This student paper was written as an assignment in the graduate course

Free Radicals in Biology and Medicine

(77:222, Spring 2001)

offered by the

Free Radical and Radiation Biology Program
B-180 Med Labs
The University of Iowa
Iowa City, IA 52242-1181
Spring 2001 Term

Instructors:
GARRY R. BUETTNER, Ph.D.
LARRY W. OBERLEY, Ph.D.

with guest lectures from:
Drs. Freya Q . Schafer, Douglas R. Spitz, and Frederick E. Domann

The Fine Print:

Because this is a paper written by a beginning student as an assignment, there are no guarantees that everything is absolutely correct and accurate.

In view of the possibility of human error or changes in our knowledge due to continued research, neither the author nor The University of Iowa nor any other party who has been involved in the preparation or publication of this work warrants that the information contained herein is in every respect accurate or complete, and they are not responsible for any errors or omissions or for the results obtained from the use of such information. Readers are encouraged to confirm the information contained herein with other sources.

Aging and Free Radicals:

Is Dietary Restriction Really a Fountain of Youth?

by

Sherry A. Rovinsky

5-607 Bowen Science Building
Department of Physiology and Biophysics
The University of Iowa
Iowa City, IA 52242-1109

For: 77:222, Spring 2001

3. May 2001

Abbreviations

8-OHdG, 8-hydroxy-2'-deoxyguanosine
AL, ad libitum
CR, caloric restriction (synonymous with DR)
DR, dietary restriction
ECD, electrochemical detection
HPLC, high performance liquid chromatography
MetR, methionine restricted
MtDNA, mitochondrial DNA
ProR, protein restricted
PUFA, polyunsaturated fatty acid
RAS, reactive aldehydic species
RBC, red blood cell
ROS, reactive oxygen species
RNS, reactive nitrogen species

Table of Contents

Abstract

The oxidative stress theory of aging states that aging occurs because of accumulated damage to cells by reactive oxygen species. Researchers have studied the aging-related oxidative damage to proteins, DNA, and lipids, and the research suggests that dietary restriction can reduce this damage. Additionally, antioxidants such as superoxide dismutase, catalase, and glutathione decrease in aging animals but are much higher in caloric restricted animals. These factors have particular importance in the mitochondria, where high concentrations of ROS are present. This mitochondrial damage is likely to account for many of the features of the aging phenotype. The purposes of this paper are (1) to introduce the idea of free radical involvement in aging, (2) to discuss the evidence for the idea that caloric restriction can reduce aging-related free radical damage, (3) describe free radical involvement in specific aging-associated pathologies, and (4) to propose experiments that will help clarify these issues.

Introduction

Caloric restriction is the only known method for slowing the aging process and extending the life span of rodents [1]. Recent work suggests that primates may also enjoy longer life spans through dietary restriction [1]. The terms “dietary restriction” (DR) and “caloric restriction” (CR) are used synonymously in this paper. DR experiments generally refer to a 30 to 40% reduction in calories compared to control animals, which are fed ad libitum (AL) [1]. Animals are supplemented with micronutrients to insure that only undernutrition, but not malnutrition occurs [1].

The first experiments examining this phenomenon in rodents were done over 60 years ago, and investigators have studied caloric restriction in primates since 1987 [1]. The phenotype

of DR primates includes shorter stature, slower maturing process, less bone mass, less fat, less muscle, and a slightly slower body temperature [1]. Interestingly, DR primates also demonstrate superior glucose tolerance and greater insulin sensitivity, suggesting that they may have a decreased aging-related diabetes risk [1]. Often, DR organisms have a decreased ability to reproduce [2].

Although this phenomenon has been known for a long time, free radical biologists have only recently proposed two models to explain it. The free radical theory of aging states that free radical sources (mainly superoxide produced by mitochondria) change the metabolic rate. Free radical damage accumulates in the cell, leading to degenerative diseases and the biological process we call aging [3].

The oxidative stress hypothesis of aging is a more recently proposed model for the production of the aging phenotype. Here, reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive aldehydic species (RAS) cause alterations to cellular structures, damage cellular defense, and damage repair machinery. These oxidants lead to abnormal protein function, imbalance of the redox environment, and accumulated damage to cell structures. All these things cause the cell to lose homeostatic control, leading to disease and aging, in a circular fashion as seen in Figure 1 [3].

Models for the Anti-Oxidative Mechanism of Dietary Restriction

There are two models for how dietary restriction slows the aging process. In the first, dietary restriction could slow production of ROS and thus slow accumulation of oxidatively damaged biological molecules [2]. In the second, DR may exert its lifespan-extending effect by

better preparing the organism to deal with oxidative stress [2]. As we will see in the following sections, it is probable that a combination of these mechanisms is at play, as depicted in Figure 2.

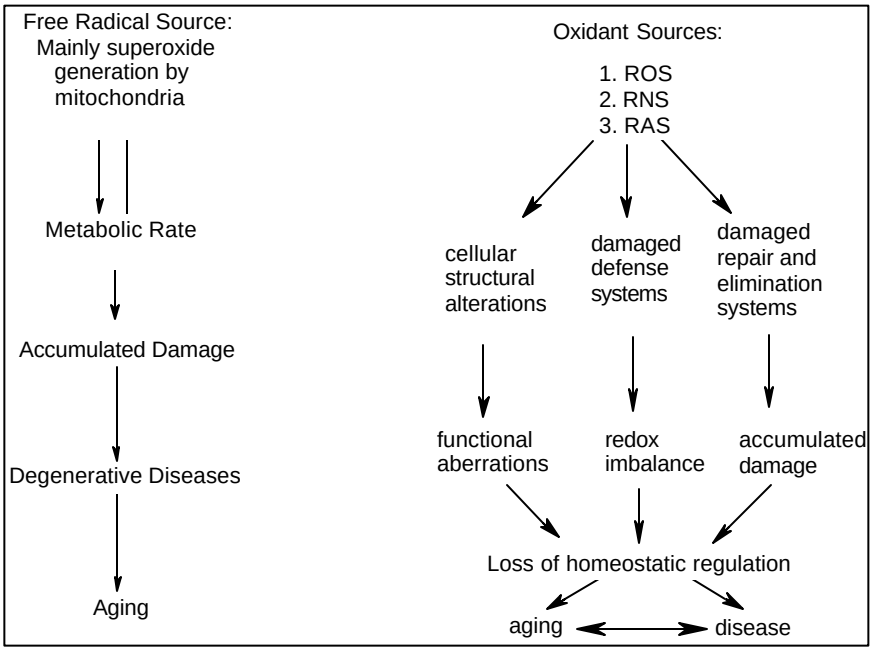

Figure 1: A Comparison of the Free Radical Theory of Aging with the Oxidative Stress Theory of Aging. Adapted from [3].

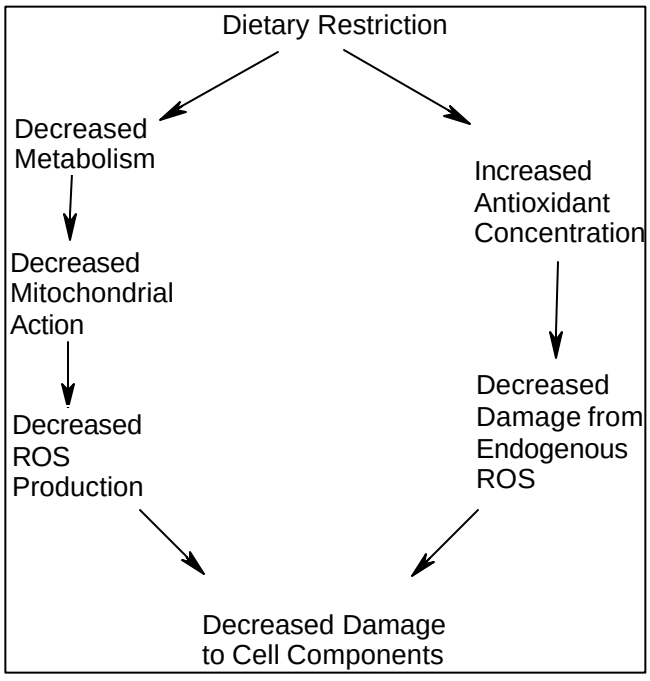

Figure 2: Dietary restriction, a reliable method for prolonging life span in laboratory animals, may affect the ROS production and/or damage by ROS through an increase in antioxidant molecules. Both models are depicted here.

Interaction of Free Radicals with Biological Molecules and Modulation by Caloric Restriction

During aging, many cellular components are damaged. As I will detail below, iron accumulates in most tissues during aging, contributing to protein oxidation, DNA damage, lipid peroxidation, and sugars.

Iron Accumulation in Aging

Total tissue iron content increases with aging [4]. Iron is known to be a pro-oxidant as in Fenton chemistry, so one might expect to see a correlation between iron accumulation and oxidative damage with aging. In aging DR rats (24 months), kidney, liver, and brain tissue iron content is significantly lower than that of AL fed rats of the same age [4].

Aging-Related Protein Oxidation

During the aging process, oxidative damage to proteins occurs, resulting in accumulation of protein carbonyls [2]. Youngman *et al.* found that oxidative stress, as measured by the accumulation of protein carbonyls, increased in Fischer 344 rats with either aging or irradiation. More importantly, they found that accumulation of protein carbonyls was significantly decreased in DR rats which had been irradiated or allowed to age [2].

Protein carbonyl formation is not simply a convenient laboratory measure of oxidative stress. It is likely to have severe consequences on protein structure and function. Protein carbonyl formation in rat brain increases with age, while activity of creatine kinase and glutamine synthetase declines [5]. One can easily imagine how oxidative damage to proteins interferes with the cell's homeostatic mechanisms and contributes to the aging phenotype.

Glucose can react nonenzymatically with certain proteins that have long half-lives, such as collagen [6], producing advanced glycosylated end-products. In a paper by Sell, an HPLC column was used to capture the intermediate Amadori product as an indicator of age-related collagen glycation. He found that collagen glycation increased dramatically with age in rodent skin, and that this increase could be inhibited by dietary restriction [6]

Aging-Related DNA damage

One marker of aging is the accumulation of oxidative damage to DNA. In one study, DNA damage in aging rats was quantified by looking at 8-hydroxy-2'-deoxyguanosine (8-OHdG) with HPLC-ECD. This group found that as rats aged, 8-OHdG accumulated first in kidney, later in heart and liver, and finally in the brain [7]. Another group found while both mitochondrial and nuclear DNA were being oxidized, mitochondrial DNA fractions contained approximately 15 times more 8-OHdG as nuclear DNA fractions [8]. Most importantly, dietary restriction slows the aging-associated accumulation of 8-OHdG [7, 8]. The Chung group found that the onset of 8-OHdG accumulation in aging DR rat liver was significantly delayed when compared to aging ad libitum fed rats [8]. A more recent paper by Kaneko et al. supports the conclusion that dietary restriction can decrease age-related 8-OHdG accumulation in kidney, heart, liver, and brain [7].

Lipid peroxidation

Lipid peroxidation is an important hallmark of aging and can be modulated by long-term DR [4]. 24-month-old AL fed rats show a much greater degree of lipid peroxidation than DR

rats of the same age [4]. Short-term DR mice do not show this decrease in lipid peroxidation, so it appears that long-term DR is necessary to receive anti-aging benefits [9].

It has been suggested that lifelong DR induces changes in the lipid composition of the membrane that compensate for the membrane fluidity changes accompanying the decreased body temperature of the animal [10]. Several groups have looked at the resistance of liver cell membranes to peroxidative stress and have concluded that the cell membranes of lifelong DR animals are less vulnerable to peroxidation as measured by malonylaldehyde production [11, 12, 13]. Hemolysis of red blood cells (RBCs) by peroxides is another model for looking at these membrane changes. When treated with 2-2'-azo-bis(2-amidinopropane) dihydrochloride, a producer of peroxyl radicals, RBCs from DR rats are more resistant to hemolysis than RBCs from ad libitum controls [10].

Modulation of Antioxidant Levels Through Caloric Restriction

There is quite a bit of contradictory evidence about the effect of DR on antioxidant levels. With short-term DR, liver SOD (type not specified), CAT, GPx, GR, cytochrome oxidase, and GSH levels are comparable to ad libitum fed mice, while ascorbate levels are actually lower [9]. However, studies of long-term DR generally imply that antioxidant levels are dramatically decreased with normal aging, and that DR significantly delays this effect [10, 12, 14, 15, 16, 17].

Superoxide Dismutase

There is some controversy regarding the issue of how SOD levels and activity are affected by aging. Semsei *et al*. reported that in rat liver, CuZn SOD steady state mRNA levels, nuclear transcription, and activity decline with age, but that these effects are reversible by

lifelong DR [14]. An earlier paper had concluded that overall mouse liver SOD activity was unchanged by lifelong DR [12].

Catalase

Catalase activity is clearly decreased in older rodents [12, 14]. CAT mRNA levels, activity, and nuclear transcription decline in rat liver with age [14]. With lifelong dietary restriction, however, this effect disappears [14]. CAT activity was also significantly decreased in older mouse livers, and increased in older, lifelong DR mice [12].

Glutathione

In rat liver, both cytosolic and mitochondrial GSH concentrations are increased in aging DR rats in comparison to aging AL rats [16]. GSH levels drop with age in both groups, but the decline is delayed in DR rats.

Orentreich *et al.* found that selective dietary restriction of methionine, a GSH precursor, increases the life span of rats [18]. This group concluded that this effect was independent of caloric restriction. Richie *et al.* later showed that blood glutathione increases in response to methionine restriction, paralleling their increased life span [15]. Interestingly, this effect cannot be recapitulated by overall protein restriction; liver GSH is significantly decreased in protein restricted rats [17].

Effects of Dietary Restriction on Mitochondria

The mitochondria are the largest source of ROS in the cell, and since location of the ROS determines where damage will occur, it seems probable that the mitochondria are particularly

susceptible to damage [19]. In this section, I will explain how the themes of DNA, lipid, and protein damage and altered antioxidant levels have particular relevance in the mitochondria. The result of oxidative lesions to the DNA, proteins, and lipids comprising the mitochondria will ultimately impair mitochondrial function. Many researchers suggest that the mitochondria themselves are the key to the understanding the aging phenotype and its modulation by DR.

Damage to Mitochondrial DNA

Not only is mitochondrial DNA (mtDNA) located right next to an ROS generator, the electron transport chain, it also lacks protection from histones, and lacks a DNA repair system [19]. Mitochondrial DNA accumulates about 15 times more age-related damage than nuclear DNA [19]. Thus, mitochondrial DNA is very susceptible to DNA mutation.

In addition to the problem of mutation, mtDNA is also susceptible to DNA deletions that can be modulated by DR [20]. Interestingly, this phenomenon appears to be tissue specific. DR prevented the age-related accumulation of mtDNA deletions in rat liver tissue, but not in brain [20].

Mitochondrial Membranes

The mitochondrial membrane is attacked by many ROS because of its close association with the proteins of the electron transport chain [19]. Lipid peroxidation is an important result of this, decreasing mitochondrial membrane fluidity and function [21]. In rat heart mitochondria, peroxidative damage to lipids accumulates with age, while DR decreases age-associated lipid peroxidation [21].

Lee *et al.* has proposed a positive feedback loop model of how ROS can cause fluidity changes in biological membranes [21]. A membrane begins with a mixture of lipids of different degrees of unsaturation. The more unsaturated a fatty acid is, the more susceptible it is to lipid peroxidation. The presence of ROS, causes peroxidation of vulnerable fatty acids, which results in decreased membrane fluidity. The cell compensates for the loss of fluidity by inserting more polyunsaturated fatty acids (PUFA) into the membrane, but these new PUFA are very peroxidizable. Dietary restriction would slow aging by reducing the initial amount of damage from ROS [21]. See Figure 3.

The other major component of the membrane is its proteins. Again, there is an age-related increase in oxidative damage to mitochondrial protein [19]. This result can again be prevented through dietary restriction [19].

Glutathione Levels

The antioxidant changes seen in mitochondria of DR animals may provide the answer for why DR is able to slow aging-related ROS damage. Normally, mitochondrial GSH decreases with age, although DR animals maintain higher levels [16, 22].

Effects of Dietary Restriction on Aging-Related Neuronal Changes

To this point, we have focused on the changes that occur on the molecular and organellar levels with aging, as well as how DR modulates many of these changes. Now we need to consider the phenotypic consequences of these changes. Dietary restriction has been demonstrated to decrease presbycusis [23] and hyperinsulinemia [24]. Additionally, the age-associated drop in immune function is delayed by DR [27]. Measures of ROS and ROS damage

show that DR animals do not experience these age-related changes as rapidly as do AL fed animals, and antioxidant levels generally increase. For illustrative purposes, we will discuss the evidence that DR may slow the onset of aging-related neuronal pathologies.

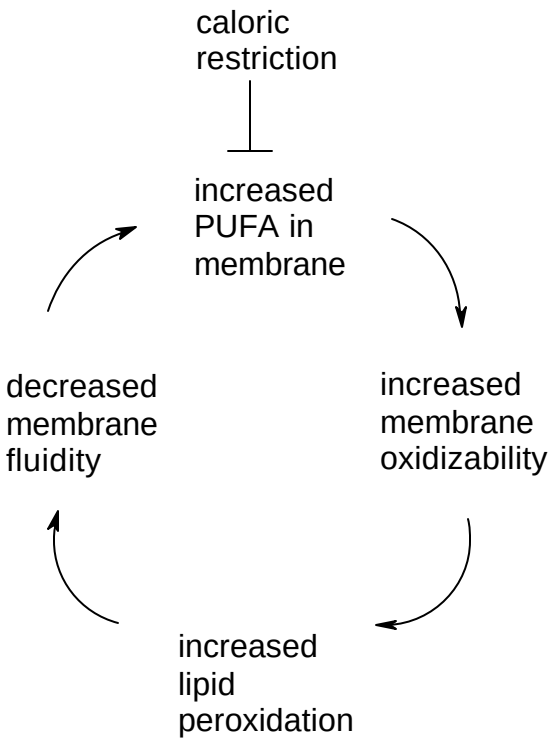

Figure 3: The vicious cycle of lipid peroxidation and changes in membrane fluidity. PUFA in membranes become oxidized, decreasing membrane fluidity, which causes more PUFA to be placed in the membrane. This lipid peroxidative damage accumulates with age, causing a feedback loop as depicted above. The frequency of PUFA insertion into the membrane can be modulated by DR. Adapted from [21].

Since neural tissue is the most active tissue in the body, with extensive mitochondria, one might expect that free radical damage here is extensive. This is generally true. For example,

Askenova *et al.* found that protein carbonyls increased with age in rat cortex, hippocampus, and cerebellum [5].

Interestingly, many researchers have found that DR can reduce the incidence of neuropathologies such as Alzheimer's disease, Parkinson's disease, and stroke [25]. Aging DR rats had lower protein carbonyl accumulation in multiple brain tissues when compared to aging ad libitum fed rats [5].

Neural tissue also contains a high lipid content, so lipid peroxidation is another potential problem. In a study of synaptosomal membrane fluidity, aging AL fed rat synaptosomal membranes were less fluid than those of same-age, DR rats [26]. Not surprisingly, ROS production (as measured by the Lebel/Bondy dichlorofluorescein diacetate method) was increased at AL fed rats, compared to DR rats [26].

Hypothesis and Future Directions for Research

Hypothesis: Although methionine restriction (MetR) increases glutathione levels [15], there are many other adaptations that occur during energy restriction. Thus, methionine restriction alone will be able to recapitulate some *but not all* of the DR-mediated effects on aging-related oxidative damage.

Aim 1: Determination of Superoxide in AL, DR, ProR and MetR Rats

Fisher 344 rats will be fed one of four diets: (1) AL: ad libitum fed, (2) DR: 40% reduction in calories from carbohydrates relative to AL, (3) ProR: 40% reduction in protein relative to AL, (4) MetR: methionine reduced to 25% of AL fed. These animals will be

sacrificed at 6 months, 12 months, or 24 months, and their livers removed, washed, and homogenized. Superoxide production will be quantified by spin-trapping.

Aim 2: Determination of Antioxidant Levels

Rats will be fed and sacrificed as described above. Although part of this experiment has been done, no one has yet done a comprehensive study comparing antioxidant levels of AL, DR, ProR, and MetR animals. Thus, total and oxidized glutathione will be measured using the method of M. E. Anderson [15]. Since total GSH has been demonstrated to decrease with age, yet is higher in DR animals, this will function as a positive control [15]. In addition to this, we will measure CAT, SOD, Thioredoxin, and GPx activity. Protein will be visually quantified by western blot, and message abundance evaluated through northern blotting.

Hypothesis: Since the mitochondria are the source of most cellular ROS, liposomal MnSOD and CAT containing the mitochondrial presequence will delay aging-related oxidative damage, recapitulating the effect of DR. Transfection with MnSOD alone may actually increase aging-related oxidative damage, especially of lipids.

Aim 1: To demonstrate that the increased antioxidant defenses protect the mitochondria and cytosol from age-related oxidative damage.

Two versions each of liposomal CAT and liposomal MnSOD will be created. In one, the SOD or CAT will contain the mitochondrial targeting presequence. The other will lack the mitochondrial presequence, so it will be primarily cytosolic. These will be transfected, samples recovered, homogenized, and separated on a sucrose gradient into cytosolic and mitochondrial

fractions. These fractions will be evaluated for lipid peroxidation, protein carbonyl formation, and 8-OHdG accumulation.

Summary

Both the free radical and oxidative stress theories of aging suggest that the aging phenotype can be explained by damage to cells by reactive oxygen species. Dietary restriction can reduce production of mitochondrial ROS, presumably by decreasing the flow of substrates through the electron transport chain. Superoxide dismutase, catalase, and glutathione decrease in aging animals but are much higher in caloric restricted animals. Future experiments are needed to determine if MetR alone may mimic all of the DR phenotype or if it only duplicates the rise in GSH.

References

1. Roth GS, Ingram DK, Black A, Lane MA. (2000) Effects of reduced energy intake on the biology of aging: the primate model. *Eur J Clin Nutr.* **54**:S15-20.
2. Youngman LD, Park JY, Ames BN. (1992) Protein oxidation associated with aging is reduced by dietary restriction of protein or calories. *Proc Natl Acad Sci*. **89**:9112-9116.
3. Yu BP. (1996) Aging and oxidative stress: modulation by dietary restriction. *Free Radic Biol Med.* **21:**651-668.
4. Cook CI, Yu BP. (1998) Iron accumulation in aging: modulation by dietary restriction. *Mech Ageing Dev.* **102**:1-13.
5. Aksenova MV, Aksenov MY, Carney JM, Butterfield DA. (1998) Protein oxidation and enzyme activity decline in old brown Norway rats are reduced by dietary restriction. *Mech Ageing Dev.* **100**:157-168.
6. Sell DR. (1997) Ageing promotes the increase of early glycation Amadori product as assessed by e-N-92-furoylmethyl)-L-lysine (furosine) levels in rodent skin collagen. The relationship to dietary restriction and glycoxidation. *Mech Ageing Dev.* **95**:81-99.
7. Kaneko T, Tahara S, Matsuo M. (1997) Retarding effect of dietary restriction on the accumulation of 8-hydroxy-2'-deoxyguanosine in organs of Fischer 344 rats during aging. *Free Radic Biol Med*. **23**:76-81.
8. Chung MH, Kasai H, Nishimura S, Yu BP. (1992) Protection of DNA damage by dietary restriction. *Free Radic Biol Med.* **12**:523-525.
9. Rojas C, Cadenas S, Perez-Campo R, Lopez-Torres M, Pamplona R, Prat J, G Barja. (1993) Relationship between lipid peroxidation, fatty acid composition, and ascorbic acid in the liver during carbohydrate and caloric restriction in mice. *Arch Biochem Biophys.* **306**:59-64.
10. Piere C, Moroni F, Marra M. (1995) Food restriction increases the protection of erythrocytes against the hemolysis induced by peroxyl radicals. *Mech Ageing Dev.* **87**:15-23.
11. Laganiere S, Yu BP. (1987) Anti-lipoperoxidation action of food restriction. *Biochem Biophys Res Commun.* **145**:1185-1191.
12. Koizumi A, Weindruch R, Walford RL. (1987) Influences of dietary restriction and age on liver enzyme activities and lipid peroxidation in mice. *J Nutr*. **117**:361-367.

13. Piere C, Falasca F, Marcheselli F, Moroni R, Recchioni R, Marmocchi F, and Lupidi G. (1992) Food restriction in female Wistar rats: V. Lipid peroxidation and antioxidant enzymes in the liver. *Arch Gerontol Geriatr*. **14**:92-99.
14. Semsei I, Rao G, Richardson A. (1989) Changes in the expression of superoxide dismutase and catalase as a function of age and dietary restriction. *Biochem Biophys Res Commun*. **164**:620-625.
15. Richie JP Jr, Leutzinger Y, Parthasarathy S, Malloy V, Orentreich N, Zimmerman JA. (1994) Methionine restriction increases blood glutathione and longevity in F344 rats. *FASEB J*. **8**:1302-1307.
16. Armeni T, Pieri C, Marra M, Saccucci F, Principato G. (1997) Studies on the life prolonging effect of food restriction: glutathione levels and glyoxalase enzymes in rat liver. *Mech Ageing Dev.*
17. Dubick MA, Heng H, Rucker RB. (1985) Effects of protein deficiency and food restriction on lung ascorbic acid and glutathione in rats exposed to ozone. *J Nutr*. **115**:1050-1056.
18. Orentreich N, Matias JR, DeFelice A, Zimmerman JA. (1993) Life span extension following decreased methionine ingestion in rats. *J Nutr*. **123**:269-274.
19. Ames BN, Shigenaga MK, Hagen TM. (1995) Mitochondrial decay in aging. *Biochim Biophys Acta*. **1271**:165-170.
20. Kang CM, Kristal BS, Yu BP. (1998) Age-related mitochondrial DNA deletions: effect of dietary restriction. *Free Radic Biol Med.* **24**:148-154.
21. Lee J, Yu BP, Herlihy JT. (1999) Modulation of cardiac mitochondrial membrane fluidity by age and calorie intake. *Free Radic Biol Med.* **26**:260-265.
22. Armeni T, Tomasetti M, Svegliati Baroni S, Saccucci F, Marra M, Pieri C, Littarru GP, Principato G, Battino M. (1997) Dietary restriction affects antioxidant levels in rat liver mitochondria during ageing. *Mol Aspects Med.* **18**:S247-250.
23. Seidman MD. (2000) Effects of dietary restriction and antioxidants on presbyacusis. *Laryngoscope*. **110**:727-738.
24. Facchini FS, Hua NW, Reaven GM, Stoohs RA. (2000) Hyperinsulinemia: the missing link among oxidative stress and age-related diseases? *Free Radic Biol Med.* **29**:1302-1306.

25. Mattson MP. (2000) Neuroprotective signaling and the aging brain: take away my food and let me run. *Brain Res.* **886**:47-53.
26. Choi JH, Yu BP. (1995) Brain synaptosomal aging: free radicals and membrane fluidity. *Free Radic Biol Med.* **18**:133-139.
27. Utsuyama M, Ichikawa M, Konno-Shirakawa A, Fujita Y, Hirokawa K. (1996) Retardation of the age-associated decline of immune functions in aging rats under dietary restriction and daily physical exercise. **91**:219-228.