, onset and duration, tissue and signaling pathway targeted, and all of these factors impose a caution for DR implementation in human practice (149). All of these beneficial but specific effects must be taken into consideration when designing an optimal dietary intervention in humans.

There is almost no treatment that has been studied as much as dietary restriction over the decades and centuries. Numerous beneficial effects on health and lifespan were proven, and underlying mechanisms of those effects discovered. However, DR as a potential panacea still fascinates us. With each new study, another missing piece of the puzzle is discovered, reminding us constantly that DR is much more than just the sum of its parts.

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Abbreviations:

AD - Alzheimer's disease

AMPK - 5' adenosine monophosphate-activated protein kinase

ARE - antioxidant response element (ARE) pathway

Aβ - amyloid-β peptide

BER - base excision repair

BMI body mass index

CALERIE - Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy

CDV - cardio vascular diseases

CR - Calorie restriction

DM - diabetes mellitus

DR - Dietary restriction

EOD - every other day feeding

FOXO - forkhead box

FR - food restriction

GCN2 - signaling network and general control nonderepressible 2

H_2O_2 -hydrogen peroxide

IF - intermittent fasting

IFR - two consecutive days a week fasting

IL-1β – interleukin 1β

MetS - metabolic syndrome

MS - multiple sclerosis

mTOR - mammalian target of rapamycin

NER - nucleotide excision repair

NO - nitric oxide

Nrf2 - nuclear factor erythroid 2-related factor 2

$O_2^{\bullet-}$ - superoxide anion

OH• - hydroxyl radical

PD - Parkinson's disease

PRXs - peroxiredoxins

ROS - Reactive oxygen species

RSV - resveratrol

GSH

SIRT - sirtuins

SOD – superoxide dismutase

TNF-α – tumor necrosis factor α

TRXs - thioredoxins

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Figure legends:

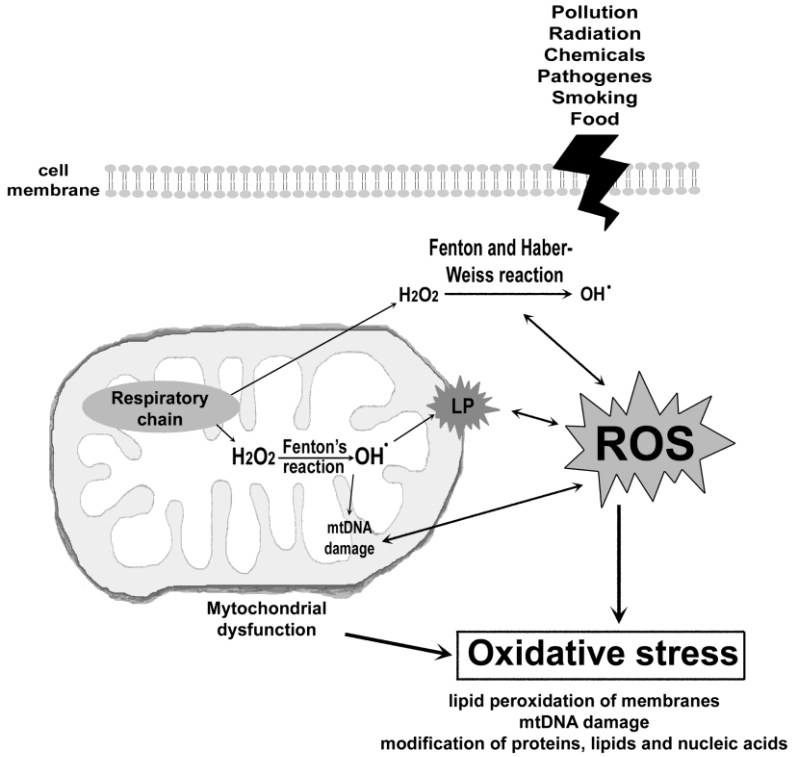

Figure 1. Sources of the reactive oxygen species (ROS). Mitochondria are primary consumers of O_2 and primary source of $O_2^{\bullet -}$ oxidants during the process of normal aerobic respiration. Mitochondrial complex III is the dominant site for the net production of ROS. ROS can also be generated from extrinsic sources, like diseases and pathology states (inflammation, obesity), during exposure to UV light, ionizing radiation, or heavy metal ions.

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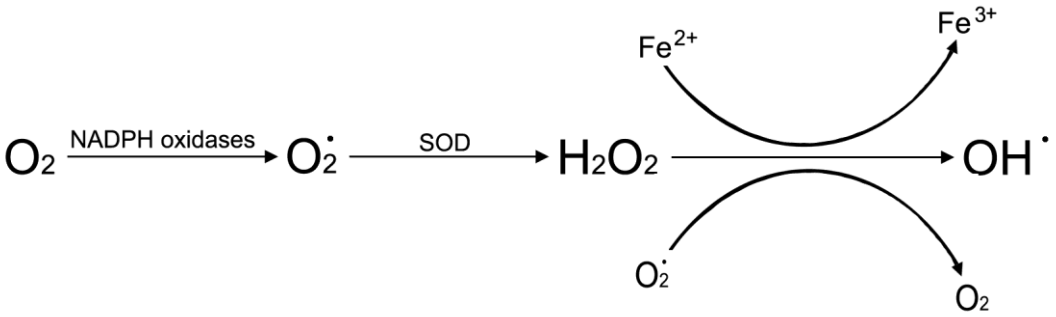

Figure 2. Generation of free radicals in the cell. Superoxide anion ($O_2^{\bullet -}$) is directly generated from the reduction of oxygen (O_2) and can be further converted to hydrogen peroxide (H_2O_2) with superoxide dismutase (SOD); H_2O_2 can be transformed to the most reactive form of oxygen, called hydroxyl radical (OH•) in a Fenton's reaction, in which ferrous iron (Fe^{2+}) reacts with hydrogen peroxide to produce the hydroxyl radical.

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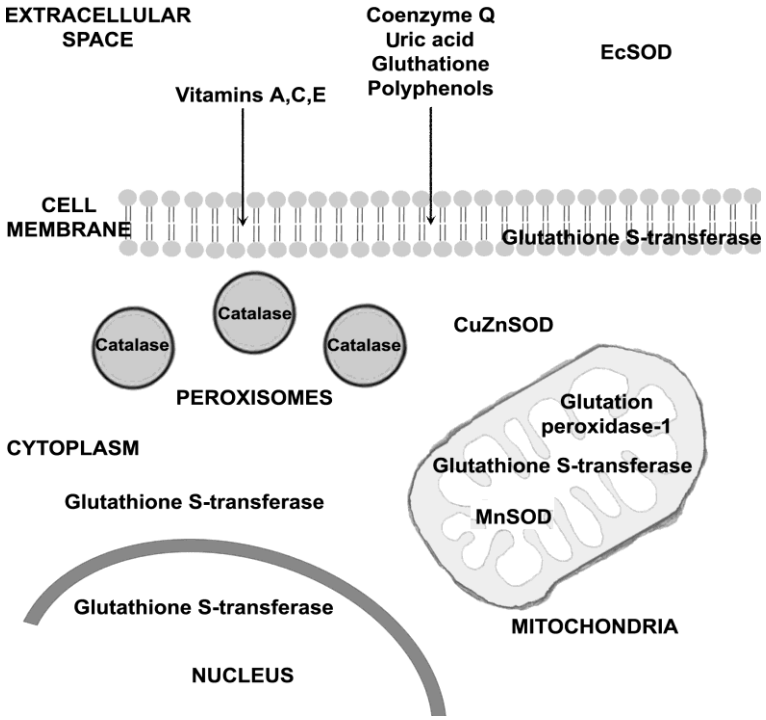

Figure 3. Simplified scheme of antioxidant defense systems in the cell. Two major classes of molecules with antioxidant properties can be found in different cellular compartments. Enzymatic component includes catalase in peroxisomes, glutathione peroxidase in both mitochondria and cytosol, glutathione S-transferase in the nucleus, cytosol and in the cell membranes, and superoxide dismutase (SOD), with 3 forms: CuZnSOD (in cytosol), Mn-SOD (in mitochondria) and EC-SOD (in extracellular space), while nonenzymatic component includes molecules like vitamins (A, C, E), Coenzyme Q, uric acid, glutathione, and polyphenols.

A)

	Effects	Consequences	References
DR	Free radical generation ↓	Decreased aging rate at cellular and organismal level	21, 22, 30, 39, 52, 93, 101, 109, 124
	Macromolecules oxidative damage ↓		
	H2O2 production ↓		
	Mitochondrial biogenesis and efficiency ↑		
	Glutathione ↑	Increased anti–oxidative defense	1, 48, 90, 132, 153, 154, 177
	Glutathione S–transferase ↑		
	DNA–repair capacity ↑	Maintenance of genome integrity	13, 15, 65,66, 131, 167

B)

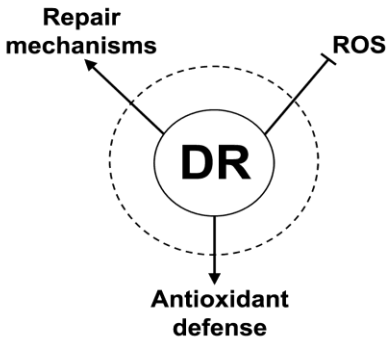

Figure 4: Beneficial effects of dietary restriction (DR) on oxidative stress. A) Overview of the main effects of DR on the ROS production and antioxidant capacity in the cell. B) Three levels of DR action are anticipated: DR decreases production of free radicals in the cells, and counteracts the consequences of oxidative stress by increasing the level and activity of various antioxidants, and by increasing the turnover of oxidized macromolecules. All of these DR beneficial pathways mutually intertwine.

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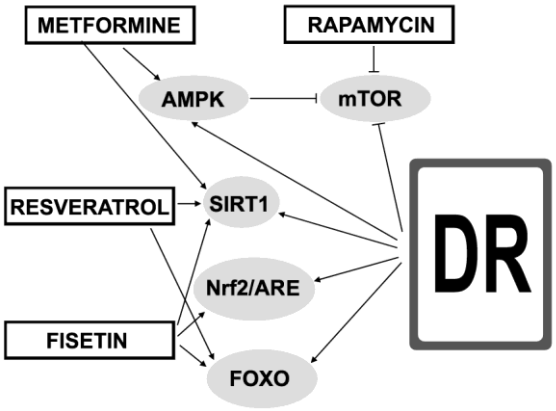

Figure 5. The downstream cellular targets shared between mimetics and dietary restriction. DR and metformin result in increased AMP:ATP ratio (not shown) that leads to AMPK activation and inhibition of mTOR. Rapamycin directly inhibits mTOR. DR, resveratrol, metformin and fisetin activate SIRT1. Both fisetin and DR exert their beneficial actions trough Nrf2/ARE pathway, while DR and fisetin enables binding of FOXO3a in the promoter of SIRT1 (not shown), further driving SIRT1 expression.

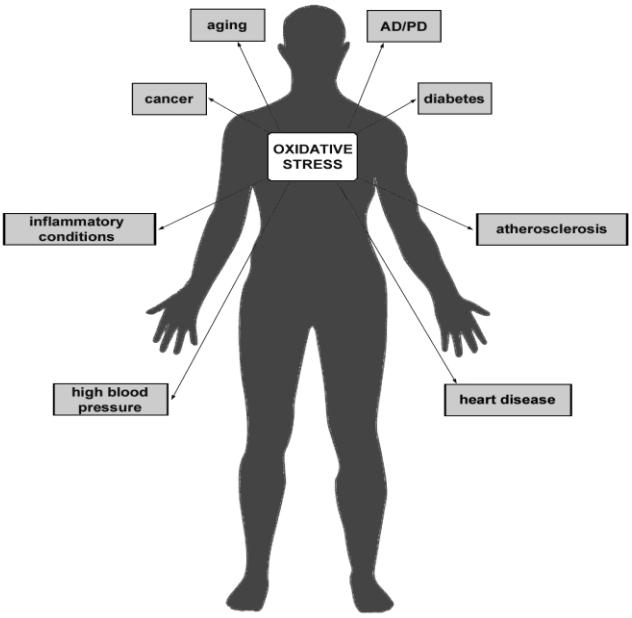

Figure 6. The role of oxidative stress in human diseases. Oxidative stress has been considered as one of the major factors involved in majority of age-related disorders, like atherosclerosis, hypertension, diabetes and neurodegenerative diseases like Alzheimer's and Parkinson's disease.

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