EFFECT OF AGING ON MITOCHONDRIAL AND NUCLEAR DNA OXIDATIVE DAMAGE IN THE HEART AND BRAIN THROUGHOUT THE LIFE-SPAN OF THE RAT

Asunción Herrero and Gustavo Barja

Department of Animal Biology-II (Animal Physiology)

Faculty of Biology

Complutense University

Madrid 28040, Spain.

ABSTRACT

The oxygen radical-induced DNA lesion 8-oxo,7,8-dihydro-2'-deoxyguanosine (8-oxodG) is the most commonly measured marker of oxidative DNA damage, which is currently considered a main cause of aging. However, a detailed study of the age-related variations of this marker in both mitochondrial (mtDNA) and nuclear (nDNA) DNA of post-mitotic organs throughout the life span has not been previously performed. In this investigation 8-oxodG steady-state levels were simultaneously measured in mtDNA and nDNA in the heart and brain of Sprague-Dawley rats at up to five different ages covering most of the adult life span, 4, 8, 12, 17 and 24 months of age, using exactly the same digestion of DNA to deoxynucleosides and chromatographic procedures for mtDNA and nDNA. 8-oxodG levels were maintained without changes during young and middle age in all cases, but showed statistically significant increases at the older ages studied in the majority of the kinds of DNA investigated. These age-related increases in oxidative damage occurred in brain nDNA at 17 and 24 months of age, in heart nDNA at 24 months of age, and in brain mtDNA at 24 months of age, whereas no significant age-related changes were detected in heart mtDNA. Besides, 8-oxodG levels were various fold higher in mtDNA than in nDNA, both in brain and heart, at all the ages studied. The results show that oxidative damage to DNA is higher in the mtDNA than in the nDNA of post-mitotic tissues throughout the whole life span of the rat and that an increase in mtDNA and nDNA oxidative stress occurs in most cases in old animals.

INTRODUCTION

Oxygen radical generation in the mitochondrial respiratory chain has been hypothesized as a major cause of aging (1,2), a concept strongly reinforced by recent findings (3,4). Oxidative damage to DNA is specially

relevant for aging (5). Recent comparative studies from our laboratory have shown that oxidative damage to mitochondrial DNA (mtDNA) is negatively correlated with maximum life span across mammalian species (6). This is consistent with many previous investigations which showed that the rate of mitochondrial oxygen radical generation is also negatively correlated with maximum life span (see 4 for review). Thus, short-lived species, like laboratory rodents, continuously produce oxygen radicals at mitochondria with high intensity throughout life (both in young and old adult animals). This would cause their comparatively higher steady-state levels of oxidative damage to mtDNA and their higher rate of accumulation of mtDNA mutations, which would explain in part their quick aging rate, the reverse being true in long-lived species (4,7). While this can be correct, the secondary additional possibility exists that the balance between the generation and elimination of oxidative DNA damage is altered during aging. An increase in the steady-state levels of oxidative DNA damage during aging would indicate that the flux of oxidative damage through DNA is higher in old than in young individuals, which would accelerate aging in the old. To clarify that possibility, oxidative damage to mtDNA and nuclear DNA (nDNA) must be measured at various ages throughout the life span in post-mitotic tissues (the ones more relevant to aging), like heart and brain.

Previous studies about age-related variations in oxidative damage to nDNA, measured as 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), are very scarce in the heart (8-10) and in the case of brain have resulted in conflicting results (8-15). In the case of heart mtDNA the scarcity of studies is also accompanied by strong discrepancies (16,17). In addition, many of those reports studied only two ages (one for young and one for old individuals), instead of measuring 8-oxodG at many time points throughout the whole life span. This limitation is specially frequent in the case of mtDNA. Moreover, studies measuring simultaneously 8-oxodG in mtDNA and nDNA are scant, are mainly limited to non-post mitotic tissue (liver, 10,18,19), and the possibility that oxidative damage is higher in mtDNA than in nDNA has never been tested throughout the aging process.

In order to contribute to overcome those limitations, in this work 8-oxodG steady-state levels were simultaneously measured by high performance liquid chromatography (HPLC) with electrochemical detection in brain

To whom all correspondence should be addressed:

Prof. Dr. G. Barja

Departamento de Biología Animal-II (Fisiología Animal)

Facultad de Biología

Universidad Complutense

Madrid 28040, Spain

Tel. 34-91-3944919

FAX: 34-91-3944935.

and heart nDNA and mtDNA of male Sprague-Dawley rats at up to 5 different ages covering most of the adult life span of this animal: 4, 8, 12, 17 and 24 months of age.

RESULTS

The values of rat brain 8-oxodG in nDNA as a function of age obtained in the present investigation cover most of the life-span of the adult rat (4, 8, 12, 17 and 24 months of age; Fig.1). Significant differences as a function of age were found by one-way analysis of variance (ANOVA). Paired post-ANOVA comparisons between selected age groups showed lack of differences at the beginning and the middle of the life span (between 4, 8 and 12 months). They also showed that the brain 8-oxodG values in nDNA started to increase at 17 months of age since at this age significantly higher values than at 12 months were already observed. At 24 months of age 8-oxodG was significantly higher than at 4, 8 and 12 months of age (Fig. 1).

Figure 1: Variation of rat heart and brain 8-oxodG/10⁵dG in nuclear DNA (nDNA) as a function of age. Values are means $\pm$ SEM from 5 to 8 animals (brain) or from 5 to 7 animals (heart). Significant differences as a function of age were observed in both organs by one-way ANOVA. Heart: * significant difference between 24 months and all the rest of the age groups. Brain: * significant difference between 17 months and 12 months; and * significant difference between 24 months and 4, 8, or 12 months (LSD post-ANOVA tests).

Analogously to what happened for brain nDNA, 8-oxodG also showed age-related significant differences in rat brain mtDNA (Fig. 2). In this case, oxidative damage did not vary between 4, 8, 12, and 17 months. At 24 months of age 8-oxodG levels in brain mtDNA were significantly higher than in all the other age groups (Fig. 2).

8-oxodG in rat heart nDNA showed a pattern of significant age-related variations (Fig. 3) similar to that observed in both kinds of brain DNAs. Whereas no significant differences were observed between 4, 8 and 17 months of age, the 8-oxodG values observed at 24 months were significantly higher than those observed at the three previous ages (Fig. 1).

Figure 2: Variation in rat heart and brain 8-oxodG/10⁵dG in mitochondrial DNA (mtDNA) as a function of age. Values are means $\pm$ SEM from 6 to 8 animals except at 12 months (4 animals) in brain, and from 5 to 7 animals except at 12 months (3 animals) in heart. Significant differences as a function of age were observed in the brain by one-way ANOVA whereas heart values did not show significant age-related differences. * significant difference between 24 months and all the rest of the age groups (LSD post-ANOVA tests).

8-oxodG was also assayed in rat heart mtDNA (Fig. 2). In this particular case, differing from what was observed in the three previous comparisons, no significant differences as a function of age were observed (Fig. 1).

The 8-oxodG values obtained in mtDNA were also compared with those of nDNA in each tissue at the different ages, when both values were available in the same individual animal (Table 1). The results showed that 8-oxodG values were always significantly higher in mtDNA than in nDNA at all the ages studied. In the case of brain, 8-oxodG was around 8 fold higher in mtDNA than in nDNA. In the heart, 8-oxodG values were around 9 fold higher in mtDNA than in nDNA. The magnitude of the difference in 8-oxodG between mtDNA and nDNA did not vary significantly as a function of age in any organ (Table 1).

Table 1. Ratio between 8-oxodG/10⁵dG in mitochondrial DNA and 8-oxodG/10⁵dG in nuclear DNA in the heart and brain of rats of different ages^a.

Age	Brain	Heart
4 months	7.1 $\pm$ 1.6 (8)	11.8 $\pm$ 3.2 (4)
8 months	7.2 $\pm$ 1.1 (7)	9.7 $\pm$ 1.5 (5)
12 months	9.6 $\pm$ 2.1 (4)	—
17 months	7.1 $\pm$ 1.7 (5)	7.8 $\pm$ 2.4 (5)
24 months	11.3 $\pm$ 3.4 (5)	7.6 $\pm$ 4.1 (5)

^aValues are means $\pm$ SEM with the number of animals in parentheses. Data were calculated dividing the 8-oxodG/10⁵ value found in the mtDNA by the 8-oxodG/10⁵dG value observed in the nuclear DNA when both values were available in the same animal. Mean $\pm$ SEM were computed at the different ages. No significant differences as a function of age were found in any organ (one-way ANOVA). 8-oxodG/10⁵dG values were significantly higher in mtDNA than in nDNA at all ages in both organs (Student's t-test).

DISCUSSION

The results obtained in this work show that steady-state oxidative damage to nuclear DNA in the brain and heart is maintained at constant levels during most of the adult life span of the rat but it increases in old individuals in relation to middle age and young adults. A similar pattern was found for brain mitochondrial DNA whereas in heart mitochondrial DNA no age-related variations were observed. The data also show that oxidative damage is various fold higher in mtDNA than in nDNA at all ages.

Previous studies about variations as a function of age in rodent or human 8-oxodG in brain nDNA are controversial, since some studies found increases in old versus young individuals (8,9,10,11) whereas other studies showed lack of age-related changes (12,13,14,15). A common characteristic of most of these studies is that they used only two (10,14) or three (8) selected ages or studied correlations with age in single individuals of different ages (11,12,13,15). Only in the study of Kaneko et al. (9) mean values were obtained at many different rat ages, and, similarly to our observations, increases in 8-oxodG in brain nDNA were observed only at the end of the life span. Increases in 8-oxodG in nDNA in the brain of humans also occur at old ages: from 60 to 97 years (11).

The situation for heart 8-oxodG in nDNA is different to that for brain nDNA. In this case, all previous studies, although less abundant, agree that age-related increases do occur. Thus, significantly higher values in heart nDNA 8-oxodG values have been found at 25 months than at 5 months in mongolian gerbils (8), at 27 months than at 8 months in C57BL/6 mice (10), and at 27 and 30 months than at all the studied younger ages in Fisher 344 rats (9). Similarly, we found in the present study significantly higher 8-oxodG values in rat heart nDNA at 24 months than at 4, 8, and 17 months of age in Sprague-Dawley rats.

Our finding of higher 8-oxodG values in the brain mtDNA of old compared with both young and middle-aged rats is also concordant with the majority of previously published studies. Thus, higher 8-oxodG levels in brain mtDNA have been observed at 24 months than at 6 months in mice (20) and at 27 months than at 4 months in Wistar rats (21). In humans, 8-oxodG in brain mtDNA also increased with age in all the four different brain regions analyzed in a study, specially when individuals younger and older than 70 years were compared (11). 8-oxodG in brain mtDNA also increased with age between 63 and 81 years in human frontal but not in parietal cortex (22).

Concerning heart mtDNA, there is a scarcity of studies compared with brain mtDNA, and they are not consistent. Strong exponential increases as a function of age have been described in the human heart in a study (16). However, the 8-oxodG values reported in that study, which used a special micro-HPLC/mass spectrometry technique, were very high, between 100

and 1,500/10⁵dG (0.1-1.5% 8-oxodG/dG; 16). It is well known that mass spectrometry techniques have resulted in the past in artifactual 8-oxodG values (23,24). In contrast to those findings, no age-related increase was observed in heart 8-oxodG in mtDNA in mice from 12 to 26 months of age in a study reporting values of 6/10⁵dG in young adults (17). This is in the range of the values obtained in the present study in rats in which we also did not found age-related differences. Thus, in the particular case of heart mtDNA, available information does not allow to conclude that oxidative damage increases with age.

Previous studies have shown that 8-oxodG is higher in mtDNA than in nDNA in the liver (10,18,19), heart (6) and brain (6,11) of young mammals. In the present study this was found in the rat not only in young, but also in middle aged and old individuals, a fact which has not been previously reported. Thus, our results show that 8-oxodG levels are continuously maintained at higher levels in the mtDNA than in the nDNA throughout the aging process in rat post-mitotic tissues, the ones more relevant for aging.

What is the significance of the age-related variations in 8-oxodG levels? The presence of similar 8-oxodG levels at young and intermediate ages suggests that the flux of oxidative damage through mtDNA is maintained essentially constant during most of the life span (in young adults and at middle age), which would lead to a constant rate of accumulation of mtDNA mutations (25-32). This would agree with the known fact that aging is a progressive phenomenon occurring at roughly the same rate throughout most of the life span of each species. In the last parts of the life span, however, an imbalance seems to exist between factors generating and eliminating 8-oxodG, increasing its levels at least in heart and brain nDNA and in brain mtDNA. This suggests that the flux of free radical damage through DNA is increased in old individuals. This, if the free radical theory of aging is correct, should increase the rate of aging at old age over that present at young and mature ages. Concerning the absolute 8-oxodG levels obtained in this investigation, they are among the lowest reported in the literature using classic methods of DNA extraction, although still lower levels have been obtained in nDNA using the chaotropic NaI method (33). Thus, the possibility remains that the real increases in 8-oxodG with age are still higher than those described in the present investigation.

EXPERIMENTAL PROCEDURES

Animals and tissue samples

Male OFA Sprague-Dawley rats of 250 g of weight were obtained from Iffa-Credo (Lyon, France) and were then maintained under barrier conditions at the university animal house at 20±1°C, 14:10 L:D cycle and 40±5% relative humidity with water and food ad libitum (B&K Universal GJ rodent toxicology diet, Humberside, UK). The diet contained the following amounts of antioxi-

dants at all ages: vitamin A 18,000 UI/Kg diet, vitamin E 24 mg/Kg diet and ethoxyquin 150 mg/Kg. The mean and maximum longevity of the male Sprague-Dawley colony were 26.9 and 36 months respectively. Rats were euthanized by decapitation at 4, 8, 12, 17 and 24 months of age. Whole ventricles and whole brains were washed free of blood, frozen in liquid nitrogen, and stored at -80°C to be analyzed simultaneously afterwards.

Isolation of mitochondrial and nuclear DNA

The nuclear DNA was isolated after homogenization, resuspension of nuclear pellets of brain and heart samples, and SDS treatment, by chloroform extraction and ethanol precipitation following the method of Loft and Poulsen (34) except that the initial homogenization buffer contained 5mM EDTA. Mitochondrial DNA, free of nDNA, was isolated by the method of Latorre et al., (35) adapted to mammals (20) as described previously (6). All subsequent steps, i.e. enzymatic digestion and 8-oxodG and deoxyguanosine (dG) HPLC analysis, were identical for mtDNA and nDNA. All aqueous solutions used for DNA isolation or digestion were prepared in HPLC-grade water (Fisher Chemicals, Loughborough, UK).

Digestion of nDNA and mtDNA

The isolated nuclear and mitochondrial DNAs were digested to deoxynucleoside level by incubation at 37°C with 5 U of nuclease P1 (in 20 µL of 20 mM sodium acetate, 10 mM ZnCl₂, 15% glycerol, pH 4.8) for 30 min and 1 U of alkaline phosphatase (in 20 µL of 1 M Tris-HCl, pH 8.0) for 1 hour (34).

8-oxodG and dG assays in nDNA and mtDNA

8-oxodG and dG were measured by HPLC with on line electrochemical and ultraviolet detection respectively. For analysis, the nucleoside mixture was injected into a reverse-phase Beckman Ultrasphere ODS column (5 µm, 4.6 mm × 25 cm), and was eluted with a mobile phase containing 2.5% acetonitrile and 50 mM phosphate buffer pH 5.0 prepared using HPLC-grade water (Fisher Chemicals, Loughborough, UK). The amount of deoxynucleosides injected was higher than the minimum amount needed to avoid potential artifacts due to injection of small amounts of deoxynucleosides in the HPLC system (36). A Waters 510 pump at 1mL/min was used. 8-oxodG was detected with an ESA Coulochem II electrochemical coulometric detector (ESA, Inc. Bedford, MA) with a 5011 analytical cell run in the oxidative mode (E1 = 0 mV, E2 = 225 mV), and dG was detected with a Biorad model 1806 UV detector at 254 nm. For quantification peak areas of dG standards and of three level calibration pure 8-oxodG standards (Sigma) were analyzed during each HPLC run. Comparison of areas of 8-oxodG standards injected with and without simultaneous injection of dG standards ensured that no oxidation of dG occurred during the HPLC run.

Statistical methods

Global comparisons between all the different age groups were analysed by one-way analysis of variance (ANOVA). When significant differences were found in the ANOVA, comparisons between pairs of groups were performed using the least significant difference (LSD) post-ANOVA test. The 0.05 level was selected as the point of minimal statistical significance in every analysis.

ACKNOWLEDGEMENTS

This work was supported by a grant from the National Research Foundation of the Spanish Ministry of Health. A. Herrero received a predoctoral grant for the Spanish Ministry of Education and Science.

ABBREVIATIONS

8-oxodG: 8-oxo,7,8-dihydro-2'-deoxyguanosine
dG: deoxyguanosine
mtDNA: mitochondrial DNA
nDNA: nuclear DNA
HPLC: high performance liquid chromatography

REFERENCES

1. Harman, D.: The biological clock: the mitochondria? J. Am. Geriatr. Soc., 20: 145-147, 1972.
2. Miquel, J.: An integrated theory of aging as a result of mitochondrial-DNA mutation in differentiated cells. Arch. Gerontol. Geriatr., 12: 99-117, 1988.
3. Sohal, R.S., and Weindruch, R.: Oxidative stress, caloric restriction, and aging. Science, 273: 59-63, 1996.
4. Barja, G.: Mitochondrial Free Radical Generation: Sites of production in states 4 and 3, organ specificity and relationship with aging rate. J. Bioenerg. Biomembr., 31: 347-366, 1999.
5. Beckman, K.B., and Ames, B.N.: The free radical theory of aging matures. Physiol. Rev., 78: 547-581, 1998.
6. Barja, G., and Herrero, A.: Oxidative damage to mitochondrial DNA is inversely related to maximum life span in the heart and brain of mammals. FASEB Journal, 14: 312-318, 2000.
7. Barja, G.: The flux of free radical attack through mitochondrial DNA is related to aging rate. Aging. Clin. Exper. Res., 12: 342-355 (2000).
8. Sohal, R.S., Agarwal, S., and Sohal, B.H.: Oxidative stress and aging in the mongolian gerbil (*Meriones unguiculatus*). Mech. Ageing Dev., 81: 15-25, 1995.
9. Kaneko, T., Tahara, S., and Matsuo, M.: Non-linear accumulation of 8-hydroxy-2'-deoxyguanosine, a marker of oxidized DNA damage, during aging. Mut. Res., 316: 277-285, 1996.

10. Sohal, R.S., Agarwal, S., Candas, M., Forster, M.J., and Lal, H.: Effect of age on DNA oxidative damage in different tissues of C57BL/6 mice. *Mech. Ageing Dev.*, 76: 215-224, 1994.
11. Mecocci, P., MacGarvey, U., Kaufman, A.E., Koontz, D., Shoffner, J.M., Wallace, D.C., and Beal, F.: Oxidative damage to mitochondrial DNA shows age-dependent increases in human brain. *Ann. Neurol.*, 34: 609-616, 1993.
12. Fraga, C.G., Shigenaga, M.K., Park, J.W., Degan, P., and Ames, B.N.: Oxidative damage to DNA during aging: 8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine. *Proc. Natl. Acad. Sci. USA*, 87: 4533-4537, 1990.
13. Sai, K., Tagaki, A., Umemura, T., Hasegawa, R., and Kurokawa, Y.: Changes of 8-hydroxydeoxyguanosine levels in rat organ DNA during the aging process. *J. Exper. Pathol. Toxicol. Oncol.*, 11: 139-143, 1992.
14. Hirano, T., Yamaguchi, R., Asami, S., Iwamoto, N., and Kasai, H.: 8-hydroxyguanine levels in nuclear DNA and its repair in rat organs associated with age. *J. Gerontol.*, 51A: B303-B-307, 1996.
15. Koppele, J.M.T., Lucassen, P.J., Sakee, A.N., Van Asten, J.G., Ravid, R., Swaab, D.F., and Van Bezooijen, C.F.A.: 8OHdG levels in brain do not indicate oxidative DNA damage in Alzheimer's disease. *Neurobiol. Aging*, 17: 819-826, 1996.
16. Ozawa, T.: Mitochondrial DNA mutations associated with aging and degenerative diseases. *Exp. Gerontol.*, 30: 269-290, 1995.
17. Muscari, C., Giaccari, A., Stefanelli, C., Viticchi, C., Giordano, E., Guarnieri, C., and Caldarera, C.M.: Presence of a DNA-4236 bp deletion and 8-hydroxy-deoxyguanosine in mouse cardiac mitochondrial DNA during aging. *Aging Clin. Exp. Res.*, 8: 429-433, 1996.
18. Richter, Ch., Park, J.W., and Ames, B.N.: Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proc. Natl. Acad. Sci. USA*, 85: 6465-6467, 1988.
19. Chung, M.H., Kasai, H., Nishimura, S., and Yu, B.P.: Protection of DNA damage by dietary restriction. *Free Rad. Biol. Med.*, 12: 523-525, 1992.
20. Asunción, J.G., Millan, A., Pla, R., Bruseghini, L., Esteras, A., Pallardo, F.V., Sastre, J., and Viña, J.: Mitochondrial glutathione oxidation correlates with age-associated oxidative damage to mitochondrial DNA. *FASEB J.*, 10: 333-338, 1996.
21. Sastre, J., Millán, A., Asunción, J.G., Plá, R., Juan, G., Pallardó, F.V., O'Connor, E., Martín, J.A., Droy-Lefaix, M.T., and Viña, J.: A ginkgo biloba extract (EGb 761) prevents mitochondrial aging by protecting against oxidative stress. *Free Rad. Biol. Med.*, 24: 298-304, 1998.
22. Lezza, A.M.S., Mecocci, P., Cormio, A., Beal, M.F., Cherubini, A., Cantatore, P., Senin, U., and Gadaleta, M.N.: Mitochondrial DNA 4977 bp deletion and OH⁸dG levels correlated in the brain of aged subjects but not Alzheimer's disease patients. *FASEB J.*, 13: 1083-1088, 1999.
23. Ravanat, J.C., Turesky, R.J., Gremaud, E., Trudel, L.J., and Stadler, R.H.: Determination of 8-oxoguanine in DNA by gas chromatography-mass spectrometry and HPLC-electrochemical detection: overestimation of the background level of the oxidized base by the gas chromatography-mass spectrometry assay. *Chem. Res. Toxicol.*, 8: 1039-1045, 1995.
24. Cadet J., D'ham, C., Douki, T., Pouget, J.P., Ravanat, J.C. and Sauvaigo, S.: Facts and artifacts in the measurement of oxidative base damage to DNA. *Free Rad. Res.*, 29: 541-550, 1998.
25. Liu, V.W.S., Zhang, C., Linnane, A., and Nagley, P.: Quantitative allele-specific PCR: demonstration of age-associated accumulation in human tissues of the A-G mutation at nucleotide 3243 in mitochondrial DNA. *Human Mut.*, 9: 265-271, 1997.
26. Wang, E., Wong, A., and Cortopassi, G.: The rate of mitochondrial mutagenesis is faster in mice than in humans. *Mut. Res.*, 377: 157-166, 1997.
27. Brierley, E.J., Johnson, M.A., Lightowlers, R.N., James, O.F.W., and Turnbull, D.M.: Role of mitochondrial DNA mutations in human aging: implications for the central nervous system and muscle. *Ann. Neurol.*, 43: 217-223, 1998.
28. Kovalenko, S.A., Kospidas, G., Kelso, J., Rosenfeldt, F., and Linnane A.W.: Tissue-specific distribution of multiple mitochondrial DNA rearrangements during human aging. *Annals New York Acad. Sci.*, 854: 171-181, 1998.
29. Nagley P., and Zhang C.: Mitochondrial DNA mutations in aging, in *Mitochondrial DNA Mutations in Aging, Disease and Cancer*, edited by Singh, K.K.. Berlin, Springer. 1998, pp. 205-238.
30. Singh, K.K.: *Mitochondrial DNA Mutations in Aging, Disease and Cancer*. Berlin, Springer, 1998.
31. Napiwotzki, J., Reith, A., Becker, A., Leist, S., and Kadenbach, B.: Quantitative analysis of mutations of mitochondrial DNA during human aging, in *Understanding the Process of Aging*, edited by Cadenas, E., and Packer, L.. New York, Marcel Dekker. 1999, pp. 251-264.
32. Ozawa, T.: Mitochondrial genome mutation in cell death and aging. *J. Bioenerg. Biomembr.*, 31: 377-390, 1999.

33. Helbock, H.J., Beckman, K.B., Shigenaga, M.K., Walter, P.B., Woodall, A.A., Yeo, H.C., and Ames, B.N.: DNA oxidation matters: The HPLC-electrochemical detection assay of 8-oxo-deoxyguanosine and 8-oxo-guanine. *Proc. Natl. Acad. Sci. USA*, 95:288-293, 1998.
34. Loft, S., and Poulsen, H.E.: Markers of oxidative damage to DNA: antioxidants and molecular damage. *Methods in Enzymol.*, 300: 166-184, 1999.
35. Latorre, A., Moya, A., and Ayala, A.: Evolution of mitochondrial DNA in *Drosophila subobscura*. *Proc. Natl. Acad. Sci. USA.*, 83: 8649-8653, 1986.
36. Beckman, K.B., and Ames, B.N.: Detection and quantification of oxidative adducts of mitochondrial DNA. *Methods Enzymol.*, 264: 442-453, 1996.