The Mitochondrial Free Radical Theory of Aging: A Critical View

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Abstract: The Mitochondrial Free Radical Theory of Aging (MFRTA) proposes that mitochondrial free radicals, produced as by-products during normal metabolism, cause oxidative damage. According to MFRTA, the accumulation of this oxidative damage is the main driving force in the aging process. Although widely accepted, this theory remains unproven, because the evidence supporting it is largely correlative. For example, long-lived animals produce fewer free radicals and have lower oxidative damage levels in their tissues. However, this does not prove that free radical generation determines life span. In fact, the longest-living rodent -*Heterocephalus glaber*- produces high levels of free radicals and has significant oxidative damage levels in proteins, lipids and DNA.

At its most orthodox MFRTA proposes that these free radicals damage mitochondrial DNA (mtDNA) and in turn provoke mutations that alter mitochondrial function (e.g. ATP production). According to this, oxidative damage to mtDNA negatively correlates with maximum life span in mammals. However, in contrast to MFRTA predictions, high levels of oxidative damage in mtDNA do not decrease longevity in mice. Moreover, mice with alterations in polymerase gamma (the mitochondrial DNA polymerase) accumulate 500 times higher levels of point mutations in mtDNA without suffering from accelerated aging.

Dietary restriction (DR) is the only non-genetic treatment that clearly increases mean and maximum life span. According to MFRTA caloric restricted animals produce fewer mitochondrial reactive oxygen species (mtROS). However, DR alters more than free radical production (e.g. it decreases insulin signalling) and therefore the increase in longevity cannot be exclusively attributed to a decrease in mtROS generation. Thus, moderate exercise produces similar changes in free radical production and oxidative damage without increasing maximum life span.

In summary, available data concerning the role of free radicals in longevity control are contradictory, and do not prove MFRTA. In fact, the only way to test this theory is by specifically decreasing mitochondrial free radical production without altering other physiological parameters (e.g. insulin signalling). If MFRTA is true animals producing fewer mtROS must have the ability to live much longer than their experimental controls.

Keywords: Aging, comparative biology of aging, mitochondrial free radical theory of aging, mitochondria, reactive oxygen species, oxidative damage, dietary restriction, exercise.

INTRODUCTION

Nowadays, aging is one of the most important socio-sanitary problems in western countries. An improving quality of life has enormously increased the number of older people, leading to augmented sanitary expenses. This has created a very serious social problem that can only be solved through better understanding of the biological mechanisms behind aging. Aging is a complicated biological process, and it must be investigated from several very different perspectives. The best definition of aging relates to survival rate: "Aging is the result of the progressive accumulation of deleterious changes that reduce an organism's ability to resist stress causing a decrease in survival possibilities" [1]. Thus, aging has four characteristics [2]: 1) progressive, 2) endogenous, 3) universal, and 4) deleterious.

The scientific method relies upon theories to explain the natural world. These theories are the basic framework upon which scientists work, using them, to make predictions that can be tested in the laboratory. Theories must be constantly tested until they are proved as valid or not. Nowadays, the most popular theory used to explain aging is the Mitochondrial Free Radical Theory of Aging.

Denham Harman originally proposed the Free Radical Theory of Aging in his seminar paper titled "Aging a theory based on free radical and radical chemistry" [3]. This paper was first published more than 50 years ago. In his article, Harman proposed that aging is the result of the accumulation of damage caused by free radicals generated as by-products during normal metabolism. Initially, Harman's theory did not obtain much support from his peers. However, the situation changed after the discovery of the anti-free radical enzyme superoxide dismutase (SOD) by McCord and Fridovich in 1969 [4]. If the cell makes something to detoxify free radicals, then free radicals must be produced *in vivo*. Only four years after the discovery of SOD, Britton Chance's group described the production of hydrogen peroxide in isolated mitochondria [5]. This demonstrated that even during normal mitochondrial respiration, oxygen is incompletely reduced and gives rise to highly reactive (and unstable) molecules termed "reactive oxygen species" (ROS; mtROS when they are produced by mitochondria). Free radicals production ranges from 0.1 to 4% of the oxygen consumed. Nowadays, it is well established that complex I [6], complex III [7], glycerol 3-phosphate dehydrogenase [8] and alpha-ketoglutarate dehydrogenase [9] produce free radicals in

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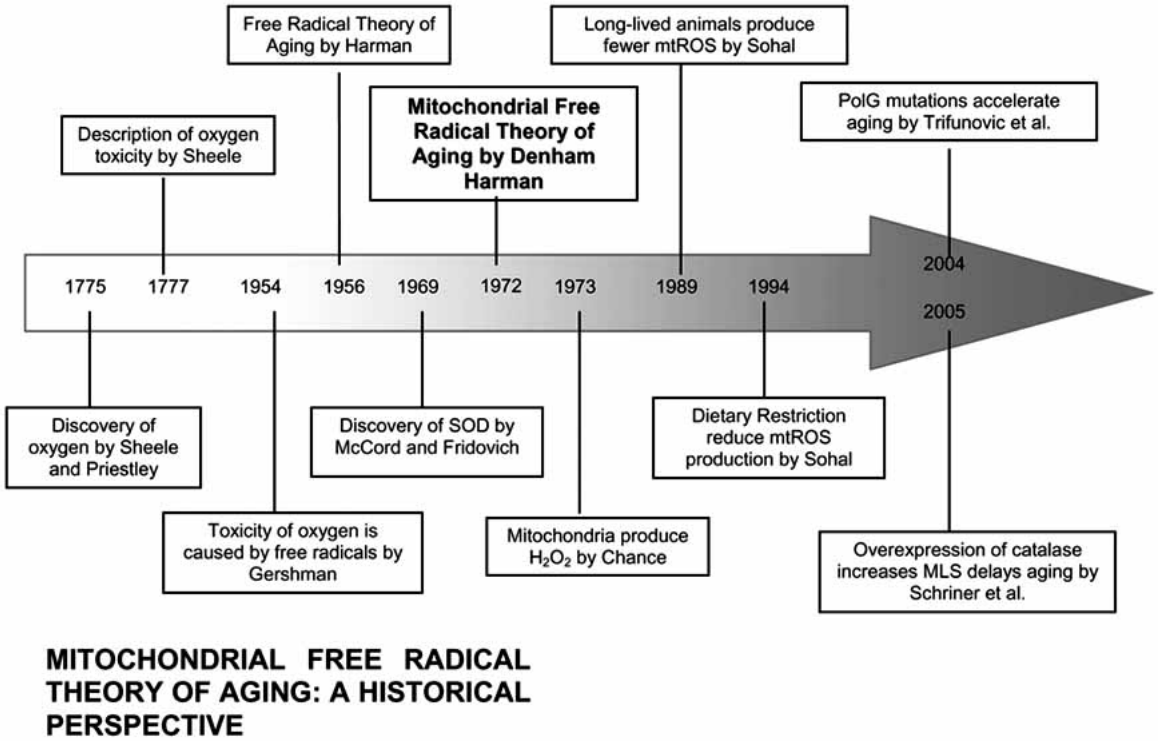

Fig. (1). Summary of the most important discoveries related with the Mitochondrial Free Radical Theory of Aging.

isolated mitochondria. In 1972, Harman himself singled out the mitochondrion as the main place where free radicals are generated and, concurrently, the main target of free radical action [10]. Since then, the Free Radical Theory of Aging has become the Mitochondrial Free Radical Theory of Aging (MFRTA), which is the most famous version of Harman's theory (Fig. **1**).

Since the discovery of SOD, numerous antioxidant systems have been found in cells. In spite of all these anti-free radical systems, there is some oxidative damage *in vivo*. This is demonstrated by the existence of oxidation-derived non-enzymatic molecular modifications such those in DNA, reactive aldehydes from glycation or lipid peroxidation reactions, and cross-linked sulphur bridges in proteins. This indicates that antioxidant systems are not 100% effective in their interception/repair capabilities. Initially, it was thought that the aging rate was controlled by endogenous antioxidants. This possibility was enormously attractive because it could provide the opportunity to increase longevity with the aid of antioxidants. In invertebrates the results of such experiments are contradictory [11]. Some studies show big changes in longevity after over-expression of antioxidants (e.g. SOD [12]), but others do not show any change [13]. However, the supplementation, induction or over-expression of antioxidants has continuously failed to significantly increase maximum life span (MLS) in mammals [14,15]. As a rule, antioxidants increase mean life span when rearing conditions are sub-optimal. However, the effect under optimal conditions is minimal on mean and inexistent on MLS. The lack of effect on MLS is quite indicative of the inefficiency of antioxidants as anti-aging therapies. It is important to distinguish between mean and MLS. Mean life span is the mean number of years than an individual can live because he is born in a specific ecosystem. For example, a human being born in Africa would live about 30 years, while a European or North American would live until the age of 70. MLS is the maximum number of years that an individual can live because he is member of a particular species. For instance, the oldest rat lives for four years, while the oldest human lives up to 122. The environment mainly determines mean longevity. On the other hand, MLS is primarily fixed in the genome. Over the course of human's history we have increased the mean life span, but have not markedly increased MLS (also in the past some people lived more than one hundred years but the number of centenarians was lower). Western countries have almost maximized mean life span so in order to delay aging (and aging associated disease, e.g., cancer or Alzheimer) we must increase MLS. In conclusion, real anti-aging therapies must therefore increase MLS (e.g. DR), if not, they cannot be considered as anti-aging therapies (e.g. antioxidants).

Most researchers interpreted the absent effect of antioxidants on the rate of aging as evidence against MFRTA. But in the nineties, a group led by Rajindar Sohal published data offering a feasible explanation for this. Mitochondrial free radical generation (or mtROS) is negatively correlated with longevity in mammals [16,17]. This indicates that long-lived animals produce fewer free radicals than short-lived ones. On the other hand, antioxidant levels in tissues do not correlate or are negatively correlated with MLS [18], which means that long-lived animals have fewer antioxidant defence mechanisms in agreement with their lower levels of endogenous damage generation. This also explains "antioxidant failure". Increasing antioxidant levels as a strategy to increase longevity is, therefore, not effective both in short- and long-lived species because they have, under normal conditions, enough to guarantee their MLS. From an evolution-

ary point of view, there are three possible ways to increase longevity: 1) decrease damage accumulation, 2) increase defences and/or 3) increase repair. Energetically the most effective way to increase longevity is to decrease endogenous damage generation and this is the pathway which evolution has favoured.

Dietary Restriction (DR) studies also provide strong support for MFRTA. Reduction of calorie intake increases mean and MLS and decreases mtROS production [19]. In the last few years, two new discoveries have reinforced MFRTA. First, it has been described that the modification of proofreading activity of polymerase gamma (the mitochondrial polymerase; PolG) increases the accumulation of mutations [20,21] and therefore accelerating aging. And second, catalase overexpression in mitochondria increases MLS in mice [22].

In summary, MFRTA is supported by evidence coming from three different experimental approaches: 1) comparative biology studies, 2) dietary restriction and 3) molecular manipulation of mitochondrial function. Although, MFRTA is probably the most supported theory today, there are some flaws that must be discussed. In fact, some data indicates that we should rethink the role of free radicals in aging.

THE PREDICTIONS OF THE MITOCHONDRIAL FREE RADICAL THEORY OF AGING

The best way to validate or discard a theory is to make predictions using it, and design experiments to test if those predictions are correct or not. The first and most obvious prediction of MFRTA is that individuals with low levels of mitochondrial free radical generation must live longer. Obviously, the best way to test MFRTA is by specifically decreasing mtROS production, and measuring the effect if any of such a modification on longevity. Only if MLS is significantly increased can it be concluded that the aging process is casually connected with mitochondrial free radicals.

Reducing electron leak from the electron transport chain (ETC) is a beautiful problem. Unfortunately, the process of electron transfer inside the chain is not totally understood yet, and any direct manipulation of the respiratory complexes would probably cause more disadvantages than advantages (e.g. an ATP crisis). Nowadays, the most feasible possibility is to marginally uncouple mitochondrial respiration. Increasing oxygen consumption during state 4 could decrease free radical production. In theory, this decrease in mtROS should increase life span. In *Drosophila melanogaster* the overexpression of human uncoupling protein 2 in the nervous system reduces ROS production and increases MLS by between 5-10% [23]. This increase is not very significant compared to the effect of DR in *Drosophila* that increases longevity by between 30-60% [24]. Paradoxically, DR does not affect mtROS production in flies [25]. One of the problems with using uncoupling proteins (UCPs) is that they would mainly decrease mtROS at complex III. Electron leak at complex III is strongly dependant on membrane potential, whereas complex I is less affected by variations in this parameter [26]. Since complex I appears to be the most relevant complex for aging, modifications in complex III will have a low impact on longevity. There are important side effects of using UCPs. They could affect ATP synthesis if they decrease the proton gradient during state 3 [27] (when ATP is synthesized). Since aging is characterized by a decrease in ATP synthesis in flies [28], mice [29] and humans [30] the use of UCPs could increase mortality at old ages. The ideal situation would be to decrease mtROS generation without altering mitochondrial respiration (especially during state 3). This is possible since during DR mtROS generation is decreased without any alteration in mitochondrial respiration [19]. The problem is to knowing how to achieve this.

The alternative, to the former approach is to compare the rates of mtROS production in species or individuals with very different longevities. Species age at different rates, making it possible to study if they produce different amounts of free radicals. If MFRTA is true, long-lived species must produce fewer mtROS and have lower levels of oxidative damage. The same can be done for a single species, if there is a possibility of increasing MLS. Since DR increases MLS between 30-60% [31] it offers an excellent opportunity to test MFRTA. Again, if Harman's theory is correct, dietary restricted animals must produce fewer mtROS.

MFRTA also makes secondary predictions. According to MFRTA, aging is caused by the accumulation of oxidative damage produced by free radicals. MFRTA predicts that long-lived animals or individuals have lower levels of oxidative damage in lipids, proteins, carbohydrates and nucleic acids. It is also possible to predict that biological molecules from long-lived animals are more resistant to oxidative stress, which seems be the rule. The mitochondrion is the only cellular structure in animals that has its own DNA. MFRTA predicts that long-lived animals or individuals must accumulate mitochondrial mutations slower than short-lived ones. Thus, MFRTA also predicts that any increase in the rate of mitochondrial mutations must accelerate the rate of aging.

MFRTA also predicts that longevity must be increased if free radicals are intercepted before they can cause damage. The failure of antioxidants to increase longevity reflects their incapacity to completely intercept and therefore prevent damage. Free radical species are extremely reactive, so if the ROS generator and the target are in close proximity (e.g. inner mitochondrial membrane and ETC); there is no time or space to intercept before damage. However, if oxidative damage is reduced (through increasing interception) longevity must be also increased.

COMPARATIVE BIOLOGY: MITOCHONDRIAL FREE RADICAL PRODUCTION, MACROMOLECULAR RESISTANCE TO OXIDATION AND MAXIMUM LIFE SPAN

MFRTA is strongly supported by the Comparative Biology of Aging (CBA) data. Undoubtedly, CBA is an excellent tool for studying aging and saves both time and money. The results are usually, correlations between some physiological parameter and MLS. It is well known that in Science correlation does not imply causation. However, correlations are useful to discard factors that cannot explain aging. It is unlikely that something not correlated with MLS can cause aging.

During the nineties, it was described that mtROS generation –both superoxide and hydrogen peroxide- negatively correlates with MLS in mammals [16,17]. Thus, the same relationship between free radical production and life span was found in different species of flies [32]. The problem of classical Sohal's studies [16,17] is that the metabolic rate of mammals studied, also correlates with MLS. This makes it impossible to distinguish which factor –metabolic rate or mtROS- is responsible for the rate of aging. The solution is to study mtROS generation in animals with the same metabolic rate but different longevity. As a general rule, birds live longer than mammals of the same size (metabolic rate). For example, pigeons live 35 years whereas rats only live four. When, free radical production is studied in the isolated mitochondria of pigeons and rats, pigeon mitochondria produce fewer mtROS [33]. Since pigeons and rats have the same metabolic rate, this factor cannot be responsible for differences in longevity. For technical reasons all those studies were carried out in isolated mitochondria using succinate as substrate. The use of succinate as the only substrate has two major problems: 1) to determine which complex (I or III) is responsible for ROS generations (both of them are producing ROS) and 2) to distinguish between forward and reverse transfer of electrons. With succinate as only substrate most of the free radicals are produce due to the reverse transfer of electrons between complex II and I. It is unknown if reverse flux is physiological, but it seems unlikely that it continually occurs *in vivo* conditions.

Other laboratories have confirmed the negative relationship between free radical production and life span [34]. The use of different substrates and inhibitors make it possible to locate in which respiratory complex mtROS are produced. When pigeon mitochondria are supplemented with pyruvate(+malate) or succinate they produce fewer mtROS than rat mitochondria. Under these circumstances both respiratory complexes I and III produce free radicals. However, differences disappear when succinate is combined with rotenone. In these conditions only complex III leaks superoxide (reverse flux is blocked due to rotenone inhibition of complex I). Since ROS production at complex III is the same, differences related with longevity are localized at complex I. Also canary and parakeet mitochondria leak fewer ROS than mice mitochondria with pyruvate (+malate) as substrate [35]. Unfortunately, no data has been reported using succinate or succinate + rotenone so it is not possible conclude that differences are exclusively located at complex I. In fact, some reports suggest that complex III can also leak fewer ROS in long-lived species. Both complexes (I and III) produce fewer ROS in human (MLS = 122 years) than in rat (MLS = 4 years) mitochondria [36]. Other study shows that complex III of short-lived snakes produce more free radicals than complex III of long-lived species [37].

From an evolutionary point of view, comparisons between flying versus non-flying mammals are especially interesting. Flying mammals live longer than non-flying mammals of similar size. This has been related with the lower predation rates enjoyed by flying animals [38]. Brunet-Rossinni [39] studied free radical production in the bat *Myotis lucifugus* (MLS= 34 years, weigh= 9 grams), the white-footed mouse *Peromyscus leucopus* (MLS = 8 years, weigh= 20 grams) and the shrew *Blarina brevicuda* (MLS= 2 years, weigh= 24 grams). Basal (without any substrate) mtROS production is higher in *B. brevicuda* than in the other two species, which agrees with its shorter longevity. However, ETC of *P. leucopus* leaks fewer electrons than that of *M. lucifigus* in spite of the longer life span (4 times more) of the bat. This is an exception to the rule "fewer mtROS production, longer life span" and it is not the only one.

The classical experiments of Sohal's group have recently been repeated by Martin Brand's laboratory at Cambridge [40]. New mammalian species and new comparisons, mammals versus birds, have been studied. Again, the results show a significant correlation between mtROS generation and life span. In their study, Lambert and collaborators used pyruvate(+malate), succinate and succinate(+rotenone). The only significant correlation between longevity and free radical production occurred when succinate (without rotenone) was used as substrate. The results pointed to complex I to be responsible for the differences. However, the lack of correlation between H_2O_2 production and MLS with pyruvate(+malate) as substrate indicate that the differences are only produced during reverse flux, and not during forward electron transfer. This contradicts former results and also questions the biological importance of mtROS generation in life span determination.

One very important exception to the rule "fewer mtROS, longer life span" is *Heterocephalus glaber* (the naked mole-rat). The naked mole rate is the longest-living rodent with a MLS of 28 years [41]. Despite its extraordinary longevity naked mole-rat mitochondria do not produce fewer free radicals than mouse (MLS = 4 years) mitochondria. Paradoxically, the activity of glutathione peroxidase is almost undetectable in naked mole-rat tissues [42]. According to their elevated production of ROS and low antioxidant activity, naked mole-rats have higher levels of oxidative damage to lipids, proteins and DNA than mice [43]. In conclusion, the longest-living rodent shows that a long life span is compatible with high levels of mtROS production and oxidative damage. The extraordinary longevity of naked-mole rats has been related with some "unusual" characteristics of this species [41]. As long-lived primates naked-mole rats are social animals (although, their societies are organized more like ant or bee colonies). Surprisingly, they have a poor endothermy tolerance as caloric restricted animals. Unfortunately, it is not possible to relate these two characteristics with the molecular mechanism responsible for naked-mole rat longevity.

Other exceptions are wild-derived mice. They live 20% longer than laboratory mice [44], but their mitochondria produce the same amount of free radicals [40]. Both wild-derived mice and naked mole-rats have low unsaturated membranes in their tissues (with the exception of the brain) [45,46]. Paradoxically, naked-mole rats tissues suffer more lipid peroxidation, probably caused by the low levels of glutathione peroxidase and high levels of mtROS [40,42].

Unfortunately, most of the work done in isolated mitochondria is not accompanied by a study of the oxidative damage markers. These measurements are very useful to confirm if low levels of free radical production *in vitro* are related with low levels of oxidative damage *in vivo*. There is a negative correlation between lipid peroxidation and MLS in mammals [47]. The same kind of correlation is found

when oxidative damage to mtDNA is studied in different mammalian species [48,49]. However, birds versus mammals comparisons are not so consistent. Different products are formed as a result of oxidative damage to DNA's nitrogen bases: 8-hydroxy-7,8-2'-deoxyguanosine (8-oxodG), 5'-hydroxy-2'-deoxycytidine, 8-oxoadenine, 2-hydroxyadenine, Fapyadenine, 5-hydroxyuracil, 5-hydroxycytosine, cytosine-glycol, etc [50]. The most commonly used biomarker of oxidative damage to DNA is 8-oxodG. Pigeons have lower levels of 8-oxodG in their mtDNA than rats both in heart and brain. However, there are no differences in oxidative damage in brain mtDNA between parakeets and mice, which indicate that high levels of 8-oxodG in the brain are compatible with long life span [51]. Unfortunately, in the same article no data about oxidative damage to mtDNA in canaries is reported [51]. On the other hand, there is no consistent association between protein damage and life span. Lipooxidative damage to proteins is negatively correlated with MLS in mammals [47], but the correlation becomes positive when specific carbonyls (glutamic semialdehyde and aminoadipic semialdehyde) are studied [52]. Strikingly, it has also been described in flies that long-lived species have higher levels of protein carbonyls [32]. Glutamic semialdehyde levels are also higher in long-lived pigeons than in short-lived rats, and the same is true for glycooxidative damage markers (carboxyethyl-lysine and carboxymethyl-lysine). However, canaries and parakeets have lower levels of lipooxidative, glycooxidative and oxidative damage in proteins than mice [53].

One of the most important criticisms of MFRTA is that, it is unlikely that the "small" differences found in mtROS generation can explain the "big" inter-specific differences in longevity. However, there are other parameters related to oxidative stress that also correlate with MLS: 1) the degree of fatty acid unsaturation in cellular and mitochondrial membranes [54], 2) the methionine content in proteins [52] and 3) the guanine + cytosine (G+C) content of mtDNA [55]. Long-lived animals do not only produce fewer mtROS but their biological molecules are also more resistant to oxidative stress. The importance of such changes in longevity has been reviewed elsewhere [56]. The existence of such correlations indicates a strong evolutionary pressure to reduce oxidative damage in long-lived animals, but it does not prove that mitochondrial free radical production is the main driving force behind the aging process.

In summary, available scientific data supports a negative correlation between free radical production in isolated mitochondria and life span both in homoeothermic vertebrates and flies (Table **1**). However, there are some important exceptions, e.g. *H. glaber*, that show that high levels of oxidative damage are compatible with a long life span. Finally, it is necessary to remember that these correlations must be carefully interpreted, as correlation does not imply causation. A good example of this is the existence of a positive correlation between coenzyme Q10 levels, MLS and superoxide production [57]. When levels of coenzyme Q10 are experimentally manipulated, both *in vivo* and *in vitro*, neither superoxide production [58] nor life span [59] is altered. This clearly shows that levels of coenzyme Q10 do not control free radical generation or life span in spite of the negative correlation between them.

DIETARY RESTRICTION AND MAXIMUM LIFE SPAN

If MFRTA is true, it must explain not only inter-specific differences in rate of aging, but also intra-specific ones. Individuals of the same species that live longer must produce fewer mtROS. The best way to prove this is, specifically decreasing mtROS production, but this is not possible today. The alternative approach is looking for models where MLS is increased. If MFRTA is correct then the longest-living individuals must produce fewer mitochondrial free radicals.

DR is the only non-genetic treatment that clearly increases mean and MLS and delays most of the age-associated diseases (e.g. Alzheimer, cancer, sarcopenia, etc.) [31]. This increase in longevity elicited by DR is highly conserved during evolution [60]. Most of the studies show a decrease in mtROS generation associated with DR [19] according to the predictions of MFRTA.

In mice 40% DR decreases mtROS in brain, heart, and kidney [61]. However, some studies report no change in skeletal muscle or liver [62]. In rats, there is a clear decrease in H_2O_2 production by mitochondria after one year of caloric restriction in liver, heart and brain [19]. Conversely, data from shorter periods (less than one year) of DR are not consistent. For example, some articles report a decrease in mtROS production after two-six weeks of caloric restriction in heart [63], liver [64] and skeletal muscle [65], but other studies do not find changes after the same period of time [63,66,67] or even longer periods [68]. In summary, calorie restriction during one year decreased mtROS production however is not clear if shorter periods have the same effect.

Most former studies have been carried out in isolated mitochondria. Under these conditions the decrease in mtROS generation during DR is located mainly in complex I, and is

Table 1. Relation between mtROS Production, Oxidative Damage to mtDNA, Lipids and Proteins and MLS

	mtROS Production	Oxidative Protein Damage	Oxidative Lipid Damage	Oxidative mtDNA Damage
Flies	Negative correlation	Positive correlation	ND	ND
Mammals	Negative correlation	Positive correlation	Negative correlation	Negative correlation
Birds versus mammals	Negative correlation	Positive or Negative	Negative correlation	Negative correlation
Heterocephalus glaber	↑	↑	↑	ND

See text for details and references. ↑ High values; ND Not determined.

associated with forward electron flux [15]. However, some reports indicate that both complexes (I and III) could reduce mtROS production during DR [69,70]. Studies carried out in cells do not allow the identification of the complex responsible for differences in free radical production. Most studies have used DCF-DA as fluorescent probe. DCF-DA has problems (e.g. it is not specific, autooxidation...) that have been reviewed elsewhere [71]. Using this probe, Lambert and Merry [72] did not find any differences in rat hepatocytes after 4 months of DR. On the other hand, the group of Rafael de Cabo at NIA has shown that DR decreases free radical production and oxidative damage both in human cells and rat hepatocytes [73]. In general, results in whole cells are more scarce and difficult to interpret than those obtained in isolated mitochondria, but they support some kind of involvement of mitochondria in the positive effect of DR.

It has been recently described that 40% protein restriction (PR) during seven weeks, decreases free radical production in liver mitochondria in a similar way to that of total calorie restriction [74]. Methionine restriction (MetR) by 80% also shows a decrease in mtROS generation, both in heart and liver [75]. Furthermore, both PR and MetR decrease oxidative damage to proteins and mtDNA [74-76]. On the other hand, neither lipid nor carbohydrate restriction alter mtROS production in liver mitochondria [77,78]. Together, these data suggest that electron leak in ETC is controlled by the level of protein or methionine in the diet. Although, both PR and MetR decrease oxidative stress in liver, in a similar way to calorie restriction, only MetR clearly increases longevity in rodents [79-81]. PR has been related with a slight increase in MLS in laboratory rodents [82]. However, all the studies showing some kind of increase (more than 5%) in MLS were carried out during the seventies. During these years rearing conditions were sub-optimal as is shown by the short mean life span of the controls. In fact, the most recent studies using 40% PR discard proteins as those responsible for life span extension in rats [83,84] or show a minimal effect on MLS (around 3-5%)[85-86]. If PR does not extend MLS, the decrease in mtROS production (elicited by PR) is against the predictions of MFRTA. Remarkably in flies, life span can be modulated by protein intake [87]. Both high and low concentrations of protein intake decrease life span [88]. DR does not change mtROS production in flies [25] and the effect of PR on ROS has not been reported. In contrast with PR, 65-80% of metR clearly increases MLS in rodents [79-81]. In contradiction to this, metR seems to have a minimum effect on *Drosophila* life span [89], whereas DR and PR are both shown to increase MLS [87]. According to MFRTA, MetR decreases mtROS generation in both heart and liver [75], and oxidative damage to proteins and mtDNA in brain [90]. Unfortunately, both DR and MeR modify a lot of different parameters other than mtROS generation and oxidative stress. For example, both dietary treatments decrease insulin, thyroid hormones and IGF-1 levels in plasma [81]. As so many parameters are altered it is not possible to identify which one (or combination) is responsible for delaying the aging process.

Oxidized proteins accumulate during the process of normal aging and seem to play some kind of role in the aging process [91]. However, the accumulation of protein carbonyls could be dissociated with mtROS generation. Low levels of calorie restriction (20%) do not change mitochondrial free radical production [92], but decrease oxidative damage to proteins, lipids and DNA [93] and significantly increase life span (25% DR increases MLS about 20%)[94]. On the other hand, short periods of 40% DR diminishes oxidative protein damage without decreasing mtROS generation [63,95,96]. This indicates that oxidative protein damage could be regulated independently of mtROS production (as it also happens in long-lived species), e.g. stimulating autophagy during DR [97]. On the other hand, oxidative damage to mtDNA depends on mtROS generation [19]. This could be explained as changes in mtROS production during DR are mainly located at complex I [15]. Complex I exclusively produces superoxide into the mitochondrial matrix. Since mtDNA is located in the mitochondrial matrix changes in mtROS generation at complex I would have a direct effect on damage levels in DNA.

In summary, most studies show a decrease in mtROS after long periods of DR, although there is no consensus on whether short periods of DR would have the same effect. mtROS production determines oxidative damage levels in mtDNA, but protein damage levels are independently regulated. The decrease in mtROS generation elicited by DR or MetR does not prove MFRTA since other multiple physiological parameters are altered at the same time. Paradoxically, PR decreases mtROS in the same way that DR or MetR but its effect in maximum longevity is scarce or null in rodents (Table **2**).

EXERCISE AND MAXIMUM LIFE SPAN

There are two types of exercise, which can be differentiated by the intensity of the activity: 1) exhaustive exercise

Table 2. Summary of Changes in Oxidative Stress and Life Span Elicited by Dietary Restriction, Methionine Restriction or Moderate Exercise in Rodents

	Oxidative Protein Damage	mtROS Production	Oxidative mtDNA Damage	MLS
Dietary Restricticion	↓	↓	↓	↑
Methionine Restriction	↓	↓	↓	↑
Protein Restriction	↓	↓	↓	=
Moderate Exercise	↓	↓	↓	=

See text for details and references. ↓ Decrease, ↑ Increase, = No change; MLS =Maximum life span.

and 2) moderate exercise. Exhaustive exercise in rodents is similar to professional sport in humans. It is quite clear that exhaustive exercise is harmful for the individual. This kind of activity has been related with over-production of free radicals both by mitochondrial and extra-mitochondrial enzymes [98-100]. This burst of free radical generation increases oxidative stress [101]. On the other hand, moderate exercise has a positive effect on health, and it could be related to moderate daily activity in humans, which is highly recommended by health professionals. Moderate exercise decreases mtROS generation and oxidative stress [102]. Endurance training decreases mitochondrial free radical production in skeletal muscle [103,104], heart [105,106] and 8-oxodG levels in liver mtDNA [107] of rats. Although, exercise and DR decrease mtROS production and oxidative stress in a similar way, they have a very different effect on life span. In rodents, DR increases MLS up 50%, however, moderate exercise have a null effect on it [108,109]. The absence of any effect of exercise on MLS contradicts MFRTA predictions (Table **2**). A possible explanation is that exercise does not alter free radical generation in brain mitochondria. Three weeks of exercise are not enough to decrease mtROS generation [110], but longer periods decrease oxidative damage [111] (including proteins, lipids and DNA) in a similar way that has been observed in skeletal muscle or heart. It is very important to study the effect of moderate exercise in mtROS production in brain using longer training periods, because many reports indicate that specific modifications in brain metabolism or signalling are enough to delay aging [112].

MUTATIONS IN MTDNA AND CATALASE OVEREXPRESSION, SUPPORTING OR DENYING MFRTA?

In 2004 and 2005 two articles were published supporting MFRTA. Trifunovic and collaborators modified the proofreading activity of PolG. This experimental manipulation accelerated the accumulation of mutations in mtDNA, decreased life span and caused an aging-like syndrome in mice [20]. One year later, Schrinner and collaborators showed that the overexpression of catalase in mtDNA increases mean and MLS in mice [22]. Both reports seemed to confirm MFRTA. However, during the past year new data has been published, and old data has been reinterpreted in such a way that now they are in direct contradiction with MFRTA.

Mitochondria have their own genome; the mutation of mitochondrial genes causes varied set diseases [113]. MFRTA predicts that free radicals damage mtDNA and cause mutations that then accumulate during aging. These mutations would in turn alter mitochondrial function, and be responsible for the aging process. According to MFRTA, oxidative damage to mtDNA negatively correlates with MLS in mammals; although the relationship in birds is not so clear. Consequently, 8-oxodG levels are regulated by mtROS production during DR, PR or MetR. Unfortunately, 8-oxodG is not a good predictor of longevity. Normal mitochondrial function is compatible with high levels of oxidative damage in mtDNA. Knockout mice for OGG1 (DNA oxoguanine glycosilase) accumulate 20 times more 8-oxodG in their mtDNA than normal controls [114]. In spite of such levels of oxidative damage they do not show any alteration either in mitochondrial oxygen consumption or in the activity of respiratory complexes. Moreover, heterozygous (SOD2(-/+)) knockout mice have very high values of 8-oxodG in mtDNA, but they live as long as normal mice with low levels of oxidation [115]. 8-oxodG is another good example that correlation does not imply causation.

The lack of effect of 8-oxodG on mortality could be because this type of alteration is easily repaired [116] and so mutations are not accumulated. In fact, MFRTA predicts that only the consequences of oxidative damage (mutations) must accumulate with age. If oxidative damage to mtDNA is repaired before causing mutations it should not have any effect on the rate of aging. Early studies on this topic showed an extremely low frequency of mutations present in older individuals, which came as a disappointment to the researchers. However, these results had two experimental biases. First, mutations were measured in homogenates, where the mutation rate is lower than 1% in normal aged individuals. And second, most of these studies analyzed only one or two types of deletions and point mutations. After these results, the main conclusion was that alterations in mtDNA could not explain aging, as an elevated degree of mutation (higher than 70%) is necessary to cause physiological alterations [117]. The former conclusion was erroneous because it assumed that mutations are homogenously distributed in all cells. However, it has been demonstrated that this is not true [118]. The combination between new PCR amplification techniques, the use of isolated cells, and the sequencing of the entire mitochondrial genome, has shown up two different issues. First, there is a distribution pattern of damage whereby within the same tissues, several cells with a high concentration of mutations (enough to provoke physiological dysfunctions) coexist with cells that posses no mutations at all in their mtDNA [119-121]. Second, each mutated cell has only one type of mutation, which is different in various cells [122]. Thus, there are hot spots in mtDNA where mutations accumulate more frequently [123].

In 2004 a paper was published that provided a causative link between mtDNA mutations and mammalian aging [20]. The authors constructed knock-in mice that expressed a proof-reading-deficient version of PolG (the mitochondrial DNA polymerase). The specific mutation increased both point mutations and deletions. The increase in somatic mtDNA mutations was associated with a severe reduction in life span and some aging-like characteristics (e.g. alopecia, osteoporosis, sarcopenia...). Thus, the accumulation of mutations in mtDNA decreases mitochondrial oxygen consumption and ATP production in some tissues for example skeletal muscle, although it does not change such parameters in others like heart or liver (Hiona, Sanz and Leeuwenburgh, unpublished data). Although, these data apparently confirm MFRTA, some authors claim that PolG alteration does not provoke "real" aging [124] since the level of mutations of knock-in PolG is abnormally elevated. Recently, Loeb's group has revaluated the data in a new study [125]. Heterozygous knock-in (PolG (-/+) mice accumulate 500 times more point mutations than normal controls, but they do not show, any reduction in life span. The study of Loeb and collaborators also suggested that the point mutations found in normal aged mice are caused by oxidative damage. Since the results suggest that mitochondrial point mutations do not

limit the natural life span of mice, they do not support a role of mtROS in the regulation of longevity. However, only point mutations were analyzed in the former study so it is not possible to discard a relevant role for deletions in the aging process with this study. In fact, it has been suggested that a cause-effect relationship between the accumulation of mitochondrial deletions and functional alteration of tissues both in brain [126] and in skeletal muscle [127]. However, experimental data does not seem to support a causative role of deletions in aging either. For example, it has been described that DR delayed the accumulation of deletions in skeletal muscle [128] and liver [129], but not in brain [129]. And more importantly, the overexpression of mutated human twinkle protein in mouse increases the accumulation of deletions in mtDNA, but does not decrease life span [130].

In 2005 it was published that overexpression of human catalase in mitochondria increases mean and MLS by around 10% in mice [20]. However, last year authors recognized that they are having problems in replicating these life span results [131]. In their paper, Rabinovitch and collaborators showed that catalase overexpression reduced mitochondrial H_2O_2 levels, oxidative damage and mutations in mtDNA. Although, catalase overexpression reduces oxidative stress in the same way than DR or MetR, it has a limited (2005 results) or null effect on MLS (2006 results), whereas DR or MetR increases MLS around 40%. Contrary to MFRTA predictions low levels of oxidative stress are not enough to strongly increase MLS.

In summary, both knock-in PolG and trangenic overexpressing catalase mice do not support a major role of mitochondrial free radical production in MLS determination. In older individuals the level of mutation in mtDNA is elevated enough to alter mitochondrial function only in a few cells. In fact, most of the alterations are concentrated in the substantia nigra [132] and in skeletal muscle [133]. Although, these alterations can be responsible for some pathology, e.g. Parkinson or sarcopenia, it is unlikely that they can explain such a complicated process as aging. Overexpression of human catalase in mitochondria provokes a decrease in mtROS generation and oxidative stress similar to DR or exercise. However, DR clearly increases MLS, whereas exercise has no effect and overexpression of catalase a very small one if any. This indicates that low levels of mtROS production could be necessary, but they are not enough to increase life span as MFRTA predicts.

CONCLUSIONS

MFRTA has become the most popular theory to explain aging over the last few years. This theory is mainly supported by the negative correlation between mtROS production and life span in some mammalian species, and low levels of mtROS generation in three bird species. In addition, caloric and methionine restricted animals produce fewer mtROS and live longer. Unfortunately, all these parameters are only correlated with longevity. Correlation does not imply causation. In fact, exercise decreases free radical production in a similar way to that of DR or MetR, but it does not affect life span. Moreover, the accumulation of oxidative stress or point mutations in mtDNA does not decrease longevity as MFRTA predicts. However, the definitive experiment has not yet been done. The only way to test MFRTA is to specifically decrease mitochondrial free radical generation. Only, if low levels of mtROS production (without major modifying of other factors) increase MLS will MFRTA be demonstrated as true.

However, even if mtROS production does not determine MLS, it is clear that free radicals play an essential role during normal metabolism and aging. Some authors point out the importance of mtROS in cellular signalling [134]. According to these authors, free radicals are "the fire employed by cells in development" [134]. Aging would not be a direct consequence of oxidative damage, if not the result of the disruption of the communication system based on free radical chemistry. Critical analyses of literature data indicate that mtROS contributes to the determination of mean life span. Excess in mtROS production or deficits in antioxidants are related with different diseases some of which are associated with aging [135]. The environment modulates mtROS production (e.g. protein or methionine intake) causing disease and accelerating aging when free radicals levels are abnormally elevated. However, aging is an endogenous process determined in the genome. Are mitochondrial free radicals the main character in aging tragedy? Or are there secondary characters playing a subtler role? Only time will answer these questions.

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ABBREVIATIONS

8-oxodG	=	8-hydroxy-7,8-2'-deoxyguanosine
CBA	=	Comparative Biology of Aging
DR	=	Dietary Restriction
ETC	=	Electron Transport Chain
G+C	=	Guanine + Citosine
MetR	=	Methionine Restriction
mtDNA	=	Mitochondrial DNA
mtROS	=	Mitochondrial Reactive Oxygen Species
MFRTA	=	Mitochondrial Free Radical Theory of Ageing
MLS	=	Maximum Life Span
Polg	=	Polymerase gamma
PR	=	Protein Restriction
ROS	=	Reactive Oxygen Species
SOD	=	Superoxide dismutase
UCPs	=	Uncoupling proteins.

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