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Mitochondrial oxidant generation is involved in determining why females live longer than males

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1. ABSTRACT

Females live longer than males in many mammalian species, including humans. This natural phenomenon can be explained on the basis of the mitochondrial theory of aging. Mitochondria are a major source of free radicals in cells. Mitochondria from female rats generate half the amount of hydrogen peroxide than those of males and have higher levels of mitochondrial reduced glutathione. The latter is due to females behaving as double transgenic in over-expressing antioxidant enzymes. Estrogens bind to the estrogen receptors and subsequently activate the mitogen activated protein (MAP) kinase and nuclear factor kappa B (NFkappaB) signalling pathways, resulting in an upregulation of antioxidant enzymes. Moreover, the 16S rRNA expression, which decreases significantly with aging, is four times higher in mitochondria from females than in those from males of the same chronological age. On the contrary, the oxidative damage of mitochondrial DNA is fourfold higher in males than in females. Ovariectomy abolishes the gender differences between males and females and estrogen replacement rescues the effect of ovariectomy. The challenge for the future is to find molecules that have the beneficial effects of estradiol, but without its feminizing effects. Phytoestrogens or phytoestrogen-related molecules may be good candidates to meet this challenge.

2. INTRODUCTION: MITOCHONDRIAL OXIDANT FUNCTION IN AGING

Denham Harman proposed in 1956 and 1972 that free radicals (1,2), especially those of mitochondrial origin (3), are causally related to the basic aging process. A series of authors and studies produced evidence in favour of the mitochondrial hypothesis of aging (4-8).

Aging is progressive, which means that the causes of aging must be present during the whole life span, both at young and old age, and at approximately the same level of intensity (9).

What is the connection between aging and oxidative stress? The rate of aging correlates with the rate formation of H_2O_2 by mitochondria. All the investigations performed to date have shown that the rate of H_2O_2 production of mitochondria isolated from postmitotic tissues (the ones more relevant to aging) is lower in long-lived than in shortlived species (10,11).

This occurs in all kinds of long-lived homeothermic animals independently of their rates of O_2 consumption, which are lower in mammals of large body size and higher in birds of small size. It is generally assumed that in homeotherms high rates of O_2 consumption

are associated with low maximal life spans. However, birds are unique because they combine a high rate of O_2 consumption at rest and under basal conditions with an extended maximal life span. Barja and his coworkers (11) studied this problem and concluded that mitochondria from birds show a rate of H_2O_2 and of free radical generation per unit of O_2 that is one order of magnitude lower than mitochondria from rats. However, the hypothesis is not free of criticism, pigeon heart mitochondria produce H_2O_2 at rates that are similar to those of rat heart (12-13).

We observed that aging causes an oxidation of mitochondrial glutathione, the co-factor of mitochondrial glutathione peroxidase which is involved in the catabolism of mitochondrial H_2O_2 and that such oxidation accounts for the total GSH oxidation that is observed in whole cells upon aging (14). It is worth noting that we were able to make such observation by developing a method to determine accurately GSSG in biological samples by preventing autoxidation of GSH (14).

Furthermore, the levels of 8-oxo-deoxyguanosine, an index of oxidative damage to mitochondrial DNA, increased with aging and this increase correlated with the oxidation of GSH mentioned above (14). The age-associated oxidation of glutathione and of mitochondrial DNA was prevented by treatment with vitamins C and E (14) and by treatment with *Gingko biloba* extract (15).

Damaged mitochondria are isolated from the organs of old animals and mitochondria are dysfunctional inside intact cells from old animals (8,16,17). Age-associated mitochondrial damage is associated to an increased rate of oxidant production and to an increased content of oxidation products (8,16,17). The observation of lower activity of the complexes of the mitochondrial respiratory chain in old animals is accompanied by the reports of a higher level of mtDNA damage (18-19).

More than 97% of the O_2 used by aerobic cells is consumed in mitochondria, and about 1% of this O_2 does not form water by the cytochrome oxidase reaction, i.e. in the main process of normal tissue respiration, but superoxide anion (O_2^-) (15, 16) (7,20). Superoxide is then converted into H_2O_2 within mitochondria, highly favoured by the action of manganese superoxide dismutase (Mn-SOD). The continuous mitochondrial generation of O_2^- and H_2O_2 throughout the life of a cell leads to "chronic oxidative stress" that plays a key role in cellular aging, and that, as it is now well established, involves oxidative damage to mitochondrial DNA, proteins, and lipids (8,15,16,18,21,22).

3. FEMALES PRODUCE LESS MITOCHONDRIAL OXIDANTS THAN MALES

The rate of oxidant production by mitochondria from females is significantly lower than that from males. We measured the rate of H_2O_2 production and found that mitochondria from female rats produce approximately half the amount of H_2O_2 generated by "male" mitochondria, as tested in liver and brain mitochondria from mice and rats (23).

Moreover, neuronal mitochondria produce much greater quantities of oxidants than do glial mitochondria, a finding that agrees with the idea that post-mitotic and non-dividing cells suffer much more age-associated damage than do cells that divide normally.

To determine whether ovarian hormones, such as estrogens, are involved in the marked differences in oxidant production between mitochondria from male and female rats, we tested the effects of ovariectomy and of estrogen replacement therapy on mitochondrial H_2O_2 production. Ovariectomy caused an increase in H_2O_2 production by liver and brain mitochondria, yielding values similar to those observed with mitochondria from males, *i.e.* an increase of more than 60% from the values found normally with mitochondria from females. Estrogen replacement therapy completely abolished the observed increase in H_2O_2 generation that was produced by ovariectomy (23-24).

4. MITOCHONDRIA ARE LESS DAMAGED IN FEMALES THAN IN MALES

Mitochondria from females suffer considerably less oxidative damage in critical molecules such as mitochondrial DNA (mtDNA) or glutathione as compared with those of males (23,25). Mitochondrial DNA is a key component of the mitochondrion (26), and its degree of oxidation is directly related to aging (18, 21,27). The observed abundance of 8-oxodeoxyguanosine, an excellent indicator of oxidative damage to DNA, is four times as great in mitochondria from males than in those from females (23,25). This is the most pronounced oxidative change we have observed in mtDNA in any physiological situation, and it indicates that the higher chronic and continuous H2O2 production that is directly related to a higher rate of damaging ROS generation in males results in marked oxidative damage to mtDNA and in mutagenic lesions DNA (28).

We will now focus in glutathione, which is the major low-molecular-weight thiol in cells (29). Its concentration is similar to that of glucose (30). Mitochondrial GSH levels are double in females than in males. The intracellular level of the oxidized form of glutathione (GSSG) is considered a biological marker of aging (31), and we have found that mitochondrial GSSG is related to the damage associated with aging (32).

5. ESTROGENS UP-REGULATE THE EXPRESSION OF MITOCHONDRIAL ANTIOXIDANT DEFENCES

In order to explain the lower oxidative stress observed in mitochondria from females as compared

Table 1. Physiological concentrations of estradiol decrease H_2O_2 levels in human MCF-7 cells mediated by a signaling pathway that includes estrogen receptors, MAPK, and NF-Kappa B

Treatment	nmol H_2O_2/mg prot
Control	1.50 ± 0.55
0.2 nM Estradiol	0.66 ± 0.14[1]
0.2 nM Estradiol + 15 microM tamoxifen 15 microM	1.72 ± 0.12
0.2 nM Estradiol + 1 microM UO126	1.06 ± 0.18
0.2 nM Estradiol + 0.2 mM PDTC	1.31 ± 0.15

Data are expressed as means ± S.D. for 8–10 different experiments, The statistical significance is expressed as [1]P < 0.01 vs. control. Reproduced with permission from 41.

with males, we examined the differences in expression and activity of mitochondrial antioxidant enzymes as a function of gender.Since the mitochondrial steady-state concentrations of O_2^- and H_2O_2 are defined by the ratio of the rates of production and utilization of these species, we determined the mitochondrial activity and expression of Mn-SOD and glutathione peroxidase (GPx) (33). Both the expression and the enzymatic activity of Mn-SOD and GPx were approximately double in females than in males (Figure 1). The fact that females have a higher GPx activity than males was already observed in the 1960s (34), but at that time the phenomenon was not related to the different longevity between genders. A few years ago Orr and Sohal (35) observed that *Drosophila* that over-xpress either SOD or catalase (they lack GPx) did not increase their average life span. However, when they overexpressed both, life span was increased. We have found that females over-express both mitochondrial antioxidant enzymes, Mn-SOD and GPx, and we hypothesized that the phenomenon depends on a hormonal regulation exerted by estrogens.

6. SIGNALLING PATHWAYS INVOLVED IN THE ANTIOXIDANT EFFECT OF ESTROGENS

Estrogens were long ago recognized as *in vitro* antioxidants (36). However, at physiological concentrations it is very unlikely that they may act as such, especially due to their low concentration in plasma. A simple calculation indicates that if the recommended dose of estradiol in estrogen replacement therapy is 50 microg/day and the recommended dose of vitamin E as supplement is 500 mg/day; estrogen ought to be ten thousand times more potent than vitamin E to have a similar antioxidant capacity and this is obviously not the case.

Yet, *in vivo* experiments show that estrogens have a powerful antioxidant effect: mitochondrial H_2O_2 production is significantly increased (by more than 50%) after ovariectomy and this is completely prevented when ovariectomised rats are treated with estradiol at doses similar to those used in estrogen replacement therapy (for details see (23)). Then, it is clear that estrogens do not act as chemical antioxidants *in vivo*, instead they exert their antioxidant effect by upregulation of the expression of antioxidant genes and antioxidant enzymes.

We used MFC-7 cells, a human mammary gland cell line, to test if the antioxidant effects of estradiol are exerted via interaction with the estrogen receptors. When these cells were incubated with estradiol, the levels of H_2O_2 were significantly decreased. However, when the cells were co-incubated with estradiol and tamoxifen (an estrogen receptor inhibitor) the levels of H_2O_2 were similar to controls, thus confiming that the antioxidant effect of estrogen is mediated by the interaction of estradiol with the estrogen receptor. This observation opened the question about the intracellular mechanism by which estradiol acts to increase the expression of mitochondrial antioxidant enzymes. A direct genomic effect of estradiol was unlikely because neither Mn-SOD nor GPx have estrogen-responsive elements in their promoter region. Thus, it was likely that the action of estradiol is mediated via intracellular signalling cascades. We tested the effect of mitogen activated protein (MAP) kinases by using UO126, an inhibitor of the phosphorylation of these kinases. Our experiments showed that UO126 completely inhibited the lowering effect of estradiol on the level of H_2O_2 in cells (Table 1). MAP kinases are known to activate the nuclear factor kappa B (NF-kappaB). Thus, we tested whether estradiol acts by activating NF-kappaB in its upregulation of the expression of both Mn-SOD and GPx genes, whose promoters contain putative NF-kappaB -binding motifs. This was indeed the case: when cells were incubated with pyrrolidine dithiocarbamate (PDTC), an inhibitor of the I-kappaB degradation, and therefore an inhibitor of the NF-kappaB translocation to the nucleus, the effect of estradiol on the upregulation of antioxidant enzyme expression was prevented (Table 1). Using these pharmacological inhibitors of the signalling pathways, we concluded that estradiol upregulates the expression of Mn-SOD and GPx mediated by the following pathway: interaction with membrane estrogen receptor, activation of MAP kinases, activation of NF-kappaB, and upregulation of gene expression (Table 1) (37).

7. PHYTOESTROGENS MIMIC THE BENEFICIAL ACTION OF ESTROGENS IN PROMOTING MITOCHONDRIAL ANTIOXIDANT DEFENCES

The effect of estradiol as an upregulator of antioxidant and longevity-related genes indicates that its administration might be beneficial to increase life span, particularly in males that should reach in that way a life span similar to females. However, considerable evidence has shown that estrogen replacement therapy after menopause may have set backs (28). Phytoestrogens constitute an interesting alternative. Their beneficial effects have been reported repeatedly (38, 39) and, to our knowledge very few, if any, serious reports have shown detrimental effects. Thus, we tested the effect of 0.5 microM genistein, one of the major phytoestrogens in soya (40) on the H_2O_2 levels in MCF 7 cells. This level of genistein can be considered as nutritionally

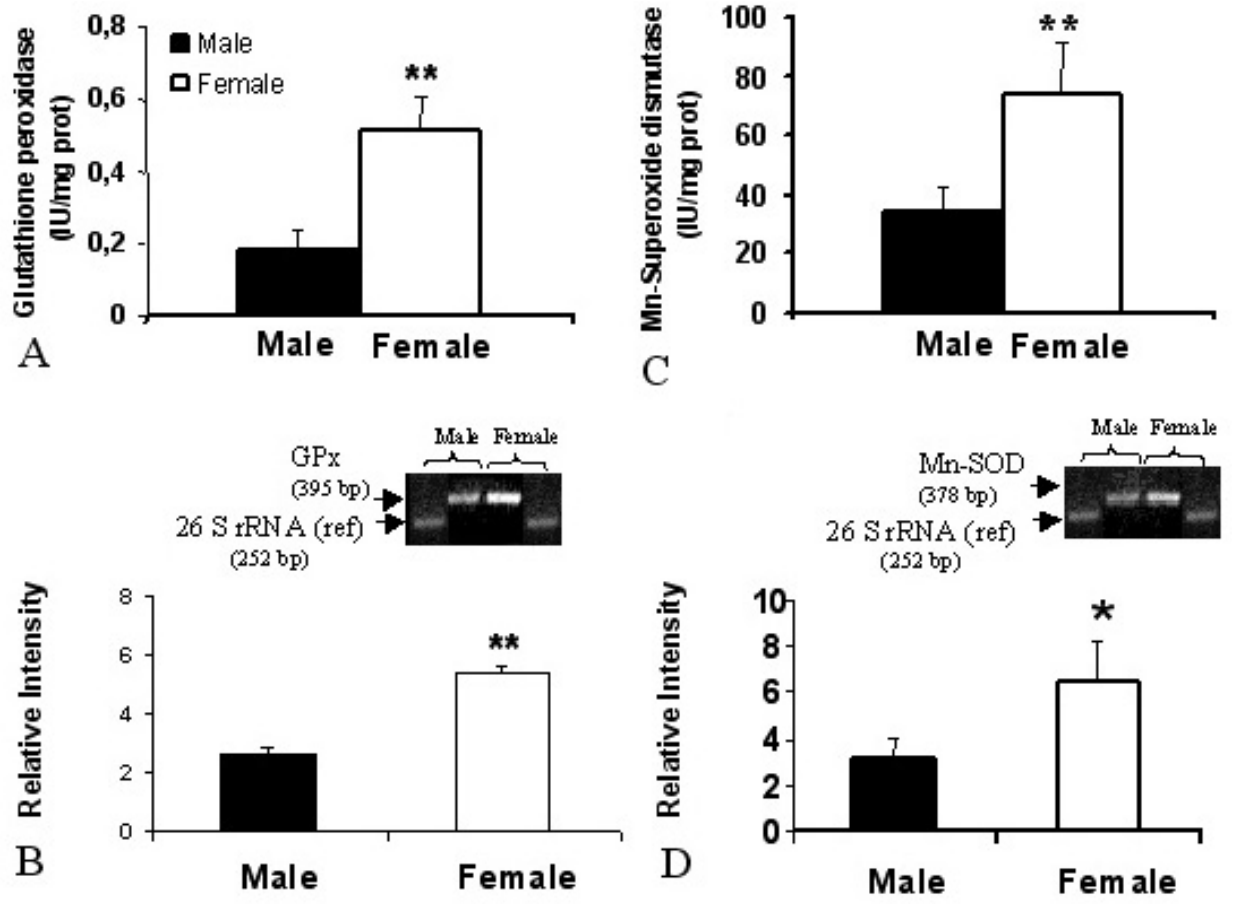

Figure 1. Expression and activity of glutathione peroxidase (GPx) and Mn-SOD in liver from male and female rats (taken from (23)). Antioxidant defence is higher in liver mitochondria from female rats than in those from males (6). Expression of antioxidant enzymes was studied by RT-PCR using specific oligonucleotides. (A) Glutathione peroxidase mRNA expression. (B) Glutathione peroxidase activity (C) Mn-SOD mRNA expression. (D) Mn-SOD activity. The statistical difference is indicated as follows: $^{**}p < 0.01$ versus male rats.

relevant as it is the concentration normally found in the blood of Far East people who eat relatively large quantities of soya in their normal diet. This concentration is, however, significantly higher than the one found in people living in the Western world. We found that genistein significantly decreased H_2O_2 levels in cells and that, just as with estradiol, this effect is mediated by estrogen receptors. We then studied if the signalling pathway that we had found to explain the antioxidant effects of estradiol also acted for genistein and found that indeed this is the case and that genistein increases MAP kinases and activates NF-kappaB resulting in an upregulation of the antioxidant gene for Mn-SOD.

8. PERSPECTIVE

Finding good models of aging is a major aim of gerontology. The different longevity between genders, *i.e.* that females live around 10% more than males in may species, including humans, offers a unique opportunity to study fundamental aspects of aging.

In the context of the mitochondrial theory of aging, we have found that mitochondrial oxidant production is approximately double in females than in males. We have traced this advantage of females to the presence of estrogens, which act via a pathway that comprises membrane estrogen receptors, MAP kinase, NF-kappaB signalling and the upregulation of the expression of the antioxidant enzymes Mn-SOD and GPx (Figure 2). Estrogen administration after menopause has clinical problems and, obviously, cannot be given to males. Phytoestrogens offer an interesting alternative. We have found that at concentrations found in plasma, they increase the expression of antioxidant enzymes without the feminizing effects of estradiol. The possibility of using those compounds to increase the longevity of males to reach the longevity of females is a fact to be seriously considered.

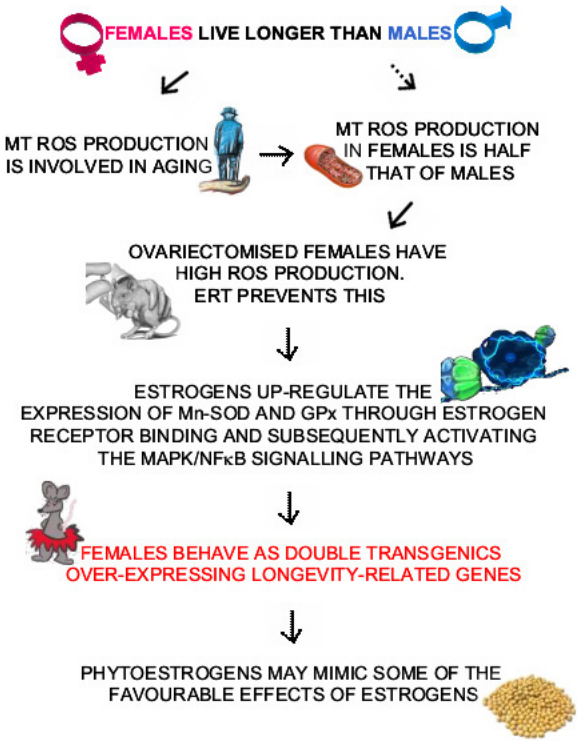

Figure 2. Molecular events that are consistent with the fact that females live longer than males in a series of mammalian species including humans.

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10. REFERENCES

1. Harman D: Aging: a theory based on free radical and radiation chemistry. *J Gerontol* 2, 298-300 (1956)
2. Harman D: The biological clock: The mitocondria. *Journal of de American Geriatrics Society* 20, 145-147 (1972)
3. Miquel J, A.C. Economos, J. Fleming, & J.E.J. Johnson: Mitocondrial role in cell aging. *Exp Gerontol* 15, 579-591 (1980)
4. Beckman KB & B.N. Ames: The free radical theory of aging matures. *Physiol Rev* 78, 547-581 (1998)
5. Barja G: Mitochondrial oxygen radical generation and leak: Sites of produccion in states 4 an 3, organ specificity and relation to aging and longevity. *J Bioenergetics Biomembranes* 31, 347-366 (1999)
6. Weindruch R & R.S. Sohal: Seminars in medicine of the Beth Israel Deaconess Medical Center. Caloric intake and aging. *N Engl J Med* 337, 986-994 (1997)
7. Boveris A, L. E. Costa & E. Cadenas: In: Undestanding the process of aging: the roles of mitochondria, free radicals, and antioxidants (Antioxidants in health and disease, 8). Eds: Cadenas, E. & Packer, L. (eds.), pp. 1-20 Marcel Decker, New York (1999)
8. Navarro A & A. Boveris: Rat brain and liver mitochondria develop oxidative stress and lose enzymatic activities on aging. *Am J Physiol Regul Integr Comp Physiol* 287, R1244-R1249 (2004)
9. Barja G: Rate of generation of oxidative stress-related damage and animal longevity. *Free Radic Biol Med* 33, 1167-1172 (2002)
10. Ku HH, U.T. Brunk, & R.S. Sohal: Relationship between mitochondrial superoxide and hydrogen peroxide production and longevity of mammalian species. *Free Radic Biol Med* 15, 621-627 (1993)
11. Barja G, S. Cadenas, C. Rojas, R. Perez-Campo, & M. Lopez-Torres: Low mitochondrial free radical production per unit O2 consumption can explain the simultaneous presence of high longevity and high aerobic metabolic rate in birds. *Free Radic Res* 21, 317-327 (1994)
12. Boveris A & B. Chance: The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem J* 134, 707-716 (1973)
13. Cadenas E & A. Boveris: Enhancement of hydrogen peroxide formation by protophores and ionophores in antimycin-supplemented mitochondria. *Biochem J* 188, 31-37 (1980)
14. Asensi M, J. Sastre, F.V. Pallardo, J. Garcia de la Asuncion, J. Estrela & J. Vina: A high-performance liquid chromatography method for measurement of oxidized glutathione in biological samples. *Anal Biochem* 217, 323-328 (1994)
15. Sastre J, A. Millan, J. Garcia de la Asuncion, R. Pla, G. Juan, F. Pallardo, E. O'Connor, J.A. Martin, M.T. Droy-Lefaix & J. Vina: A Ginkgo biloba extract (EGb 761) prevents mitochondrial aging by protecting against oxidative stress. *Free Radic Biol Med* 24, 298-304 (1998)
16. Sastre J, F.V. Pallardo, R. Pla, A. Pellin, G. Juan, J.E. O'Connor, J.M. Estrela, J. Miquel & J. Vina: Aging of the liver: age-associated mitochondrial damage in intact hepatocytes. *Hepatology* 24, 1199-1205 (1996)
17. Hagen TM, D.L. Yowe, J.C. Bartholomew, C.M. Wehr, K.L. Do, J.Y. Park & B.N. Ames: Mitochondrial decay in hepatocytes from old rats: membrane potential declines, heterogeneity and oxidants increase. *Proc Natl Acad Sci USA* 94, 3064-3069 (1997)
18. Shigenaga MK & B.N. Ames: Assays for 8-hydroxy-2'-deoxyguanosine: a biomarker of in vivo oxidative DNA damage. *Free Radic Biol Med* 10, 211-216 (1991)
19. Lezza AM, D. Boffoli, S. Scacco, P. Cantatore & M.N. Gadaleta: Correlation between mitochondrial DNA 4977-bp deletion and respiratory chain enzyme activities in aging human skeletal muscles. *Biochem Biophys Res Commun* 205, 772-779 (1994)
20. Chance B, H. Sies & A. Boveris: Hydroperoxide metabolism in mammalian organs. *Physiol Rev, 59*, 527-605 (1979)
21. de la Asuncion JG, A. Millan, R. Pla, L. Bruseghini, A. Esteras, F.V. Pallardo, J. Sastre & J. Vina: Mitochondrial glutathione oxidation correlates with age-associated

oxidative damage to mitochondrial DNA. *Faseb J* 10, 333-338 (1996)
22. Benzi G & A. Moretti: Age- and peroxidative stress-related modifications of the cerebral enzymatic activities linked to mitochondria and the glutathione system. *Free Radic Biol Med* 19, 77-101 (1995)
23. Borras C, J. Sastre, D. Garcia-Sala, A. Lloret, F.V. Pallardo & J. Vina: Mitochondria from females exhibit higher antioxidant gene expression and lower oxidative damage than males. *Free Radic Biol Med* 34, 546-552 (2003)
24. Vina J, C. Borras, J. Gambini, J. Sastre & F.V. Pallardo: Why females live longer than males: control of longevity by sex hormones. *Sci Aging Knowledge Environ 2005*, e17 (2005)
25. Vina J, J. Sastre, F. Pallardo & C. Borras: Mitochondrial theory of aging: importance to explain why females live longer than males. *Antioxid Redox Signal* 5, 549-556 (2003)
26. Wallace DC: Mitochondrial DNA sequence variation in human evolution and disease. *Proc Natl Acad Sci USA* 91, 8739-8746 (1994)
27. Richter C, J.W. Park & B.N. Ames: Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proc Natl Acad Sci USA* 85, 6465-6467 (1988)
28. Kasai H, P.F. Crain, Y. Kuchino, S. Nishimura, A. Ootsuyama & H. Tanooka: Formation of 8-hydroxyguanine moiety in cellular DNA by agents producing oxygen radicals and evidence for its repair. *Carcinogenesis* 7, 1849-1851 (1986)
29. Jocelyn PC & A. Kamminga: The non-protein thiol of rat liver mitochondria. *Biochim Biophys Acta* 343, 356-362 (1974)
30. Nina J, R. Hems & H.A. Krebs: Maintenance of glutathione content is isolated hepatocyctes. *Biochem J* 170, 627-630 (1978)
31. Hazelton GA & C.A. Lang: Glutathione levels during the mosquito life span with emphasis on senescence. *Proc Soc Exp Biol Med* 176, 249-256 (1984)
32. Sastre J, F.V. Pallardo & J. Vina: Mitochondrial oxidative stress plays a key role in aging and apoptosis. *IUBMB Life* 49, 427-435 (2000)
33. Boveris A & E. Cadenas: Mitochondrial production of hydrogen peroxide regulation by nitric oxide and the role of ubisemiquinone. *IUBMB Life* 50, 245-250 (2000)
34. Pinto RE & W. Bartley: The nature of the sex-linked differences in glutathione peroxidase activity and aerobic oxidation of glutathione in male and female rat liver. *Biochem J* 115, 449-456 (1969)
35. Orr WC & R.S. Sohal: Extension of life-span by overexpression of superoxide dismutase and catalase in Drosophila melanogaster. *Science* 263, 1128-1130 (1994)
36. Ruiz-Larrea MB, A.M. Leal, C. Martin, R. Martinez & M. Lacort: Antioxidant action of estrogens in rat hepatocytes. *Rev Esp Fisiol* 53, 225-229 (1997)
37. Borras C, J. Gambini, M.C. Gomez-Cabrera, J. Sastre, F.V. Pallardo, G.E. Mann & J. Vina: 17beta-oestradiol up-regulates longevity-related, antioxidant enzyme expression via the ERK1 and ERK2[MAPK]/NFkappaB cascade. *Aging Cell* 4, 113-118 (2005)
38. Munro IC, M. Harwood, J.J. Hlywka, A.M. Stephen, J. Doull, W.G. Flamm & H. Adlercreutz: Soy isoflavones: a safety review. *Nutr Rev* 61, 1-33 (2003)
39. Mahn K, C. Borras, G.A. Knock, P. Taylor, I.Y. Khan, D. Sugden, L. Poston, J.P. Ward, R.M. Sharpe, J. Vina, P.I. Aaronson & G.E. Mann: Dietary soy isoflavone induced increases in antioxidant and eNOS gene expression lead to improved endothelial function and reduced blood pressure in vivo. *Faseb J* 19, 1755-1757 (2005)
40. Park D, T. Huang & W.H. Frishman: Phytoestrogens as cardioprotective agents. *Cardiol Rev* 13, 13-17 (2005)
41. Vina J, C. Borras, J. Gambini, J. Sastre & F.V. Pallardo: Why females live longer than males? Importance of the upregulation of longevity-associated genes by oestrogenic compounds. *FEBS Lett.* 579, 2541-2445 (2005)

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