THE THEORIES OF AGING: REACTIVE OXYGEN SPECIES AND WHAT ELSE?

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This manuscript is a short review on the theories of aging, focusing mainly on the balance between the nutrient and the oxygen intake necessary for energy metabolism and the processes for neutralizing the negative consequences of energy production. The first section entitled "Why" provides brief historical details regarding the main group of aging theories, firstly the evolutionary theories and secondly the theories of aging related to humans, cells and biomolecules are discussed. The second section entitled 'Where' includes brief summaries of the many cellular levels at which aging damage can occur: replicative senescence with its genetic and epigenetic implications, cytoplasmic accumulation, mitochondrial respiratory chain dysfunction, peroxisome and membrane activity. In the third section entitled 'How' the linking mechanisms between the caloric restriction and the antioxidant intake on lifespan and aging in experimental models are discussed. The role of ROS is evaluated in relation to the mitochondria, the AMPK activated sirtuins, the hormesis, the target of rapamicin and the balance autophagy/apoptosis.

Lifespan is an intriguing concern, the boundaries of which are life and death; this is the reason why science and myth have shared their challenges on this topic since the beginning of human history. Senescence, illness and death are the origin of pain for the young Siddhartha and, before him, the reason for the research of the Sumeric hero Gilgamesh and surely also of many unknown men from the past to the present. This argument reflects the history of philosophic and scientific ideas as diffusely described in a number of reviews. The lengthening of the average lifespan is producing a growing interest concerning elderly people for a variety of reasons. The aim of this paper is to organize a part of the scientific literature about aging theories and the role of free radicals, permitting an easier understanding for many health operators who are dealing with the age-related needs of patients without necessarily being a mathematician, evolutionist or gerontologist.

1. Why: Evolutionary theories

The evolutionary biologists explain aging and longevity through the process of mutations and natural selection using mathematic tools. After Darwin and Wallace, Weismann in 1881 (1) hypothesized that the longevity of the organism was not dependent on its physiological constitution but the result of natural selection. He outlined that immortality is futile for nature, and senescence and death are a necessity for the species. In 1941, Haldane, during his studies on Huntington's disease, observed that natural selection acts with declining force at late ages and in 1946 the

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Nobel prize Medawar, developing the idea, formulated the "mutation accumulation" theory based on the hypothesis that evolution allows the accumulation of deleterious alleles in the elderly. In fact, natural selection is negligent in opposing events that occur in only a few long living animals that are able to provide small additional contribution to offspring numbers. In 1957, Williams summarized some critical arguments against Weismann's programmed aging theory and stated that natural selection would actively benefit genes having aging as a side-effect, provided they had a beneficial effect during youth, a concept which was then named antagonistic pleiotropy by Rose 25 years later (2). The two elements of this theory are that genes can simultaneously affect several traits (pleiotropy) and that their effects can be opposite on the individual fitness (antagonistic) at different ages. Since reproduction is a cost for species longevity, any mutation favoring more offspring would be propagated in future generations. Williams predicted that rapid individual development and reproductive maturation would be correlated to early senescence (3).

Hamilton in 1966 transformed all these ideas into a mathematical function describing the specific force of selection on mortality and fecundity. Charlesworth, between 1970 and 1980, developed a mathematical analysis demonstrating that, both mutation accumulation and antagonistic pleiotropy could lead to the evolution of aging thanks to the failing force of natural selection. The disposable soma theory proposed by Kirkwood in 1977 is a special case of the pleiotropic genes theory where the reduced investment of resources in the somatic cells permits reproduction that often occurs at the expense of survival (4).

In 1980, Rose demonstrated that when the first age of reproduction is delayed, aging is retarded. Moreover, in 1992 mortality rate plateaus were demonstrated in a variety of organisms, including humans, suggesting that a deceleration in aging occurs at a late stage (late life) (2).

2. Where: localization of the damage

Although evolutional biologists explain aging as a Darwinian phenomena, biological researches were progressively focused on individual physiology, involving more and more smaller structures, from organism to cell and bio-molecules. In fact, molecular alterations related to aging can produce cellular, organ and systemic failure with subsequent evolutionary implications (survival and reproduction) (5).

Historically the "rate of living" theory, proposed by Pearl in 1928, was focused on organisms, hypothesizing that they have a finite number of breaths, and therefore a slow metabolism could be linked to a long life (6).

With regard to the cell, the initial idea for the existence of a cell division limit was from Weismann and only later was confirmed by Hayflick (7) (1961) in cellular cultures.

In 1956, Harman realized that the reactions initiated by oxygen free radicals (ROS) could be the driving force of the increasing macromolecular damage underlying the aging process (8, 9). He translated his experience in biology, starting from a professional background acquired at Shell Oil Company, working on the chemical properties of free radicals. The "free radical theory of aging" is a mechanistic theory that focuses on a single basic cause of damage unifying organisms, cells and molecules. The damage is the result of the two major oxidative processes employed by living organisms to produce energy (photosynthesis and phosphorylation) together with the effects of ionizing radiations.

Nucleus

The DNA integrity is dependent on exogenous elements (physical, chemical and biological) and on endogenous factors. The latter are represented not only by the direct damage generated by ROS on the DNA molecule but also by the spontaneous mutations deriving from replication errors.

The replicative senescence is caused by a progressive shortening of the telomeres (10). These structures are located at the terminal part of the chromosomes and are composed by DNA sequences that cannot be completely copied during mitotic division, resulting in a progressive shortening of the chromosomes to the point that cellular division becomes impossible, as theorized by Olovnikov in 1971 (11) and demonstrated by the Nobel prize 2009 Blackburn in 1978 (12). The telomere dysfunction produces a cellular growth arrest which is mediated

by the protein p53, an important tumor suppressor factor acting through the peroxisome proliferator-activated receptor gamma, co-activator 1alpha and beta (PGC) (13).

Epigenetic factors (14) play a further role in DNA damage. Among these, the histone methylation that impacts on the insulin/IGF1 signaling pathway and the histone deacetylation that is affected by the sirtuins. A further insight into these mechanisms will be shown in the third section of this review. Changes in the chromatin architecture are characteristic features in aging. Furthermore, age-related transcriptional changes affect non-coding RNAs (gero-miRs) related to the aging process and to stem cell behavior. Finally, an important role is played by the proteomic control: the systems for the stabilization of correctly folded proteins (particularly the heat shock family) and the proteolytic systems (15).

Cytoplasmic accumulation

Exogenous toxicants may affect the onset, rate, and extent of the cellular growth curves by directly increasing mortality, interfering with nutrient uptake and with the cross-membrane transport, disrupting the protein and enzyme function. In addition, exposure can lead to an increase in energy required for cellular maintenance processes. The damaged parts of the cells cause the production of 'incorrect' proteins which can accumulate, damage the cell itself and increase death probability (16).

Cellular aging can result from accumulation of endogenous protein aggregates, as observed in many age-related diseases. In an experimental study on *Escherichia coli*, a fluorescent marker was used to identify *in vivo* the localization of the protein aggregates, revealing their accumulation during the cell division in the cells with older poles. This suggests an asymmetric strategy whereby the dividing cells can segregate damage at the expense of the oldest individuals, resulting in the perpetuation of the population. Differentiated post-mitotic cells, such as neurons, cannot segregate the damage and consequently the protein aggregate accumulation is related to diseases (17, 18).

Mitochondria

In humans, 13 essential proteins of the respiratory chain, the ATP synthase, a set of mitochondrial tRNAs and the small and large subunit of the mitochondrial ribosomal RNA are encoded on mitochondrial DNA. The vast majority of mitochondrial proteins are nuclear encoded, synthesized in the cytoplasm, imported into mitochondria and sorted to the different sub-compartments where they may be assembled into macromolecular complexes on the basis of their function (e.g. respiratory chain complexes) (19).

The "mitochondrial free radical theory of aging" is based on the idea that ROS production, by mitochondrial respiratory chain, progressively damages the constituents of mitochondria itself, inducing mitochondrial DNA mutations and a subsequent respiratory chain dysfunction with a supposed further increase in ROS production in a vicious circle, leading to cellular senescence. The accumulation of mitochondrial DNA mutations can be generated by replication errors. A single mutation in the mitochondrial DNA can self-amplify. In fact the replications of the mitochondrial DNA and of the nuclear DNA are not coordinated. Thus the mitotic segregation produces further cells that exhibit homo- or hetero-plasmy (they can contain only normal or mutated DNA or a mixture of both). During embryogenesis a massive DNA replication occurs and this results in errors, segregation and clonal expansion in postnatal life. Furthermore, accumulated damage can overwhelm the repair systems (excision-repair mechanism) and result in accumulation of mutations in the mitochondrial DNA (20). Although it is clear that mitochondria play important roles in aging, the situation appears more complex than originally proposed in the mitochondrial free radical theory of aging. Aging and death are not necessarily equivalent. The involvement of mitochondria in cell death, and its being the cause or the consequence, remain issues that are complex to address. For example, they contain and release proteins that are involved in the apoptotic cascade, such as cytochrome c, moreover, the mitochondrial permeability transition pore (PTP) is a key effector of cell death. The opening of the PTP has been demonstrated to be causally involved in cell death associated with many diseases, including heart ischemia (21).

Peroxisomes

The peroxisomes, and not only the mitochondria,

produce significant amounts of ROS, and peroxisomal dysfunction may also contribute to cell death and to aging. The peroxisomes are ubiquitous eukaryotic organelles which perform a plethora of functions, including hydrogen peroxide metabolism and the β-oxidation of fatty acids. In man, peroxisomes are involved in the α- and β-oxidation of very long chain fatty acids, in the biosynthesis of phospholipids and bile salts and acids. Recently, new non-metabolic functions have been identified for the mammalian peroxisomes, among which the anti-viral innate immunity and the anti-viral signaling. Peroxisomes and mitochondria share some key components of their fission machineries. The autophagy can result in a reduction in the number of peroxisomes This mechanism is consistent with the idea that the timely rejuvenation of peroxisomes is vital for cellular viability and survival (13).

Membrane

Phospholipids containing highly polyunsaturated fatty acids are particularly prone to peroxidation and membrane composition may contribute to explain the special role played by peroxisomes in extending longevity (22). The "membrane theory of aging" is based on the observation that the number of double bonds in membrane phospholipids is inversely correlated with lifespan. Among tissue macromolecules, unsaturated fatty acids are those most sensitive to oxidative damage. This is due to the presence of species-specific desaturation pathways and to a cycle of deacetylation-reacetylation, that is fundamental in determining the membrane composition and, probably, in maintaining a low degree of fatty acid unsaturation in long-living animals (23, 24). This decrease in membrane unsaturation may be able to extend the longevity by increasing the resistance of membranes to the lipid peroxidation (25).

All the functions of the cell strongly depend on phospholipids, whose fatty acid side chains are an important contributory factor to membrane structure. A variety of stimuli utilizes an increase of the $Ca^{(2+)}$ concentration as a second messenger to transmit signals, through release of $Ca^{(2+)}$ from the endoplasmic reticulum or opening of $Ca^{(2+)}$ channels across the plasma membrane. Mitochondria contribute to the tight spatiotemporal control of this process by accumulating $Ca^{(2+)}$, thus permitting the return of cytosolic $Ca^{(2+)}$ to resting levels. The rise of the $Ca^{(2+)}$ concentration in the mitochondrial matrix stimulates oxidative metabolism. In the presence of a variety of factors of physiological relevance, the matrix $Ca^{(2+)}$ increase can also lead to opening of the permeability transition pore (PTP), the transient opening of which can provide a fast $Ca^{(2+)}$ release, whereas a persistent PTP opening is followed by deregulated release of matrix $Ca^{(2+)}$, termination of oxidative phosphorylation, matrix swelling with inner membrane unfolding and eventually outer membrane rupture with the release of apoptogenic proteins and cell death. Thus, a rise in mitochondrial $Ca^{(2+)}$ can convey both apoptotic and necrotic death signals by inducing opening of the PTP (26).

3. How: Experimental models

Studies using model organisms such as yeasts, *Cernorabtidis elegans*, Drosophila, and mice have shown that both inhibition and activation of mitochondrial function can extend lifespan. A mild increase in ROS may underlie a common mechanism in these seemingly paradoxical findings (27). This increase in ROS is supposed to mediate retrograde signaling, from mitochondria to the nucleus, and be able to elicit protective cellular responses. A mild inhibition in mitochondrial respiration is able to extend the lifespan of various species and this longevity is not caused by a simple slowing of metabolism (28). The Hormesis (from Greek: to excite) (29) is based on the idea that the feedback regulation gives rise to a better answer to oxidative stress when there is chronic exposure to a limited amount of free radicals and this would permit an improvement of scavenging and an adaptive beneficial effect on the cell and the organism. In other words, a stressor may have beneficial effects at relatively low doses and deleterious effects at high doses. This model (30) was first applied by Southam and Ehrlich (1943) to better describe the dose–response curve in toxicology. A typical hormetic curve is an inverted U-shape if the endpoint is growth or longevity (whereas if the endpoint is disease incidence, the dose–response would be described as U- or J-shaped). In this context, as the dose decreases there are not only quantitative changes in the response measured but also qualitative changes,

so a mild ROS increase would be able to boost the defenses against oxidative stress, achieving a new hormetic steady state.

Dietary restriction

Experiments conducted on animal models demonstrated that a restricted diet produced a longer lifespan than in *ad libitum* fed animals. In fact, the yeast *Saccharomyces cerevisiae*, the nematode *Cernorabditis elegans,* Drosophila, mice, and primates have been shown to live longer with caloric restriction (CR). Even in humans, health and potentially the lifespan seem to be positively affected by CR which, among its effects, improves the nitric oxide bio-availability in the cardiovascular system resulting in a decrease in blood pressure (31). Many investigations agreed that long-term CR of 40% significantly decreases the rate of mitochondrial ROS generation in rat organs, demonstrating that mitochondria in CR are different and the main decrease in ROS production occurs at the level of complex I (32).

During CR, the mitochondrial respiration is enhanced and ATP production does not decrease, probably because it improves the activity of many important factors in mitochondrial biogenesis and respiration, stimulating the mitochondria to work in a more effective way. Electron microscopy analysis showed that the number of mitochondria is increased in liver tissues of CR mice. Thus, CR may increase the mitochondrial mass by altering the expression level of genes that promote mitochondrial biogenesis, to assure that the net level of respiration is maintained and the amount of energy produced by cells is sufficient. In an experimental model, the glucose restriction, through inhibition of glycolysis in *Cernorabtidis elegans*, stimulated mitochondria to function more efficiently but simultaneously to produce more ROS. The increased ROS production seems to be required for lifespan extension; in fact, antioxidant treatment suppressed the lifespan elongation resulting from glucose restriction (28). A literature analysis described (32) a negative correlation between endogenous tissue antioxidant level and lifespan, and outlined that long-living animals did not need to maintain high antioxidant enzyme levels but simply were able to induce antioxidant production when needed.

Insulin-like signaling pathway

The lifespan extension associated with CR in model organisms is believed to be related to the insulin-like growth factor (IGF-1) and insulin levels and signaling. IGF-1 mediates many of the effects of the growth hormone; it is a single chain of 70 amino acids that displays homology to insulin. In mice, low levels of IGF-1 and insulin are able to cause the longest lifespan extension but also a reduction of age-related pathologies, including cancer and insulin resistance or diabetes (33). Some studies on animal models and on centenarians demonstrated a correlation between the reduction of GH or IGF-1 and length and quality of life (34).

The decrease in energy availability can represent the metabolic common denominator both for the impaired insulin/IGF-1 signaling and for the CR. Unlike glucose, ATP generation from fatty acids and/or amino acids can only take place in the mitochondrial compartment, and requires the oxidative phosphorylation. The combined gene expression analysis, using data from three different animal models with impaired insulin/IGF-1 signaling, demonstrated the activation of mitochondrial L-proline metabolism and a transiently increased ROS level. This probably induces an adaptive response that culminates in increased stress resistance and lifespan extension (35).

Moreover, the increase in the AMP/ATP ratio activates a protein kinase (AMPK) which increases the mitochondrial biogenesis. The pathway is through the activation of sirtuin 1 (SIRT1).

Seven different sirtuins or silent information regulators, with distinct subcellular localization, were identified in mammals: they are NAD+-dependent protein-deacetylases. Experimental evidence supports that SIRT1 (present in nucleus and cytoplasm) can mediate an oxidative stress response through direct de-acetylation of some transcription factors that regulate antioxidant genes.

SIRT3 is present in mitochondria; its role is to boost the catalytic activity of SOD2. SIRT3 is necessary during caloric restriction to mitigate oxidative stress. Additionally, SIRT3 stimulates the activity of the mitochondrial isocitrate dehydrogenase (IDH2) during caloric restriction. IDH2 promotes the conversion of NADP+ to NADPH, which in turn provides the reducing equivalents for conversion of

the oxidized glutathione to reduced. This protein is stabilized by the presence of ROS and activates a gene expression program that enhances survival and growth in hypoxic environments, as typically found inside solid tumors (36).

A further element is a family of evolutionary conserved protein kinases that regulate the balance between protein synthesis and degradation named TOR, or targets of rapamicin, (a lipophilic macrolide, isolated from a strain of *Streptomyces hygroscopicus* indigenous from Easter Island or Rapa Nui). In the presence of sufficient nutrients to fuel protein synthesis, TOR provides a permissive signal to translation, to ribosome biosynthesis, and to the amino acid permeases. In the absence of TOR signaling, the translation of mRNAs is specifically inhibited, the ribosome biosynthesis is blocked, and the autophagy is activated. A rapamycin supplementation to yeast cultures, or to mammalian cells in culture, induces autophagy, even in a nutrient-rich medium. In mammalian cells this autophagy is inhibited by amino acids and insulin (37). A recent research on telomere dysfunctional mice (38), demonstrated how the beneficial effects of glucose substitution on mitochondrial function and glucose metabolism are blocked by TOR inhibition but mimicked by IGF-1 application.

Autophagy and apoptosis

Autophagy and apoptosis control the turnover of organelles and proteins within cells, and of cells within organisms, respectively. Many stress pathways sequentially elicit autophagy and apoptosis within the same cell. Generally, autophagy blocks the induction of apoptosis, and apoptosis-associated caspase activation shuts off the autophagic process. The dialogue between autophagy and cell death pathways influences the normal clearance of dying cells, as well as the immune recognition of dead cell antigens. However, in the majority of cases, it seems that apoptosis and autophagy are mutually inhibitory. As many cellular stress pathways sequentially induce autophagy (at early stages and low doses of stress) and apoptosis (at late stages and high doses of stress). In addition, it seems that activation of the apoptotic caspase can degrade proteins that are essential for autophagy, shutting down the autophagic process and convert pro-autophagic proteins into pro-apoptotic ones (39). Healthy aging depends on the removal of damaged cellular material that is in part mediated by autophagy. The nutritional status of cells affects both aging and autophagy through acetylation, a process that rivals phosphorylation in importance. It has been demonstrated that the nucleocytosolic acetyl-coenzyme A (AcCoA) acts as a metabolic repressor of autophagy during aging in yeast, and this is associated to a reduced lifespan (40). The caspases, far from being merely cell death effectors, exhibit a wider range of functions. In fact, they have an essential role in cell proliferation, migration, and differentiation. There is also evidence that apoptotic cells can direct the behavior of nearby cells through the caspase-dependent secretion of paracrine signaling factors (41).

CONCLUSION

A mechanistic insight identifies the main causal factor of aging in the metabolism: oxygen and food and subsequent handling waste. The fuel of the metabolism is introduced into a machine made of interconnected systems of organs and cells. Every machine can have a different durability related to the quality of its components given by its DNA, but during the cellular replication a part of DNA cannot be copied to the point that cellular division becomes impossible. If this is caused by error or necessity is matter that can be better explained in evolutionistic terms.

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