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ORIGINAL CONTRIBUTION

Catherine M. Gedik
George Grant
Philip C. Morrice
Sharon G. Wood
Andrew R. Collins

Effects of age and dietary restriction on oxidative DNA damage, antioxidant protection and DNA repair in rats

Summary *Background* Experimentally imposed dietary restriction is known to extend the lifespan of rodents, perhaps by slowing the accumulation of oxidative damage that is thought to be one of the causes of aging. *Aim of the study* We examined the effects of restricted total food intake, and protein and calorie restriction, on DNA oxidation and related biomarkers in rats. *Methods* From 1 to 17 months, rats in group 1 received normal diet *ad libitum*. Group 2 received 70 % of the quantity consumed by the first group. Group 3 had the same quantity as group 2, but with a reduction in protein (from 18 % to 10 % of the diet by weight), and group 4 were further restricted with a 30 % decrease in calories. Lymphocytes were isolated from blood samples taken every two months. DNA breaks, oxidised pyrimidines, resistance to H_2O_2-induced damage, and strand break repair were measured with the comet assay. Organs were isolated from rats killed at 17 months, with 1 month-old rats for comparison; DNA oxidation and antioxidant enzyme activities were measured. *Results* DNA breaks in lymphocytes increased from 1 to 3 months but thereafter declined with age, except in *ad libitum* fed rats. Oxidised pyrimidines did not change significantly. Resistance to H_2O_2-induced damage was least at 3 months, and increased with age. Repair of DNA strand breaks was efficient at all ages. Diet had little effect on these endpoints. Diet had no influence on 8-oxo-7.8-dihydroguanine levels in DNA from liver, testis and brain of 17 month-old rats. Combining data from all four groups, the levels in brain and liver were significantly higher at 17 months compared with 1 month. Antioxidant enzyme activities tended to increase between 1 and 17 months; effects of diet were not so consistent. *Conclusions* While DNA damage shows a modest increase with age in some organs, antioxidant status and DNA strand break repair do not decline with age. Restricted diets (including protein and calorie restriction) have no effect on any of these markers of genetic stability.

Key words diet restriction – DNA repair – aging – 8-oxo-7.8-dihydroguanine – antioxidant enzymes

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C. M. Gedik · G. Grant · P. C. Morrice · S. G. Wood · A. R. Collins
Rowett Research Institute
Greenburn Road
Aberdeen AB21 9SB, Scotland

Present address:
A. R. Collins (✉)
Dept. of Nutrition
University of Oslo
PO Box 1046 Blindern
0316 Oslo, Norway
E-Mail: a.r.collins@basalmed.uio.no

Introduction

One theory of aging proposes that it results from the accumulation of oxidative damage caused by the action of free radicals (reactive oxygen species, ROS) on macromolecules such as DNA, proteins and lipids, leading to a loss of function of these molecules [1]. A decline in the efficiency of mitochondrial action with age would cause release of higher concentrations of reactive oxygen. This would be exacerbated by a decline in the effectiveness of antioxidant defences or DNA repair pathways with age. 8-oxo-7.8-dihydroguanine (8-oxoGua) is the most studied marker of oxidative DNA damage because of its potential mutagenicity and apparent ease of measurement. Increases in 8-oxoGua with age have been reported in various tissues of rats and mice [2–6] but not in all organs or to the same extent in each organ [2, 3, 5,

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6]. Other studies reported no such increase [7, 8]. Oxidation of guanine to 8-oxoGua can occur during DNA extraction, and derivatisation (if using GC/MS), prior to chromatographic analysis [9] and so any high values reported have to be treated with caution. Recent improvements in the DNA extraction method, for example by using chelators, spin-trap reagents and antioxidants, have decreased the amount of spurious oxidation [10, 11]. Using such methods, significant age-related increases of 8-oxoGua were seen in nuclear DNA in *ad libitum* (AL) fed rodents (mouse and rat liver, brain, kidney, heart and muscle [12]; rat heart [13]; rat liver [14]).

Dietary restriction (DR) has been reproducibly shown to increase lifespan in various rodent species [15, 16]. DR in rats and mice ameliorates various diseases and reverses most physiological changes that are associated with aging (reviewed in [17]). If an age-related increase in DNA oxidation is important in aging, then DR should retard or limit this increase. DR to 60 % of AL intake has been reported to decrease [5, 12, 18], or retard [19], the age-related accumulation of 8-oxoGua residues in nuclear DNA; however, the effectiveness of DR varied between organs [5] and depended on strain and sex of animal [12]. Protein restriction (PR) to 60 % of an AL diet is reported to cause a small increase in longevity of rats [16] and to retard the age-related lesions of chronic nephropathy and cardiomyopathy [20]. The effects however are smaller than those seen with DR, where both calories and protein are restricted.

Antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPx) and Cu/Zn- and Mn-superoxide dismutases (Cu/ZnSOD and MnSOD) react with ROS and so prevent oxidative damage of DNA. Glutathione reductase (GR) is involved in the antioxidant process by catalysing the reduction of the antioxidant, glutathione. A decline in activity with age in these enzymes could result in an accumulation of DNA damage. Comparisons of enzyme activities in young and old mammals [12, 21–27] give varying indications as to whether a particular antioxidant enzyme's activity decreases with age, or not, in a specific organ. A similarly confused picture is seen for the effects of DR on enzyme activity [21–23, 25–27].

Increasing damage in DNA with age could be due to a decline in the efficiency of repair mechanisms. Cockayne syndrome, characterised by premature aging, is apparently associated with defective repair of oxidative DNA damage in nuclear DNA [28]. Various studies have given conflicting results on the effect of age (± DR) on repair, but the doses of damaging agent generally used are far in excess of anything likely to be encountered *in vivo* and so results have to be treated with caution. King et al. [29] found that repair of a low level of H_2O_2-induced strand breaks in lymphocytes was not significantly different in 75–80 year old volunteers from that measured in a 35–39 age group. The rate of removal of 8-oxoGua induced by whole body γ-irradiation (2 Gy) was similar (in liver, heart and brain) for young and old mice [12] and the activity of OGG1 (an 8-oxoGua glycosylase/AP lyase) in rat heart and liver was unaffected by age [30].

Our aim was to investigate the effect of age and various dietary restrictions – including PR, which has not been widely researched – on the activities of antioxidant enzymes, damage in DNA and the efficiency of repair of induced damage. One group of rats (group 1, AL) was fed *ad libitum* from the time of weaning at one month. A second group (group 2, DR) were given 70 % of the food consumed by the first group. Two further groups of rats were maintained on this restricted diet with additional protein restriction to 55 % (group 3, PR); or with 55 % protein restriction and calorie restriction to 70 % (group 4, PC). Thus groups 1 to 4 represent increasing severity of restriction. This design contrasts with other reported studies on PR, in that the rats on PR were also diet restricted (rather than fed AL), so they could not compensate for the lack of protein by increasing their daily food intake.

Materials and methods

Animals and diets

Thirty-two male specific pathogen-free Hooded-Lister rats (Rowett strain) were reared and housed in the small animal unit of the Rowett Research Institute. The rats were weaned at 19 days and given free access to a non-purified diet (SDS, Witham, UK) for 10 days. They were separated into 4 weight-matched groups of 8, housed individually and fed exclusively with semi-synthetic diets (Table 1) essentially as described in Grant et al. [31] until 17 (calendar) months old. Distilled water was freely available at all times.

Group 1 (control, AL) rats were given free access to diet A (180 g protein/kg, energy: 17.5 MJ/kg). Group 2 (DR) rats were fed a fixed amount of diet A daily, equivalent to 70 % of the daily intake of group 1. Group 3 (PR) rats were fed a fixed daily amount as group 2, but with the protein content reduced from 18 % to 10 % (diet B: 100 g protein/kg, energy: 17.5 MJ/kg). Group 4 (PC) rats were fed a fixed daily amount as Group 2, but with both protein and calories restricted (diet C: 100 g protein/kg, 12.5 MJ/kg). The available calorie content was reduced by 30 % in diet C. Every diet contained at least the daily requirement of vitamins and minerals.

The free intake of Group 1 was initially 15 g/rat/day, rising to 19 g/rat/day by week 8 and remaining at that level until week 24. The intake decreased to 17 g/rat/day for weeks 24 to 40 and to 16 g/rat/day thereafter. The amounts given to Groups 2, 3 and 4 were initially 11 g/rat/day, increasing to 14 g/rat/day by week 8. This in-

Table 1 Composition of experimental diets (g/kg)

Diet	A	B	C
Lactalbumin	195	108	108
Maize starch	305	392	317
Potato starch	100	100	300
Glucose	150	150	75
Glycerol	0	0	50
Corn oil	150	150	50
Vitamins	50	50	50
Minerals	50	50	50
Silicic acid	0.4	0.4	0.4
Available protein (g/kg)	180	100	100
Available energy (MJ/kg)	17.5	17.5	12.5

Mineral mix (1 kg) comprises copper sulphate 400 mg, ferrous sulphate 5000 mg. manganous sulphate 4000 mg, zinc sulphate 3600 mg, potassium iodate 40 mg, potassium iodide 40 mg, sodium fluoride 120 mg, ammonium vanadate 10 mg, nickel chloride 80 mg, stannous chloride 120 mg, sodium selenate 6 mg, chrome alum 960 mg, calcium carbonate 420 g, potassium di-hydrogen orthophosphate 314 g, potassium chloride 22 g, magnesium sulphate 102 g and di-sodium hydrogen orthophosphate 142 g. *Vitamin mix (1 kg) contains* thiamine 200 mg, pyridoxine 200 mg, riboflavin 200 mg, p-amino benzoic acid 200 mg, nicotinic acid 600 mg, calcium pantothenate 400 mg, folic acid 100 mg, biotin 100 mg, inositol 8000 mg, α-tocopherol 5000 mg, retinyl acetate 230 mg, cholecalciferol 300 mg, cyanocobalamin 5 mg, menadione 100 mg, choline chloride 20 g made up to 1 kg with maize starch

take was maintained until week 24 and was then decreased to 13 g/rat/day for weeks 24 to 40 and to 12 g/rat/day thereafter. All food offered to animals in Groups 2, 3 and 4 was consumed each day. The weights of the rats were recorded regularly as part of a check on health (Fig. 1).

All management and experimental procedures conducted during this study were done in strict accordance with the requirements of the UK Animals (Scientific Procedures) Act 1986 and current ethical review procedures.

Separation of lymphocytes for the comet assay

Blood was taken from the tail vein of 16 one month-old rats. We also collected blood from the rats in Groups 1–4 at 3 months old, and thereafter at 2 monthly intervals until 17 months old. (These intervals are in calendar months.) Four aliquots of 30 ml were each added to 1 ml RPMI 1640 (Sigma-Aldrich Co. Ltd., Poole, UK) containing 10 % fetal calf serum and kept on ice for 30 min. A volume of 200 µl histopaque-1077 (Sigma-Aldrich Co. Ltd., Poole, UK) was added below the mixture and the samples were centrifuged at 200 x g for 4.5 min at 4 °C. Lymphocytes were collected in a volume of 100 µl taken from near the interface of the solutions.

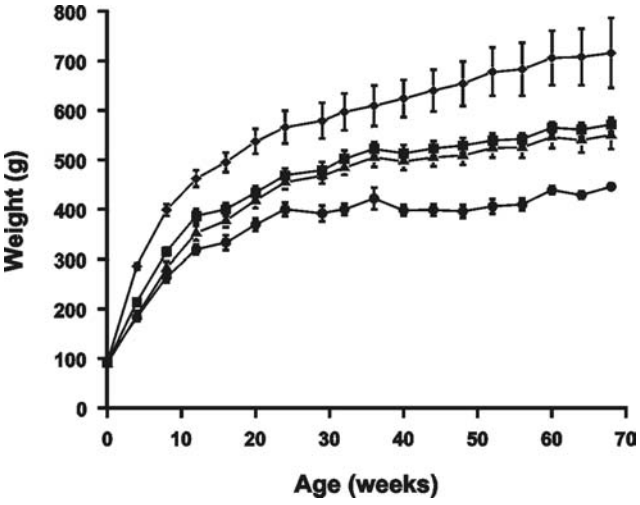

Fig. 1 Weight of rats maintained on 4 different diets: an *ad libitum* diet (AL) (◆; Group 1), with diet restriction (DR) (■; Group 2), with diet restriction and protein restriction (PR) (▲; Group 3), and with diet, protein and calorie restriction (PC) (●; Group 4). There were 6–8 animals per group. The weights were recorded each week; mean weights per group at 4-week intervals are shown ± SD

Measurement of endogenous strand breaks and endonuclease III-sensitive sites in lymphocyte DNA using the comet assay

Two aliquots of lymphocytes, isolated as above, were added to 1 ml PBS and centrifuged at 200 x g for 3 min at 4 °C. The cells were dispersed in 1 % low melting point agarose (LMP agarose: Life Technologies, Paisley, UK) in phosphate-buffered saline (PBS) at 37 °C, placed on a microscope slide and processed using the comet assay as described previously [32]. The lymphocytes in one gel were incubated with endonuclease III and in the other gel with enzyme buffer alone. After electrophoresis, comets were analysed visually, giving an overall score for each gel (100 comets) of between 0 and 400. The measure of oxidised pyrimidines (in arbitrary units) was obtained by subtraction of the comet assay score with buffer alone (due to background strand breaks) from that with endonuclease III.

Measurement of hydrogen peroxide-induced strand breaks in lymphocyte DNA and their repair by the comet assay

Two aliquots of lymphocytes, isolated as above, were resuspended in 50 µM hydrogen peroxide (H_2O_2) in PBS, kept for 5 min on ice and then centrifuged at 200 x g, 3 min at 4 °C. Cells from one tube were dispersed in LMP agarose to measure breaks induced by H_2O_2. To determine the ability of the lymphocytes to repair these breaks, H_2O_2 was removed from the other aliquot of cells by washing the cell pellet with 1 ml PBS, followed by cen-

trifugation as before. The lymphocytes were re-suspended in 1 ml RPMI with 10 % fetal calf serum and were incubated for 2 h at 37 °C in a 6 % CO_2 atmosphere. The cells were harvested by centrifugation as above and re-suspended in LMP agarose for analysis by the comet assay.

Recovery of rat tissues

Two rats in Group 4 were killed between 11 and 13 calendar months of age (owing to loss of condition), as was one rat in Group 1 just before the planned end of the experiment at 17 months (because of declining food intake). The remaining rats were sacrificed at 17 months old. In addition 16 rats were killed at 10 days after weaning; they had had free access to the non-purified diet (as for Groups 1 to 4) after weaning. These rats were killed by exsanguination under deep terminal anaesthesia with Isofluorane. Organs were removed and immediately frozen in liquid nitrogen, except for the liver, which was perfused with saline and then cut into small pieces before freezing. Each hemisphere of the brain was stored separately. The median life span of Hooded-Lister rats (Rowett strain) given free access to standard rat chow is 18 months, 25 % remaining by 20 months.

Isolation of nuclear DNA from rat organs

Nuclear DNA was isolated from brain, testis and liver of the rats in Groups 1 to 4 and of the one month-old rats 1 to 8. The tissues were thawed in the cryovials on ice and immediately added to buffer after weighing, to minimise exposure to atmospheric oxygen. One hemisphere of the brain was homogenised in 3 ml 10 mM Tris-HCl, 0.4 M NaCl, 5 mM deferoxamine mesylate pH 8.0 with 0.5 % Triton X-100 and a volume of the homogenate containing 300 mg of tissue was added to 2 ml buffer. This was kept for 5 min on ice before sedimenting the nuclei at 1200 x g for 10 min at 4 °C. The pelleted nuclei were washed in buffer and nuclear DNA was isolated as described [33] and stored at –80 °C under nitrogen until use. For testis and liver, 200 mg and 150 mg of tissue respectively were homogenised in 3 ml buffer containing Triton X-100 and kept for 5 min on ice. Following cell lysis, nuclear DNA was isolated as above and stored under nitrogen at –80 °C until use.

DNA hydrolysis and HPLC analysis

DNA was hydrolysed by incubation with DNase I (bovine pancreas), phosphodiesterases I and II (*C. durissus* and calf spleen respectively) and alkaline phosphatase (calf intestine) in the presence of $MgCl_2$ for 2 h at 37 °C. The method was based on that of Richter et al. [34] and the enzymes were obtained from Roche Diagnostics Ltd. (Lewes, UK).

The DNA hydrolysate was applied to an Apex ODS 3 mm C18 (150 x 4.6 mm) column (Capital Analytical, London, UK) with a pellicular LC18 guard column (Supelco, Poole, UK). The mobile phase was 50 mM potassium phosphate buffer containing 8 % methanol (HPLC-grade water and methanol: Rathburn Chemicals, Walkerburn, UK) and the flow rate was 0.5 ml/min. A Coulochem II electrochemical detector (ESA, Aylesbury, UK) with an ESA 5021 conditioning cell were used and the nucleosides 8-oxodGuo and dGuo were measured at 400 mV on an ESA 5011 analytical cell and at 254 nm by UV detection respectively.

Measurement of antioxidant enzymes

Liver, testis, brain, heart and kidney tissue of all rats in Groups 1 to 4 and of one month-old rats 1 to 8 were thawed on ice and homogenised with a Potter-Elvehjen homogeniser on ice in 50 mM potassium phosphate buffer pH 7.4; aliquots were snap frozen in liquid nitrogen and stored at –80°C until use. The methods for measuring GR activity and GPx activity were based on those of Galbraith and Watts [35] and Paglia and Valentine [36]. Measurements of CAT and SOD activities were based on the methods of Aebi [37] and Flohé and Ötting [38] respectively. Enzyme activities were expressed relative to protein concentration, which was measured by the biuret method [39].

Statistical analysis

Data were analysed by one-way ANOVA using Genstat 6 (Release 6.1, Lawes Agricultural Trust, Rothamsted, Herts, UK). Comparison of treatments was done by calculating the t-statistic from the ANOVA output.

Data collected every 2 months from 3 to 17 months from individual rats were subjected to linear regression analysis; mean slopes were calculated for each dietary group. One-way ANOVA was used to ascertain whether these mean slopes were significantly different from zero slope and from the slopes of the other groups. (In Group 4, two rats died before 13 months; data collected from them at earlier time points were not included in the slope analysis.)

Results

Levels of 8-oxoGua in organ DNA

DNA oxidation, in the form of 8-oxoGua, was measured by HPLC in DNA isolated from liver, brain and testis (Table 2). No significant differences in the levels of 8-oxoGua in brain, testis or liver DNA were observed between the groups fed on different diets (the apparent trend to lower 8-oxoGua in brain with increasing restriction of diet is not significant); therefore we could compare the 8-oxoGua levels in rats from the four diet groups combined with the 1-month old rats. It was found that 8-oxoGua was significantly higher in the 17-month than in the 1-month old rats for brain ($P < 0.05$) and liver ($P = 0.005$).

Endogenous strand breaks and endonuclease III-sensitive sites in lymphocyte DNA

Very low levels of DNA strand breaks (measured by the comet assay) were seen throughout the study. However, a significant increase in background strand breaks was seen when comparing rats aged 1 and 3 months (Fig. 2) (Group 1: $P = 4 \times 10^{-6}$, Group 2: $P = 1 \times 10^{-6}$, Group 3: $P = 0.001$, and Group 4: $P < 0.01$). Strand breaks then decreased up to 17 months, significantly for groups 2, 3 and 4 ($P = 0.02$, < 0.01 and < 0.001 respectively). The slope of the decrease for Group 4 was significantly greater than that for Group 1. At 15 months, strand breaks were significantly lower in groups 3 and 4 (relative to group 1: $P < 0.005$). At 17 months, strand breaks were significantly lower in group 4 relative to group 1 ($P < 0.05$).

There were no significant changes in oxidised pyrimidine levels in DNA from 1 to 3 months (Fig. 3). At 13 and 15 months, lower levels were seen in Group 4 than in the other diet groups, but these were only significantly lower than Group 1 at 15 months ($P < 0.05$). At 17 months these differences were not seen. Overall, from 3 to 17 months, there was no significant change in amounts of oxidised pyrimidines in any group and no differences between diet groups.

Table 2 Levels of 8-oxoGua/106Gua in DNA from rat organs

	Brain	Testis	Liver
1 month	2.58 ± 0.29	4.51 ± 0.48	2.25 ± 0.30
Group 1 (AL)	4.01 ± 0.46*	5.98 ± 1.35	3.80 ± 0.40
Group 2 (DR)	3.77 ± 0.49*	5.55 ± 0.90	5.22 ± 0.76**
Group 3 (CR)	3.42 ± 0.28	7.03 ± 1.08	4.61 ± 0.75*
Group 4 (PC)	2.92 ± 0.52	6.65 ± 1.38	4.22 ± 1.12

8-oxoGua/10^6Gua levels in nuclear DNA of brain, testis and liver of one month-old, and 17 month-old rats (6–8 rats per group) . DNA hydrolysates were analysed by HPLC in duplicate to quadruplicate. Mean values ± SEM for each group are shown. * and ** indicate significant differences between one month-old and old rats ($P < 0.05$, $P < 0.01$ respectively) for a particular organ

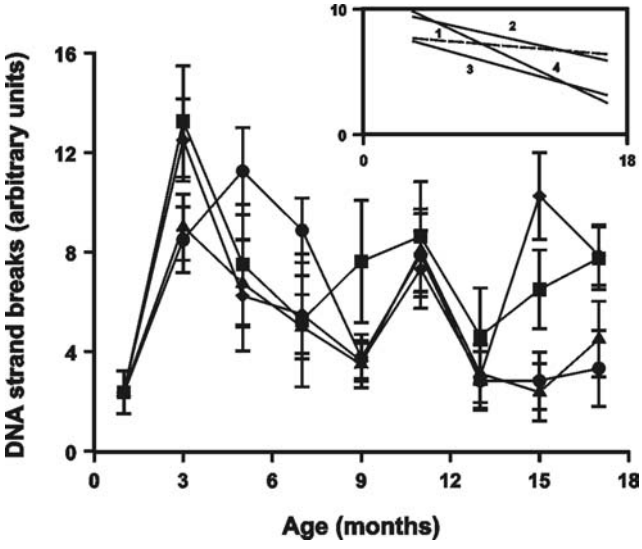

Fig. 2 Background DNA strand breaks in rat lymphocyte DNA measured by the comet assay. Breaks are expressed as arbitrary units (± SEM) for 16 one-month old rats and for groups of 6–8 rats fed on different diets as described in Fig. 1, sampled at 2 monthly intervals from 3 to 17 months. Groups: AL (◆; Group 1), DR (■; Group 2), PR (▲; Group 3), and PC (●; Group 4). The inset shows regression lines for the data for the four groups of rats (between 3 and 17 months). Solid lines; slopes differ significantly from zero (Group 2, $P = 0.02$; Group 3, $P < 0.01$; Group 4, $P < 0.001$). Broken line: no significant slope

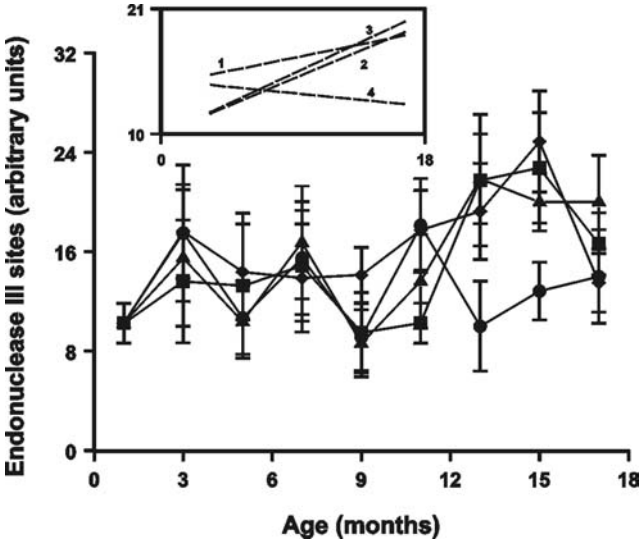

Fig. 3 Endonuclease III-sensitive sites (oxidised pyrimidines) in rat lymphocyte DNA measured by the comet assay. The mean endonuclease III sites are given as arbitrary units (± SEM) for 16 one-month old rats and for groups of 6–8 rats fed on different diets as described in Fig. 1, sampled at 2 monthly intervals from 3 to 17 months. Groups: AL (◆; Group 1), DR (■; Group 2), PR (▲; Group 3), and PC (●; Group 4). The inset shows regression lines for the data for the four groups of rats (between 3 and 17 months). Slopes did not differ significantly from zero

H_2O_2-induced breaks in lymphocyte DNA and their repair

Antioxidants present in the cell react with ROS, such as H_2O_2, preventing damage to DNA. The number of breaks induced by H_2O_2 (Fig. 4) is therefore an inverse measure of the antioxidant protection in the cell. A large increase in DNA damage was seen from 1 to 3 months for all diet groups ($P < 3 \times 10^{-7}$) (implying less antioxidant protection) followed by a steady decrease in damage until 13 months. Overall, from 3 to 17 months, the increase in antioxidant protection was highly significant for all groups. Slopes differed from zero with P values of $< 1 \times 10^{-8}$, in all groups. There was no effect of diet on this increase in antioxidant protection with age.

When H_2O_2 was removed from the lymphocytes and the cells were incubated in medium, they could begin to repair induced breaks. Repair was very efficient in rats of all ages, with approximately 75 % of breaks repaired within 2 h (Fig. 5). There was no effect of diet on the rate of repair at any age. There was, however, a decrease in repair of damage from 1 to 3 months (Groups 1 and 4: $P < 0.001$, Group 2: $P < 0.01$, Group 3: $P = 0.02$). Changes over the period 3 to 17 months were not significant except for Group 1, in which there was an increase in repair capability ($P < 0.01$). There was no effect of diet.

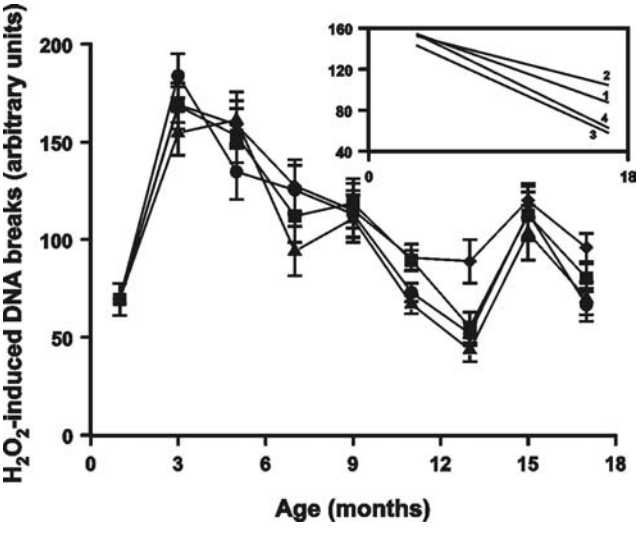

Fig. 4 DNA breaks in lymphocyte DNA induced by treatment with 50 μM H_2O_2 as measured by the comet assay. The mean breaks are shown as arbitrary units (± SEM) for 16 one-month old rats and for groups of 6–8 rats fed on different diets as described in Fig. 1, sampled at 2 monthly intervals from 3 to 17 months. Groups: AL (◆; Group 1), DR (■; Group 2), PR (▲; Group 3), and PC (●; Group 4). The inset shows regression lines for the data for the four groups of rats (between 3 and 17 months). Solid lines; slopes differ significantly from zero ($P < 10^{-8}$ in all cases)

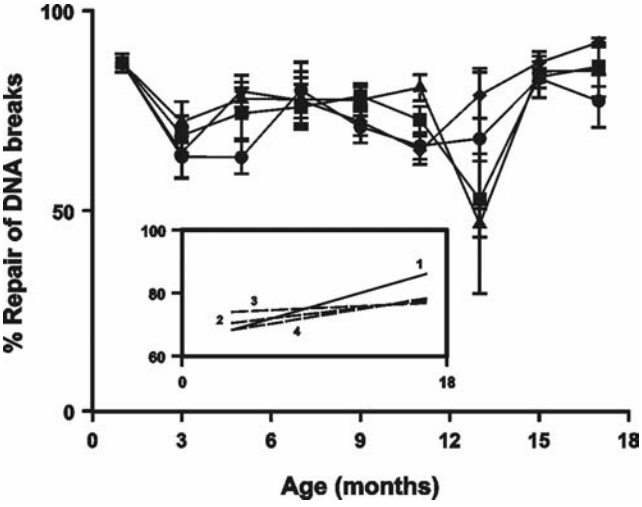

Fig. 5 Percentage repair of H_2O_2-induced DNA strand breaks in lymphocytes. The mean (± SEM) is shown for 16 one-month old rats and for groups of 6–8 rats fed on different diets as described in Fig. 1, sampled at 2 monthly intervals from 3 to 17 months. Groups: AL (◆; Group 1), DR (■; Group 2), PR (▲; Group 3), and PC (●; Group 4). The inset shows regression lines for the data for the four groups of rats (between 3 and 17 months). Solid line; slope differs significantly from zero (Group 1, $P < 0.01$). Broken lines; no significant slope

Antioxidant enzymes in various organs – effect of diet

Activities of GR, GPx, SOD (Cu/ZnSOD and MnSOD) and CAT were measured in brain, testis, liver, kidney; GR and GPx were also measured in heart (Tables 3–6). There were some significant differences in enzyme activities between 17 month-old rats on different diets. GR activity was lower in heart in Group 1 than in Groups 2 and 4; in liver, this activity in Group 4 was higher than in the other groups; in brain, it was higher in Group 4 than in Group 2. GPx was higher in liver of Groups 1 and 4 than in Group 3; in kidney it was higher in Groups 3 and 4 than in Group 2, and higher in Group 3 than Group 1; in heart it was lower in Group 3 than in Groups 1 and 4. MnSOD activity showed no differences between the four groups in any organ. Cu/Zn SOD activity (which was calculated by subtraction of MnSOD – from total SOD – activity) in brain was significantly lower in rats on the severely restricted diet than in Group 3. CAT activity was significantly lower in Group 1 than in Groups 2 and 4 for kidney and liver respectively. There was no pattern in a particular organ or for an individual enzyme in the various organs.

Antioxidant enzymes in various organs – effect of age

There were several effects of age on enzyme activity (Tables 3–6); in particular GPx activity increased significantly in all organs examined for all the diet groups. (Al-

Table 3 Activities of glutathione reductase in rat organs

	Brain	Testis	Liver	Kidney	Heart
1 month	12.1±1.1	8.8±1.1	31±2	48±2	4.7±0.2
Group 1 (AL)	15.1±0.7*	6.0±0.5**	31±2[b]	52±1	4.7±0.2[e, f]
Group 2 (DR)	13.8±0.9[a]	6.1±0.4**	30±1[c]	51±2	5.5±0.2[e, *]
Group 3 (CR)	14.6±0.6*	6.4±0.4**	31±2[d]	50±2	5.0±0.3
Group 4 (PC)	16.6±0.9[a, ***]	6.1±0.5**	37±2[b, c, d]	53±1	5.7±0.3[f, **]

Values shown (mU/mg protein) are means ± SEM for data from 6–8 rats per group: 1 month- and 17 month-old rats (Groups 1–4)
Identical letter superscripts indicate significant differences due to diet ([a, b, c, d, e] $P < 0.05$ and [f] $P = 0.01$).
*, **, *** indicate significant differences between one month old and old rats ($P < 0.05$, $P \leq 0.01$, $P \leq 0.001$ respectively)

Table 4 Activities of glutathione peroxidase in rat organs

	Brain	Testis	Liver	Kidney	Heart
1 month	0.08±0.01	0.12±0.01	0.63±0.03	0.86±0.04	0.35±0.01
Group 1 (AL)	0.16±0.01***	0.15±0.01**	0.90±0.04[a, ***]	1.70±0.06[c, ***]	0.63±0.02[f, ***]
Group 2 (DR)	0.15±0.01***	0.15±0.01**	0.86±0.05***	1.68±0.07[d, e, ***]	0.62±0.04***
Group 3 (CR)	0.15±0.01***	0.15±0.01**	0.74±0.04[a, b]	1.87±0.07[c, d, ***]	0.54±0.03[f, g, ***]
Group 4 (PC)	0.15±0.01***	0.16±0.01***	0.88±0.05[b, ***]	1.86±0.04[e, ***]	0.66±0.06[g, ***]

Values shown (U/mg protein) are means ± SEM for data from 6–8 rats per group: 1 month- and 17 month-old rats (Groups 1–4)
Identical letter superscripts indicate significant differences due to diet ([a, b, c, d, e, f] $P < 0.05$ and [g] $P = 0.01$)
*, **, *** indicate significant differences between one month old and old rats ($P = 0.05$, $P \leq 0.01$, $P \leq 0.001$ respectively)

Table 5 Activities of superoxide dismutase in rat organs

(a) total superoxide dismutase (total SOD)

	Brain	Testis	Liver	Kidney
1 month	1.24±0.07	1.32±0.07	2.84±0.22	1.42±0.06
Group 1 (AL)	1.85±0.08[a, ***]	1.30±0.14	4.20±0.21**	1.60±0.03
Group 2 (DR)	1.72±0.09**	1.61±0.18	4.15±0.35**	1.56±0.07
Group 3 (CR)	1.89±0.19[b, ***]	1.41±0.04	4.61±0.31***	1.58±0.09
Group 4 (PC)	1.45±0.10[a, b]	1.51±0.17	4.53±0.31***	1.68±0.06**

(b) Mn superoxide dismutase (MnSOD)

	Brain	Testis	Liver	Kidney
1 month	0.37±0.04	0.07±0.04	0.10±0.02	0.19±0.02
Group 1 (AL)	0.46±0.05	0.03±0.02	0.27±0.07**	0.40±0.04***
Group 2 (DR)	0.34±0.05	0.10±0.05	0.23±0.02*	0.34±0.04**
Group 3 (CR)	0.34±0.03	0.11±0.05	0.24±0.05*	0.36±0.05**
Group 4 (PC)	0.35±0.05	0.06±0.03	0.27±0.06*	0.42±0.04***

(c) Cu/Zn superoxide dismutase (Cu/ZnSOD)

	Brain	Testis	Liver	Kidney
1 month	0.87±0.07	1.25±0.05	2.75±0.23	1.23±0.07
Group 1 (AL)	1.40±0.10**	1.27±0.14	3.92±0.21**	1.20±0.06
Group 2 (DR)	1.38±0.07**	1.50±0.13	3.95±0.34**	1.20±0.06
Group 3 (CR)	1.55±0.18[c, ***]	1.30±0.04	4.38±0.30***	1.22±0.07
Group 4 (PC)	1.11±0.08[c]	1.45±0.15	4.27±0.32***	1.26±0.05

Values shown for (a) total SOD (b) MnSOD and (c) Cu/ZnSOD are means ± SEM (μg/mg protein) for data from 6–8 rats per group: 1 month- and 17 month-old rats (Groups 1–4)
Identical letter superscripts indicate significant differences due to diet ([a, b] $P < 0.05$ and [c] $P = 0.01$)
*, **, *** indicate significant differences between one month old and old rats ($P < 0.05$, $P \leq 0.01$, $P \leq 0.001$ respectively)

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Table 6 Activities of catalase in rat organs

	Brain	Testis	Liver	Kidney
1 month	1.60 ± 0.27	4.24 ± 1.14	154.11 ± 17.11	83.76 ± 9.64
Group 1 (AL)	1.61 ± 0.36	4.03 ± 0.40	223.44 ± 30.63[a]	60.06 ± 7.92[b]
Group 2 (DR)	1.11 ± 0.14	6.03 ± 0.49	248.60 ± 8.53**	92.29 ± 9.82[b]
Group 3 (CR)	1.52 ± 0.29	5.14 ± 0.70	279.29 ± 36.96**	85.72 ± 11.25
Group 4 (PC)	1.11 ± 0.31	6.29 ± 1.16	318.00 ± 32.66[a, ***]	85.70 ± 9.56

Values shown (K/g protein) are means ± SEM for data from 6–8 rats per group: 1 month- and 17 month-old rats (Groups 1–4) .
Identical letter superscripts indicate significant differences due to diet ([a, b] $P < 0.05$)
, * indicate significant differences between one month old and old rats ($P \leq 0.01$, $P < 0.001$ respectively)

though no significant increase was found by ANOVA for liver in Group 3, if compared alone with young rats the increase is significant: $P = 0.03$.) For the other enzymes the effect of age varied from organ to organ; most changes were seen in liver where, as well as GPx, the activities of Mn- and Cu/Zn-SOD increased with age in all groups; CAT activity increased in the 3 diet-restricted groups but not in Group 1. In brain there was no effect of age on CAT and MnSOD activities but Cu/ZnSOD increased in Groups 1 to 3, and GR increased in Groups 1, 3 and 4. In testis there were no changes in CAT, MnSOD or Cu/ZnSOD activities with age, but GR significantly decreased (against all other trends) in all old rats. There was no effect of age on levels of GR, CAT and Cu/Zn SOD activities in kidney, but MnSOD increased significantly in all 4 diet groups. Only GR and GPx activities were measured in heart; GR significantly increased in old rats in Groups 2 and 4 and as mentioned above GPx increased in all diet groups.

Discussion

In humans, genetic instability increases with age. *HPRT* mutation frequencies in lymphocytes increase with age [40], as do frequencies of micronuclei and chromosome aberrations [41]. These effects might result from increased free radical attack on DNA, diminished antioxidant defences, less efficient repair of the damage, or a combination of these. In rats, the only way to prolong lifespan so far known is dietary restriction. We therefore set out, using a rat model, to test the hypotheses that link dietary restriction, DNA damage, DNA repair and aging.

For a period of ten days between weaning and the start of dietary restriction, animals were fed on normal rat chow. One group of rats was sacrificed at this stage, for tissue analysis in comparison with the aged rats. The remaining animals were then transferred to semi-synthetic diets, made up from individual purified ingredients. The defined diets were in force from 1 month until the experiment finished at 17 months. Apparent changes in comet assay endpoints from 1 to 3 months, and differences in tissue parameters between 1 and 17 months, might simply reflect the different diets. In particular, the high resistance to oxidative damage seen in 1 month-old animals (Fig. 4) may be explained by residual antioxidant protection from maternal milk. The changes with time in the parameters measured in repeated samples from the same rats while on the semi-synthetic diets (i. e. from 3 to 17 months) are likely to be more informative.

Antioxidant micronutrients α-tocopherol and retinyl acetate were maintained at above the daily requirements in all the diets, while rats are able to synthesise vitamin C, so any effects of the various diets can be attributed solely to macronutrient provision. Predictions that the restricted diets would affect genetic stability were disappointingly not fulfilled. Patterns of DNA strand breakage, base oxidation, resistance to damage by H_2O_2 and repair capability were remarkably similar for the four different diets. The few differences that did appear, in a large number of potential comparisons, could well have arisen by chance. In general, however, antioxidant enzyme activities tended to increase the more severe the dietary restriction was.

There were significant age-related changes in various parameters, although they tended to be in unexpected directions. We looked for deterioration in antioxidant defences, but found that, in general, antioxidant enzyme activities in tissues tend either to remain constant with age or to increase in older animals (comparing only 17-month and 1-month-old animals, since tissues were taken from sacrificed animals). This effect is particularly marked for GPx, for CAT in liver, for MnSOD in liver and kidney, and Cu/ZnSOD in brain and liver. Although these results might just reflect the difference in diets at the different ages (as discussed above), they are supported by the very marked increase in resistance to H_2O_2-induced DNA strand breakage (a measure of antioxidant status) in lymphocytes between 3 and 17 months.

We also looked for an age-related decline in DNA repair. The capacity of lymphocytes to repair H_2O_2-induced strand breaks is not impaired as adult rats age. During the time from 3 to 17 months, repair if anything drifts to a higher efficiency (significant for group 1). It

should be noted that the repair we have measured is just one of several modes of repair; we have not measured base excision repair or nucleotide excision repair.

Is there any indication that the steady-state level of DNA damage increases with age? We measured endogenous strand breaks and oxidised pyrimidines (endonuclease III-sensitive sites) in lymphocyte DNA using the comet assay, and 8-oxoGua in various tissues by HPLC. After 3 months, strand breaks *decreased* significantly with age in groups 2–4, while oxidised pyrimidines showed no change. Strand breaks and oxidised pyrimidines were present at low levels compared with the levels we see in human cells [42], and we should be wary of over-interpreting these variations. Using a method designed to minimise adventitious oxidation during preparation of DNA for analysis, we found low values of 8-oxoGua in nuclear DNA from all three organs studied and no effect of diet, and a significant increase in liver and brain at 17 months compared with 1 month – though as mentioned above these results should be treated with caution. Even though DNA repair is apparently very efficient, an exiguous amount of unrepaired damage, over time, would increase the background level of lesions. In addition, the possibility remains that mitochondrial aging in tissues results in greater release of ROS and therefore a greater input of damage.

To sum up, in a very detailed and comprehensive study of diet, oxidative DNA damage and aging, we find that antioxidant defences are actually enhanced as rats age, while oxidative DNA damage accumulates in certain tissues. We have not found any convincing evidence of differential effects of restricted diets on markers of genetic instability and oxidative stress.

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References

1. Harman D (1956) Aging: a theory based on free radical and radiation chemistry. J Gerontol 2:298–300
2. Fraga C, Shigenaga MK, Park J, Degan P, Ames BN (1990) Oxidative damage to DNA during aging; 8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine. Proc Nat Acad Sci USA 87: 4533–4537
3. Sai K, Takagi A, Umemura T, Hasegawa R, Kurokawa Y (1992) Changes of 8-hydroxydeoxyguanosine levels in rat organ DNA during the aging process. J Environ Pathol Toxicol Oncol 11: 139–143
4. Kaneko T, Tahara S, Matsuo M (1996) Non-linear accumulation of 8-hydroxy-2'-deoxyguanosine, a marker of oxidized DNA damage, during aging. Mutat Res 316:277–285
5. Sohal RS, Agarwal S, Candas M, Forster MJ, Lal H (1994) Effect of age and calorie restriction on DNA oxidative damage in different tissues of C57BL/6 mice. Mech Ageing Dev 76:215–224
6. Izzotti A, Cartiglia C, Taningher M, De Flora S, Balansky R (1999) Age-related increases of 8-hydroxy-2'-deoxyguanosine and DNA-protein crosslinks in mouse organs. Mutat Res 446:215–223
7. Anson RM, Sentürker S, Dizdaroglu M, Bohr VA (1999) Measurement of oxidatively induced base lesions in liver from Wistar rats of different ages. Free Radic Biol Med 27:456–462
8. Hudson EK, Hogue BA, Souza-Pinto NC, Croteau DL, Anson RM, Bohr VA, Hansford RG (1998) Age-associated change in mitochondrial DNA damage. Free Rad Res 29:573–579
9. Collins A, Cadet J, Epe B, Gedik C (1997) Problems in the measurement of 8-oxoguanine in human DNA. Report of a workshop, DNA Oxidation, held in Aberdeen, UK, 19–21 January 1997. Carcinogenesis 18:1833–1836
10. Hofer T, Möller L (1998) Reduction of oxidation during the preparation of DNA and analysis of 8-hydroxy-2'-deoxyguanosine. Chem Res Toxicol 11: 882–887
11. Ravanat J-L, Douki T, Duez P, Gremaud E, Herbert K, Hofer T, Lasserre L, Saint-Pierre C, Favier A, Cadet J (2002) Cellular background level of 8-oxo-7.8-dihydroguanosine: an isotope based method to evaluate artefactual oxidation of DNA during its extraction and subsequent work-up. Carcinogenesis 23:1911–1918
12. Hamilton ML, Remmen HV, Drake JA, Yang H, Guo ZM, Kewitt K, Walter CA, Richardson A (2001) Does oxidative damage to DNA increase with age? Proc Nat Acad Sci USA 98:10469–10474
13. Suh JH, Shigeno ET, Morrow JD, Cox B, Rocha AE, Frei B, Hagen TM (2001) Oxidative stress in the aging rat heart is reversed by dietary supplementation with (R)-a-lipoic acid. FASEB J 15:700–706
14. Helbock HJ, Beckman KB, Shigenaga MK, Walter PB, Woodall AA, Yeo HC, Ames BN (1998) DNA oxidation matters: the HPLC-electrochemical detection assay of 8-oxo-deoxyguanosine and 8-oxo-guanine. Proc Nat Acad Sci USA 95:288–293
15. Yu BP, Masoro EJ, Murata I, Bertrand HA, Lynd FT (1982) Life span study of SPF Fischer 344 male rats fed ad libitum or restricted diets: Longevity, growth, lean body mass and disease. J Gerontol 37:130–141
16. Yu BP, Masoro EJ, McMahan CA (1985) Nutritional influences on aging of Fischer 344 rats: I physical, metabolic and longevity characteristics. J Gerontol 40:657–670
17. Masoro EJ (1993) Dietary restriction and aging. J Am Geriatr Soc 41:994–999
18. Chung MH, Kasai H, Nishimura S, Yu BP (1994) Protection of DNA damage by dietary restriction. Free Radic Biol Med 12:523–525
19. 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
20. Maeda H, Gleiser CA, Masoro EJ, Murata I, McMahan CA,Yu B P (1985) Nutritional influences of aging of Fischer 344 rats. II. Pathology. J Gerontol 40: 671–688
21. 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

22. Mura CV, Gong X, Taylor A, Villalobosmolina R, Scrofano MM (1996) Effects of calorie restriction and aging on the expression of antioxidant enzymes and ubiquitin in the liver of Emory mice. Mech Ageing Dev 91:115–129
23. Sohal RS, Ku H-H, Agarwal S, Forster MJ, Lal H (1994) Oxidative damage, mitochondrial oxidant generation and antioxidant defenses during aging and in response to food restriction in the mouse. Mech Ageing Dev 74:121–133
24. Cand F, Verdetti J (1989) Superoxide dismutase, glutathione peroxidase, catalase, and lipid peroxidation in the major organs of the aging rats. Free Radic Biol Med 7:59–63
25. Laganiere S, Yu BP (1989) Effect of chronic food restriction in aging rats II liver cytosolic antioxidants and related enzymes. Mech Ageing Dev 48:221–230
26. Rao G, Xia E, Nadakavukaren MJ, Richardson A (1990) Effect of dietary restriction on the age-dependent changes in the expression of antioxidant enzymes in rat liver. J Nutr 120: 602–609
27. Xia E, Rao G, van Remmen H, Heydari AR, Richardson A (1995) Activities of antioxidant enzymes in various tissues of male Fischer 344 rats are altered by food restriction. J Nutr 125:195–201
28. Bohr V, Anson RM, Mazur S, Dianov G (1998) Oxidative DNA damage processing and changes with aging. Toxicol Lett 103:47–52
29. King CM, BristowCraig HE, Gillespie ES, Barnett YA (1997) In vivo antioxidant status, DNA damage, mutation and DNA repair capacity in cultured lymphocytes from healthy 75- to 80-year-old humans. Mutat Res 377:137–147
30. De Souza-Pinto NC, Hogue BA, Bohr VA (2001) DNA repair and aging in mouse liver: 8-oxodG glycosylase activity increase in mitochondrial but not in nuclear extracts. Free Radic Biol Med 30: 916–923
31. Grant G, Dorward P, Pusztai A (1993) Pancreatic enlargement is evident in rats fed diets containing raw soybeans (Glycine max) or cowpeas (Vigna unguiculata) for 800 days but not in those fed diets based on kidney beans (Phaseolus vulgaris) or lupin seed (Lupinus angustifolius). J Nutr 123:2207–2215
32. Collins AR, Dušinská M, Gedik CM, Štětina R (1996) Oxidative damage to DNA: Do we have a reliable biomarker? Environ Health Perspect 104 supplement 3:465–469
33. Wood SG, Gedik CM, Vaughan NJ, Collins AR (2000) Measurement of 8-oxo-deoxyguanosine in lymphocytes, cultured cells, and tissue samples by HPLC with electrochemical detection. In: Barnett YA, Barnett CR (eds) Aging Methods and Protocols, Humana Press Inc., Totowa NJ, pp 171–178
34. Richter C, Park J-W, Ames BN (1988) Normal oxidative damage to mitochondrial and nuclear DNA is extensive. Proc Nat Acad Sci USA 85:6465–6467
35. Galbraith DA, Watts DC (1980) Changes in some cytoplasmic enzymes from red cells fractionated into age groups by centrifugation in Ficall/Triosil gradients. Biochem J 191:63–70
36. Paglia DE, Valentine WN (1967) Studies on the quantitative and qualitative characterisation of erythrocytes glutathione peroxidase. J Lab Clin Med 70: 158–169
37. Aebi H (1985) Catalase in vitro. In: Packer L (ed) Methods in Enzymology 105:121–126
38. Flohé L, Ötting F (1985) Superoxide dismutase assays. Methods in Enzymology 105:101–104
39. Gornell AG, Bardawill CJ, David MM (1949) Determination of serum proteins by means of the biuret reaction. J Biol Chem 177:751–766
40. Robinson DR, Goodall K, Albertini RJ, O'Neill JP, Finette B, Sala-Trepat M, Moustacchi E, Tates AD, Beare DM, Green MHL, Cole J (1994) An analysis of in vivo hprt mutant frequency in circulating T-lymphocytes in the normal human population: a comparison of four datasets. Mutat Res 313:227–247
41. Bolognesi C, Abbondandolo A, Barale R, Casalone R, Dalpra L, De Ferrari M, Degrassi F, Forni A, Lamberti L, Lando C, Migliore L, Padovani P, Pasquini R, Puntoni R, Sbrana I, Stella M, Bonassi S (1997) Age-related increase of base-line frequencies of sister chromatid exchanges, chromosomal aberrations and micronuclei in human lymphocytes. Cancer Epid Biomarkers & Prev 6: 249–256
42. Duthie SJ, Aiguo M, Ross MA, Collins AR (1996) Antioxidant supplementation decreases oxidative DNA damage in human lymphocytes. Cancer Res 56: 1291–1295