Elevated DNA damage in a mouse model of oxidative stress: impacts of ionizing radiation and a protective dietary supplement

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Transgenic growth hormone (Tg) mice express elevated free radical processes and a progeroid syndrome of accelerated ageing. We examined bone marrow cells of Tg mice and their normal (Nr) siblings for three markers of DNA damage and assessed the impact of free radical stress using ionizing radiation. We also evaluated the radiation protection afforded by a dietary supplement that we previously demonstrated to extend longevity and reduce cognitive ageing of Nr and Tg mice. Spectral karyotyping revealed few spontaneous chromatid aberrations in Nr or Tg mice, however, had significantly greater constitutive levels of both γH2AX and 8-hydroxy-deoxyguanosine (8-OHdG) compared to Nr. When exposed to a 2-Gy whole-body dose of ionizing radiation, both Nr and Tg mice showed significant increases in DNA damage. Compared to Nr mice, irradiated Tg mice had dramatically higher levels of γH2AX foci and double the levels of chromatid aberrations. In unirradiated mice, the dietary supplement significantly reduced constitutive γH2AX and 8-OHdG in both Nr and Tg mice (normalizing both γH2AX and 8-OHdG in Tg), with little difference in γH2AX and 8-OHdG over constitutive levels. Induced chromatid aberrations were also reduced, and in Nr mice, virtually absent. Remarkably, supplemented mice expressed 6-fold lower levels of radiation-induced chromatid aberrations compared to unsupplemented Nr or Tg mice. Based on our data, the dietary supplement appeared to scavenge free radicals before they could cause damage. This study validates Tg mice as an exemplary model of oxidative stress and radiation hypersensitivity and documents unprecedented radioprotection by a dietary supplement comprised of ingredients available to the general public.

Introduction

Oxidative stress arises from diverse causes such as xenobiotics, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, hypoxia, ageing, disease processes, mitochondrial dysfunction and exposure to ionizing radiation (1–4). Free radicals damage lipids, proteins, DNA and cellular structures. Oxidative DNA base damage, chromosome aberrations, genomic instability and telomere alterations are among the most deleterious consequences of oxidative processes consequently making high doses potentially mutagenic (1–13).

Several studies investigating various indicators of DNA damage, including oxidative base damage, telomere shortening, chromosome fragments, chromosome loss and aneuploidy have consistently shown that DNA damage increases proportionately with age (5,8–10) and are considered valid markers of ageing (7). Genomic aberrations increase the risk of carcinogenesis and may actually accelerate the ageing process by contributing to cell dysfunction and death (14–17). Chronic oxidative stress such as that associated with ageing is a significant factor in the progression of the disease (19–23). The age-related increase in DNA damage is also associated with progressive mitochondrial dysfunction and rising free radical production, which may contribute to progressive physiological dysfunction, deterioration and mortality (16,17,24–27). Cellular responses mediating defence, repair and replacement also appear to attenuate with age and contribute to ageing (7,17,28).

Transgenic growth hormone (Tg) mice express elevated free radical processes (29) and accelerated ageing (30–34). Tg mice live only half as long as normal mice (29,30,35,36) and experience elevated superoxide radical production and lipid peroxidation in several tissues (29,37,38). They have age-related, early onset arthritis, increased incidence of catacares, reduced motor activity and muscle wasting (30,35,36,39,40). Brown-Borg et al. (31) found elevation of hepatic carboxyls at 12 months. Tg mice also express reduced levels of hepatic antioxidants (38,41,42). Young Tg mice display greatly enhanced cognitive abilities (43,44), but experience a rapid age-related decline in learning abilities (43).

Chronically elevated free radical production in Tg mice would imply that they may display more constitutive chromosomal aberrations and oxidative base damage. We further hypothesized that Tg mice would show greater radiosensitivity to DNA damage than age-matched Nr mice and that the (anti-ageing) dietary supplement would reduce DNA damage in Tg mice. Limoli et al. (45) found that neuronal precursor cells subjected to an oxidative environment showed increased radiosensitivity. To assess these ideas, we examined metaphase chromosomes from bone marrow cells using spectral karyotyping (SKY) and γH2AX to examine double-strand breaks (DSBs) and repair kinetics and 8-OHdG as an indicator of oxidative base damage. It is established that antioxidants can reduce the severity of DNA damage and diminish chromosome aberrations (46–49). Development of radioprotective diets is a high priority for individuals exposed to ionizing radiation. For example, diets could protect astronauts from cosmic radiation and normal tissue in patients undergoing radiation therapy. Numerous materials show potential value, but effective protection remains elusive (46–57).

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Multiple cellular mechanisms defend against oxidative stress (58–61). Antioxidants typically spare, synergize or recycle one another, and if administered singly, they may become pro-oxidants. Various antioxidants occupy different cellular compartments, so general protection requires multiple components. For example, lipophilic antioxidants like vitamin E protect cell membranes but are less effective for hydrophilic components of the cytosol. Antioxidants that intercept free radicals generated by the mitochondria or other cytosolic sources may prevent DNA damage, but those that closely associate with nuclear DNA may be most effective. Materials that activate cellular defence systems or provide support or substrates for enzymatic antioxidant systems and DNA repair processes are also of significant importance. Multiple pharmaceuticals are required to serve all these criteria, even though some may have multiple functions. Since no one antioxidant can fill all these roles, the ideal supplement must combine compounds that possess all these characteristics. Following oxidative insults like radiation, cells may express elevated free radical production for weeks or even months (62). Mechanisms include cell membrane NADPH oxidases and (associated) potassium channels, mitochondrial dysfunction and inflammatory processes (3,12,63–65). NAPDH oxidases both produce and are activated by free radicals. This can perpetuate chronic free radical production (64). Mitochondrial dysfunction induced by irradiation (Ca$^{2+}$ elevation, depolarization of membrane potential, low respiration rate and reduced activity of manganese superoxide dismutase (MnSOD)) likely contribute to sustained free radical production generation, even among clonal cell descendants (65,66). Leach et al. (66) highlighted Ca$^{2+}$ inter-mitochondrial signalling in amplifying radiation-induced free radical generation.

The chronically elevated free radical production characteristic of Tg mice indicates that older Tg mice may experience increased constitutive structural chromosome aberrations and oxidative base damage. Consequently, Tg mice should have reduced ability cope with acute free radical insult from radiation exposure, resulting in greater radiation-induced genomic aberrations compared to age-matched normal mice. If these hypotheses are supported, then supplementation with our anti-ageing/antioxidant supplement should reduce the level of DNA damage in Tg mice. This study tested these hypotheses by examining metaphase chromosomes from bone marrow to look at DSB levels using γH2AX foci formation, DNA repair fidelity using SKY and assessing oxidative base damage by measuring 8-OhdG levels.

### Materials and methods

#### Animals

Our Tg mice (C57BL/6J-SJL background) have transgenes with metallothionein promoters fused to rat growth hormone (GH) structural regions (67). This results in a >100-fold elevation of plasma GH. Tg mice were reliably identified by their significantly larger size by 28 days of age (68), as well as morphological differences (increased body length, significantly broader nose). All mice used in this study were derived from a closed breeding colony where Tg sires were bred to Nrdams providing equal numbers of Nrd and Tg mice of similar genetic background. The chromosomal aberration assessment used 24 female mice aged 11–12 months, evenly divided into four experimental groups (see below). The γH2AX and 8-OHDG experiments also used 24 female mice in evenly divided into four experimental groups (11–12 months). For all assays, experimental groups included unsupplemented Nr mice, unsupplemented Tg mice, diet-supplemented Nr mice and diet-supplemented Tg mice. A maximum of four mice were housed in cages (27 × 12 × 15.5 cm) bedded with wood-chip (Harlan Sani-Chips, 7090). A stainless steel hopper provided food ad libitum (Harlan Teklad 8640 22/5 rodent chow) and supported a water bottle.

The housing room maintained a 12:12 h light:dark photoperiod at 22 ± 2°C. All protocols met the standards of McMaster University’s Animal Research Ethics Board and the Canadian Council on Animal Care.

#### Dietary supplement

The dietary supplement was designed to simultaneously ameliorate several processes implicated in ageing (oxidative stress, inflammatory processes, insulin resistance and mitochondrial dysfunction). Criteria for selecting specific ingredients for the supplement were as follows: (i) scientifically documented evidence as effective for one or more of the targets, (ii) oral administration and (iii) approved for human use.

Dosages and preparation of the supplement were described by Lemon et al. (36, 43). Dosages for mice were based on amounts prescribed to humans. Values were adjusted for the smaller body size of the mice and then increased by a factor of 10 based on the higher gram-specific metabolic and utilization rates of mice compared to humans (69). Ingredients and associated targets are outlined in Table I. The supplement was prepared in liquid form and 0.4 ml was allowed to absorb into a 1 × 1.5 × 1 cm piece of bagel (bread product). This was air-dried and then refrigerated for immediate use. The mice were given the bagel pieces midway through the photoperiod. The bagel pieces were immediately and completely ingested by the mice, ensuring mice obtained full and equivalent doses. All supplemented mice began receiving the supplement prior to 3 months of age.

#### In vivo irradiation

During irradiation, mice were placed in a polycrylonitrile (PVC) tube (5 × 12.5 cm) with PVC mesh endcaps. Each mouse was given a 2-Gy whole-body dose of gamma radiation from a 137Cs source (dose rate: 0.5 Gy/min). After irradiation, each mouse was returned to its housing cage. Non-irradiated sham control mice were otherwise exposed to exactly the same conditions as irradiated mice.

#### SKY

Sample collection and preparation. Four hours after irradiation, mice were anesthetized with isoflurane™ and euthanized via cervical dislocation. Both femurs of each mouse were excised and a 3-ml syringe equipped with a 23-g needle containing 1 ml heparinized complete RPMI 1640 [10% foetal bovine serum (FBS), 1% L-glutamine, 1% penicillin–streptomycin] was used to flush the bone marrow from the femurs. Each femur was flushed three times with the same 1 ml of complete RPMI to ensure complete removal of bone marrow. The cell suspension was then repeatedly drawn through the syringe gently, 10 times, to break up any remaining cell clumps. The bone marrow cell suspension was immediately placed in a 15-ml centrifuge tube containing 4 ml complete RPMI, 2 ml FBS and 0.1 μg/ml colcemide. Cells were incubated at 37°C (5% CO2, 98% humidity) for 4 h. The cells were centrifuged at 200 × g for 8 min, the supernatant aspirated and the cells were re-suspended in ~100 μl of remaining supernatant by gentle vortexing.

A total of 10 ml of 0.075 M KCl was added to each sample and incubated at 37°C for 20 min. The samples were then centrifuged at 200 × g for 8 min, the supernatant was aspirated and the cells were re-suspended. A total of 10 ml of 3:1 methanol:acetic acid fix was added to each sample and incubated at room temperature for 15 min. The samples were centrifuged at 300 × g for 8 min, the supernatant was aspirated and the cells were re-suspended. A total of 10 ml of 3:1 methanol:acetic acid fix was added to each tube and samples were stored at −20°C until analysis.

Slide preparation. Samples were allowed to warm to room temperature, then the assay tubes were centrifuged at 300 × g for 7 min and the supernatant was aspirated to 0.5 ml above the cell pellet. The cells were re-suspended and 5 ml of freshly prepared 3:1 methanol:acetic acid fix was added to each assay tube. Slides were dropped in a humidity- and temperature-controlled chamber (Thermotron®, Holland, MI) onto acid cleaned slides. Prepared slides were examined under a phase-contrast microscope to determine quality of the chromosome spreads and the mitotic index of each sample. Slides were aged overnight at 37°C in a dry oven prior to preparation.

#### Hybridization

The slides were prepared for SKY with a slightly modified version of the instructions supplied by manufacturer, Applied Spectral Imaging® (ASI, Vista, CA). The slides were treated as follows: the slides were treated with pepsin solution for 6 min at 37°C and then washed twice in phosphate-buffered saline (PBS) at room temperature. The slides were washed in 1× PBS/MgCl2 at room temperature, then placed in a 1% formaldehyde solution and incubated for 10 min at room temperature and were washed once in 1× PBS. At this point, 10 μl of the SkyPaint® mixture (ASI) was denatured by incubating the mixture at 80°C for 7 min and then placed at 37°C for 90 min.

The slides were incubated in 2 μM sodium chloride/sodium citrate (SSC) at 70°C for 30 min and then allowed to cool to room temperature (~20 min). The slides were washed once in 0.1× SSC and denatured in 0.07 M NaOH at room temperature for 1 min. The slides were then washed once in 0.1× SSC at 4°C,
Table I. Components of the dietary supplement and associated cellular targets

<table>
<thead>
<tr>
<th>Component</th>
<th>Target</th>
<th>Component</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin B1</td>
<td>Insulin sensitivity, anti-inflammatory</td>
<td>Flax seed oil</td>
<td>Omega fatty acids for membrane support</td>
</tr>
<tr>
<td>Vitamin B3</td>
<td>Insulin sensitivity, anti-inflammatory</td>
<td>Folic acid</td>
<td>Antioxidant, maintains glutathione levels, endothelial support</td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>Insulin sensitivity, anti-inflammatory, scavenges O$_2^-$</td>
<td>Garlic</td>
<td>Antioxidant in lipid membrane, scavenges O$_2^-$, H$_2$O$_2^-$</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>Insulin sensitivity, anti-inflammatory</td>
<td>Ginger</td>
<td>Antioxidant in cytosol, scavenges OH$^-$, O$_2^-$, ONOO$^-$</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>Antioxidant in cytosol, scavenges O$_2^-$, H$_2$O$_2$</td>
<td>Gingko biloba</td>
<td>Antioxidant in cytosol, scavenges NO$^-$</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>Antioxidant in lipid membrane</td>
<td>Ginseng</td>
<td>Antioxidant in cytosol and nucleus, scavenges OH$^-$, O$_2^-$, ONOO$^-$</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>Antioxidant in lipid membrane, scavenges O$_2^-$, H$_2$O$_2$</td>
<td>Green tea extract</td>
<td>Antioxidant in cytosol, scavenges H$_2$O$_2$, OH$^-$</td>
</tr>
<tr>
<td>Acetyl l-carnitine</td>
<td>Mitochondrial support, antioxidant in mitochondria, insulin sensitivity</td>
<td>l-Glutathione</td>
<td>Enzymatic antioxidant support, antioxidant in cytosol</td>
</tr>
<tr>
<td>Alpha lipoic acid</td>
<td>Mitochondrial support, antioxidant in mitochondria, insulin sensitivity</td>
<td>Magnesium</td>
<td>Insulin sensitivity, cellular support</td>
</tr>
<tr>
<td>ASA</td>
<td>Anti-inflammatory, scavenges NO$^-$</td>
<td>Melatonin</td>
<td>Antioxidant in cytosol and nucleus, scavenges OH$^-$, H$_2$O$_2$, O$_2^-$, NO, ONOO$^-$</td>
</tr>
<tr>
<td>Beta carotene</td>
<td>Antioxidant in lipid membrane, scavenges O$_2^-$, H$_2$O$_2$</td>
<td>N-Acetyl cysteine</td>
<td>Mitochondrial support, antioxidant in mitochondria</td>
</tr>
<tr>
<td>Bioflavonoids</td>
<td>Antioxidant in cytosol and nucleus, scavenges OH$^-$, O$_2^-$, metal chelator</td>
<td>Potassium</td>
<td>Insulin sensitivity, cellular support</td>
</tr>
<tr>
<td>Chromium</td>
<td>Insulin sensitivity, scavenges H$_2$O$_2$</td>
<td>Rutin</td>
<td>Antioxidant in lipid membrane, scavenges OH$^-$, O$_2^-$, metal chelator</td>
</tr>
<tr>
<td>Cod liver oil</td>
<td>Omega fatty acids for membrane support</td>
<td>Selenium</td>
<td>Scavenges H$_2$O$_2$, enzymatic antioxidant support, insulin sensitivity</td>
</tr>
<tr>
<td>CoEnzyme Q10</td>
<td>Mitochondrial support, antioxidant in mitochondria</td>
<td>Zinc (chelated)</td>
<td>Neural support (zinc + antioxidants), insulin sensitivity</td>
</tr>
<tr>
<td>DHEA</td>
<td>Antioxidant, endocrine support</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Please note that this is not meant to be a complete list of functions for each of the supplement components, the functions of each ingredient are presented only in the context of the supplement. Synergistic and recycling interactions among supplement components are not included. Ingredient concentrations can be found in Lemon et al. (36,43).

followed by one wash in 2 × SSC at 4°C. The slides were dehydrated in an ethanol series (70, 80, then 100%) at −20°C and allowed to air-dry.

The denatured SkyPaint® was added to the denatured chromosome preparation and a 22 × 22-mm glass cover slip was placed over the probe mixture. The edges of the cover slip are sealed with rubber cement and the slides were incubated in a humidified chamber at 37°C for 60–64 h. The slides were washed three times in 50% formamide/2 × SSC at 45°C, followed by two washes in 1 × SSC at 45°C. Slides were then washed once in 4 × SSC/0.1% Tween 20 at room temperature. Blocking reagent (80 l) was applied to each slide and covered with a plastic cover slip. The slides were incubated for 30 min in a humidified chamber at 37°C. The slides are then dried and 80 l of Cy5 buffer (CAD 03 buffer; ASI) was added to each slide. The slides were washed at 37°C for 45 min. The slides were then washed three times in 4 × SSC/0.1% Tween 20 at 45°C. Slides were drained and 80 l of Cy5.5 buffer (CAD 04 buffer; ASI) was added to each slide and incubated at 37°C for 40 min. The slides were washed three times in 4 × SSC/0.1% Tween 20. The slides were finally washed in distilled water to remove detergent residue and allowed to air-dry. Once the slides were fully dry, 15 μl 4,6-diamidino-2-phenylindole (DAPI)/Antifade solution was applied to each slide and a 22 × 22-mm glass cover slip was placed over the cell spreads. The slides were allowed to incubate for 5 min, the slides were gently pressed between two Kimwipes to remove excess solution and the edges of the cover slips were sealed with clear nail polish. The slides were then ready for analysis.

Fluorescence microscopy. The SKY slides were scored using the SpectraCube® system (ASI). Suitable metaphases (i.e. those with good chromosome quality and sufficient chromosome separation) were assessed with a DAPI (excitation: 310 nm, emission: 450 nm) filter. Once such a metaphase was found, a black and white band image was captured, followed by a spectral image with the SKY filter (proprietary filter). Image acquisition is based on a spectral imaging system using an interferometer and a CCD camera. Both the band and spectral images were then used to determine the karyotype of each cell, with SkyView EXPOTM, the image analysis software. A minimum of 30 suitable metaphases were scored for each mouse.

γH2AX and 8-OHdG

Cell collection. Immediately after irradiation, mice were euthanized via cervical dislocation and bone marrow cells were obtained from both femurs as described above. The cell suspension was added to 6 ml of 0°C complete RPMI 1640 and placed on ice for the duration of sample preparation. Cell concentrations were determined using the Z2 Coulter particle count and size analyser (Beckman-Coulter, Miami FL) and adjusted to 1 × 10$^6$ cells/ml with 0°C complete RPMI 1640. A 500-μl aliquot of cell suspension was removed for the 0 h time point from each sample and placed in 5 ml polypropylene tube for the γH2AX assay. A 3 ml ice cold 70% ethanol was immediately added to each tube and all tubes were placed on ice.

Antibody staining. The remaining bone marrow samples were incubated in a 37°C water bath. For the γH2AX assay, 500-μl aliquots were removed at 15, 30, 60, 120 and 240 min and placed in 5-ml tubes. For the 8-OHdG assay, 500-μl aliquots were removed at 240 min and placed in 5-ml tubes. A 3 ml of 0°C 70% ethanol was immediately added to each sample. All tubes were incubated on ice for 1 h. Samples were stored at −20°C until analysis.

Bone marrow cells fixed in 70% ethanol were allowed to warm to room temperature prior to the start of antibody staining. Cells were incubated in Tris-buffered saline (TBS; Trizma base + NaCl, Sigma Aldrich, Mississauga, Ontario) for 10 min to rehydrate cells. The tubes were centrifuged and resuspended in 4% FBS (VWR International, Mississauga, Ontario) for 10 min to permeabilize cells. The cells were incubated in Tris-buffered saline (TBS; Trizma base + NaCl, Sigma Aldrich, Mississauga, Ontario) for 10 min to rehydrate cells. The cells were incubated in Tris-buffered saline (TBS; Trizma base + NaCl, Sigma Aldrich, Mississauga, Ontario) for 10 min to rehydrate cells.

Bone marrow cells were incubated in 37°C water bath. For the γH2AX assay, 500-μl aliquots were removed at 15, 30, 60, 120 and 240 min and placed in 5-ml tubes. For the 8-OHdG assay, 500-μl aliquots were removed at 240 min and placed in 5-ml tubes. A 3 ml of 0°C 70% ethanol was immediately added to each sample. All tubes were incubated on ice for 1 h. Samples were stored at −20°C until analysis.

Bone marrow cells fixed in 70% ethanol were allowed to warm to room temperature prior to the start of antibody staining. Cells were incubated in Tris-buffered saline (TBS; Trizma base + NaCl, Sigma Aldrich, Mississauga, Ontario) for 10 min to rehydrate cells. The tubes were centrifuged and resuspended in Tris– saline– triton [TST; TBS + 4% FBS (VWR International, Mississauga, Ontario) + 0.1% Triton X-100 (Sigma Aldrich)] and incubated on ice for 10 min to permeabilize cells. The cells were centrifuged and resuspended in 200 μl of Bufferation© H2AX (ser139) antibody (γH2AX; Upstate Cell Signaling, Charlotteville, VA) or anti-8-OHdG antibody (Chemicon International, Temecula, CA).

Both primary antibodies were diluted 1:100 in TST and incubated at room temperature for 2 h. The cells were washed with TST and re-suspended in 200 μl of either AlexaFluor® 488-conjugated goat anti-rabbit IgG F(ab)2 antibody (γH2AX) or AlexaFluor® 488-conjugated goat anti-rabbit IgG F(ab)2 antibody (8-OHdG). Both secondary antibodies were diluted 1:500 in TST (Invitrogen Canada, Burlington, Ontario) and incubated at room temperature for 1 h. The cells were then washed in TBS and re-suspended in 300 μl TBS + 5 μl propidium iodide (1 mg/ml; Sigma Aldrich). Samples were put on ice and immediately run on the Epics XL flow cytometer (Beckman Coulter; Mississauga, Ontario).

Statistics

All values were represented as the mean and standard error of the mean. Student’s t-tests were performed to determine if significant differences existed between groups.

Results

Chromosome aberrations

Constitutive. Few spontaneous structural chromosome aberrations were found in any of the experimental groups (Figure 1).
There were no significant differences in number of chromosome aberrations between unsupplemented Nr (2.33 ± 0.48%) and unsupplemented Tg (1.76 ± 0.99%). The number of constitutive chromosome aberrations in supplemented Nr (0.56 ± 0.56%) did not differ from supplemented Tg (0.63 ± 0.63%). Supplemented mice had the same number of spontaneous aberrations as unsupplemented mice. Despite a trend, levels of spontaneous aberrations between supplemented and unsupplemented mice were not statistically resolved.

Radiation induced. The percentage of cells containing aberrations increased significantly in unsupplemented Nr exposed to 2 Gy (27.53 ± 5.29%; Figure 1) compared to spontaneous aberrations in unsupplemented Nr (P < 0.009). Unsupplemented Tg also had a significant elevation in radiation-induced chromosome aberrations (46.60 ± 5.33%) compared to constitutive levels (P < 0.0012). Also, unsupplemented Tg mice were more sensitive to radiation and had significantly more cells with radiation-induced chromosome aberrations compared to unsupplemented Nr mice (P < 0.024; compare in Figure 1). Diet-supplemented Nr had a significant reduction in radiation-induced chromosome aberrations (5.27 ± 4.33%) and became resistant compared to unsupplemented Nr (P < 0.031).

Supplemented Tg also had significantly fewer radiation-induced aberrations compared to both unsupplemented Tg (P < 0.0031) and unsupplemented normals (P < 0.033). There was no difference in radiation-induced chromosome aberrations between diet-supplemented Nr and diet-supplemented Tg. Supplemented animals exposed to 2 Gy dose had similar levels of chromosome aberrations as unirradiated mice, suggesting nearly complete protection from the effects of a large dose of radiation. 

Chromosome-type structural aberrations predominate in both constitutive and radiation induced for all groups of mice (Table II). Unrepaired chromatid arm breaks and the corresponding acentric fragments comprise 100% of constitutive aberrations in unsupplemented and supplemented mice. The variety and complexity of radiation-induced aberrations was higher than constitutive aberrations (Table II), chromatid arm breaks and corresponding acentric fragments were still the dominant type of aberration, followed by translocations. Although it was apparent that some chromosomes were involved in structural aberrations more frequently than others (Figure 2), R² values (Table III) indicated that the frequency with which each chromosome was involved in chromosome aberrations does not correlate to chromosome length, number of genes per chromosome or gene density (genes/Mbp) of the chromosome.

DSB repair

Kinetics. There was a reduction in constitutive γH2AX foci formation and corresponding fluorescence observed in both groups of unsupplemented mice during the incubation period (Figure 3A); however, this trend was only significant in unsupplemented Tg mice (P < 0.025). The kinetics of radiation-induced γH2AX was the same for all groups of mice, with supplemented mice demonstrating significantly reduced γH2AX levels (P < 0.045) at all time points (Figure 3B). The level of γH2AX fluorescence increased from 0 to 30 min, where it peaked, followed by a decrease in fluorescence at each of the following time points out to 240 min, when γH2AX fluorescence returned to baseline levels. The level of γH2AX fluorescence peaked at 30 min after irradiation in all groups of mice; to simplify analysis, statistics in the following sections was based only on the 30-min incubation data.

Constitutive foci formation. The background level of γH2AX foci was significantly lower in unsupplemented Nr (1.27 ± 0.13) compared to unsupplemented Tg (1.60 ± 0.14; P < 0.047). Constitutive expression of γH2AX in supplemented mice

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### Table II. The types and percentages of constitutive (0 Gy) and radiation-induced (2 Gy) chromosome aberrations in the bone marrow of each group of mice

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>Chromosome type</th>
<th>rings</th>
<th>Chromatin type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Breaks +ACF</td>
<td>translocs</td>
<td>dels</td>
</tr>
<tr>
<td>0 Gy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>US Norm</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>US Tg</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sup Norm</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sup Tg</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2 Gy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>US Norm</td>
<td>72.3</td>
<td>12.3</td>
<td>4.6</td>
</tr>
<tr>
<td>US Tg</td>
<td>69.5</td>
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<td>Sup Norm</td>
<td>85.7</td>
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</tr>
<tr>
<td>Sup Tg</td>
<td>84.2</td>
<td>5.3</td>
<td>10.5</td>
</tr>
</tbody>
</table>

ACF, acentric fragments; transloc, translocations; dels, deletions; US Norm, unsupplemented normal mice; US Tg, unsupplemented Tg mice; Sup Norm, diet-supplemented normal mice and Sup Tg, diet-supplemented Tg mice.

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**Fig. 1.** The average number of constitutive (0 Gy) and radiation-induced (2 Gy) chromosome aberrations in bone marrow of unsupplemented and diet-supplemented mice. Asterisks indicate values significantly different from unirradiated normal mice (*P < 0.009).
Nr (1.10 ± 0.12) was lower than unsupplemented Nr; however, this reduction was also not significant. There was a reduction of background cH2AX levels in supplemented Tg (0.93 ± 0.09), significantly lower than both unsupplemented Nr (P < 0.0001) and unsupplemented Tg (P < 0.0001). However, there was no difference in γH2AX levels between supplemented Nr and supplemented Tg (Figure 3A).

**Radiation-induced foci formation.** Radiation-induced γH2AX foci was significantly higher in unsupplemented Nr (2.37 ± 0.016; P < 0.0052) and unsupplemented Tg (2.95 ± 0.19; P < 0.0018) compared to their respective background levels (Figure 3B). Unsupplemented Nr had significantly lower radiation-induced γH2AX levels compared to unsupplemented Tg (P < 0.038). Both supplemented Nr (1.77 ± 0.080; P < 0.0066) and supplemented Tg (1.28 ± 0.10; P < 0.0001) demonstrated significant reductions in radiation-induced γH2AX fluorescence, so that radiation-induced γH2AX fluorescence did not differ significantly in supplemented mice from constitutive levels. The level of γH2AX in the supplemented groups of mice did not differ from each other.

**Oxidative base damage**

**Constitutive levels of 8-OHdG.** The constitutive level of 8-OHdG was significantly lower in unsupplemented Nr (1.92 ± 0.08) compared to unsupplemented Tg (3.82 ± 0.10; P < 0.0001). There was no significant difference in constitutive
8-OHdG levels between unsupplemented and supplemented Nr (1.63 ± 0.12). Supplemented Tg (1.83 ± 0.09) had a dramatic reduction in 8-OHdG levels compared to unsupplemented Tg ($P < 0.0001$); however, there was no difference in 8-OHdG levels between supplemented Nr and supplemented Tg (Figure 4).

**Radiation-induced levels of 8-OHdG.** There was a significant increase in radiation-induced 8-OHdG levels in unsupplemented Nr (2.53 ± 0.19) and Tg (4.87 ± 0.27) compared to constitutive 8-OHdG expression ($P < 0.0052$ and $P < 0.0018$, respectively). Unlike with radiation-induced chromosome aberrations or γH2AX levels, the amount of radiation-induced 8-OHdG increased by the same proportion in unsupplemented Nr and Tg (Figure 4). The level of radiation-induced 8-OHdG in supplemented Nr (1.85 ± 0.23) was significantly lower than unsupplemented Nr ($P < 0.028$). Supplemented Tg also had a significant reduction in radiation-induced 8-OHdG (2.13 ± 0.21) compared to unsupplemented Tg ($P < 0.0001$). Levels of 8-OHdG in supplemented mice were actually slightly lower than constitutive levels observed in unirradiated unsupplemented mice. There was also no significant difference in radiation-induced 8-OHdG fluorescence between supplemented Nr and supplemented Tg.

**Discussion**

Our Tg mice have significantly elevated free radical production and lipid peroxidation in several tissues (29). These biomarkers strongly increase with age, correlate with longevity and indicate that Tg mice are under chronic oxidative stress and have accumulated significantly greater cellular oxidative damage compared to Nr. Although spontaneous structural chromosome aberrations in Tg were no greater than in Nr, unsupplemented Tg expressed dramatically elevated levels of γH2AX (DSB) and 8-OHdG (oxidative base damage). These results indicate that there is DNA damage produced by elevated free radicals in Tg mice, but this does not manifest into genomic DNA damage seen at the chromosome level. H2AX has been termed the ‘histone guardian of the genome’ and may function in chromatin structure alterations at sites of DNA damage (70). In contrast, Brown-Borg et al. (31) found elevated oxidative stress to proteins in liver and brain of Tg, but DNA base damage (8-OHdG) did not differ from controls. However, inter-individual variation in oxidative biomarkers was very large. Moreover, oxidized proteins increased with age in liver, but appeared to decline with age in brain. There are a number of possible explanations for oxidative stress observed in Tg mice. GH axis distortion could increase oxidative stress via alterations in mitochondria, especially via increased coupling or altered ion channels (33,71–73). As in many models of extended longevity such as birds and dietary restricted rodents (74), dwarf (GH deficient) mice were shown to generate lower amounts of mitochondrial free radicals (31). In this case, reduced metabolic rate rather than changes in mitochondrial coupling may be the cause. Sanz et al. (75) showed that mitochondrial DNA damage was also reduced in dwarfs, especially in brain.

Antioxidant defences may also be reduced in Tg mice (38,41,42). GH receptor knockout mice show strongly elevated hepatic superoxide dismutase that likely reflects down-regulated phoshoinositide-3 kinase (PI3K)/Akt signaling and elevated forkhead box transcription factors (FOXO) activity (76). GH administration also up-regulates enzymes involved in glutathione degradation and down-regulates those involved in synthesis (76). Since the glutathione system is crucial for cellular redox control, redox-regulated signalling, xenobiotic and stress resistance, impacts on radiosensitivity might be expected in Tg mice.

Membrane- and receptor-associated NAD(P)H oxidases generate considerable free radicals that are essential for
mitogenic signalling (33,77,78). There is also a linkage
between NAD(P)H oxidase activity and mitochondrial func-
tion. Thus, growth itself may be associated with free radical
generation and this would likely be greatly accentuated in Tg
mice (33,73). Free radical-induced DNA damage can also
derive a state of genomic instability (increased rate of acquiring
DNA modifications) which may well occur in Tg mice (18).
Persistently elevated free radical processes associated with
 genomic instability may be associated with dysregulated
mitochondria and reduced antioxidant activity (18,65).
NAD(P)H oxidase is also a candidate for the sustained
 elevation of free radicals and genomic stability associated with
radiation exposure and the bystander effect (79).

The absence of increased spontaneous chromosome aberrations
would suggest that high-fidelity repair processes dominate in the bone marrow cells of our mice; however, non-homologous end joining (NHEJ), an inherently error-
prone mechanism, is known to predominate in G0/G1 cells.
SKY is unable to discriminate the small deletions that typically
occur during NHEJ. Further examination, using techniques that
can detect small-scale deletions will be necessary to verify this
hypothesis. Depending on the location of DSBs, alterations or
loss of genetic material can increase cell dysfunction, genomic
instability and carcinogenesis (80,81). It is interesting to note
that constitutive levels of γH2AX in Tg mice are initially higher
than other groups, indicating some residual damage, but decrease
significantly over the incubation time, no other group shows this
trend (Figure 3A). We suggest that increased numbers of DSBs
are being generated in Tg mice with endogenously elevated
reactive oxygen species (ROS), but when cells are removed from
the organismal milieu, DSB production declines and existing
DSBs are repaired. Also, the significant increase in constitutive
8-OHdG levels indicates unsupplemented Tg mice have
dramatically elevated oxidative base damage compared to Nr
mice (see Figure 4). This supports the contention that chronic
oxidative stress in Tg mice results in the accumulation of considerable DNA damage with ageing (82,83).

We considered two possible outcomes of increasing oxidative
stress in Tg mice using ionizing gamma radiation. Adjustments
to offset endogenous free radical elevation could pre-adapt Tg
mice, so they might better cope with additional stress (the radio-
biology adaptive response). Alternatively, endogenous oxidative
stress in Tg mice may overtax defensive and repair abilities
making the mice hypersensitive to radiation-induced damage.
The dramatic increase in structural chromosome aberrations,
oxidative base damage and DSBs in unsupplemented irradiated
Tg versus comparable controls indicates that Tg may function
close to upper levels of manageable oxidative stress, indicating
Tg mice are a good model of elevated free radical processes.

All the spontaneous structural aberrations on both Tg and Nr
mice were chromosome-type aberrations with the apparent
absence in chromatid-type alterations. In radiation-induced
aberrations, unrepairred arm breaks with corresponding acentric
fragments comprised between 69.5 and 85.7% of all aberra-
tions depending on the experimental group. Translocations
were the next most frequent, followed by dicentric and ring
chromosomes. A small number of chromatid-type aberrations
were present in cells from irradiated mice; however, they only
occurred in unsupplemented animals. Since chromosome-type
aberrations occur in G1 phase of the cell cycle and the majority
of cells in exponentially growing stem cells (i.e. bone marrow)
are in G1, these results correspond well with what is currently
known in stem cell cycle processes.

The frequency of breaks in each chromosome does not appear to be correlated to chromosome length, gene density or
total genes per chromosome (Table III); however, it must be
emphasized that given the elapsed time after irradiation, we
were only looking at misrepaired breaks. The total number of
breaks induced per chromosome by the radiation exposure
would be proportional to size. When total breaks per
chromosome are correlated to the number of chromosome
aberrations remaining after repair, a relationship to the effect of
Tg or diet may become apparent. Gene activity must be altered
in the different groups and one could argue this changing repair
bias on different chromosomes (84,85).

Untreated Nr and Tg mice had significantly higher 8-OHdG
levels after 2-Gy irradiation; however, both groups increased
by the same relative amount over constitutive levels (131 and
129% for Nr and Tg, respectively). This is not surprising given
both groups of mice should have approximately the same
number of free radicals generated within their tissue when given
the same dose of radiation. This finding also indicates that while
Tg mice have increased endogenous ROS production, it does not
appear to exacerbate the radiation-induced oxidative base
damage (i.e. is an additive effect, not synergistic).

The kinetics of radiation-induced γH2AX fluorescence are
the same in Nr and Tg mice, which indicates the rate of DSB
repair are similar in Nr and Tg mice. At the peak fluorescence
for γH2AX (30-min incubation), it was readily apparent that
unsupplemented Tg mice had significantly higher levels of
γH2AX fluorescence. Although absolute levels of γH2AX
were greater in Tg mice, the relative change from constitutive
levels did not differ from unsupplemented Nr mice (increases
of 54 and 56%, respectively). This suggests that the chronically
elevated free radicals in Tg mice did not exacerbate the
radiation-induced DSB production.

Similar to Tg mice, diseases that manifest as accelerated
ageing (i.e. Werner’s, Hutchinson–Gilford progeria syndromes)
and ‘senescence-accelerated mice’ exhibit elevated free radical
production and/or increased chromosome aberrations (86–89).
These syndromes also display hypersensitivity to mutagens and
ionizing radiation, which cause significant increases in chro-
mosome instability compared to controls. Although Tg mice
show radiation-induced increases in all parameters of DNA
damage, but relatively minor constitutive DNA damage, they
demonstrate the hypersensitivity to ionizing radiation that is
characteristic of other accelerated ageing phenotypes. There-
fore, a critical attribute of the dietary supplement is that it not
only protects from direct effects of radiation but also over-rides
the ROS processes that influence the accelerated ageing
phenotype.

Antioxidants can protect against chronic oxidative stress
associated with ageing or disease (3,90) and acute oxidative
insult such as those induced by irradiation or xenobiotics
(56,91,92). Limoli et al. (93) found that antioxidants reduced
radiation-induced cell mortality, the degree of DNA damage
and genomic instability. Antioxidants, including α-lipoic acid,
offset free radical elevations and stem cell dysfunctioning
(94,95). Many individual ingredients in our supplement have
been tested as radioprotective agents (56), including most of
the components that have been postulated as potentially useful
in protecting astronauts from high-level radiation exposure
(55). Single antioxidants (i.e. ascorbate) administered after
irradiation have been shown to suppress long-lived radicals that
can otherwise cause mutations contributing to genomic
instability (57).
Gaziev et al. (50,51) found that a multiple-ingredient supplement was radioprotective for mice and human lymphocytes (vitamins E, C, β-carotene, rutin, selenium and zinc). Significant radioprotection of BALB/c mice and cells from bone marrow and bladder were obtained with a combination of vitamins E, C and vitamin A/β-carotene (52,53). A vitamin E and C combination reduced bleeding and diarrhoea in patients subjected to pelvic irradiation (54). Given that the free radical theory of ageing was born in radiation science (24), it is not surprising that the 31 ingredients in our supplement include most materials considered to be radioprotective (Table I). Our results show that a combinatorial approach with multiple targets and ingredients greatly enhances the radioprotection afforded by dietary supplements.

Although a reduction in constitutive 8-OHdG and γH2AX levels was evident in all diet-supplemented mice, it was only significant in Tg mice. This shows that the dietary supplement can provide protection from DNA damage resulting from constitutive cellular processes in normal mice but is particularly effective in systems that generate excessive amounts of ROS.

The dietary supplement provided considerable protection in all parameters of radiation-induced DNA damage examined in both Nr and Tg mice. Radiation-induced chromosome aberrations and oxidative base damage did not differ from constitutive levels in both groups of diet-supplemented animals, indicating that even after considerable exposure to radiation the dietary supplement was able to protect cells from radiation-induced damage. Since the majority of cellular damage produced by gamma radiation is indirect, through the production of free radicals via the hydrolysis of water, rather than by the energy tract itself, it is likely that the levels of cellular antioxidants are high enough in supplemented mice to effectively scavenge the excess ROS produced by the radiation dose (57). The small number of radiation-induced chromosome aberrations in supplemented mice was likely produced by unscavenged free radicals or could result directly from the radiation tract.

In conclusion, Tg mice have significantly increased constitutive DNA damage compared to normal mice and based on chromosome aberration induction are more vulnerable to radiation-induced exposure compared to age-matched normal mice. Our complex dietary supplement provided substantial protection from constitutive DSBs and oxidative base damage in both groups of supplemented mice, a strong indication that the supplement provides meaningful protection from damage induced by endogenous ROS production.

Even after a substantial dose of ionizing radiation, the dietary supplement provided significant protection to DNA, preventing DSBs, chromosome aberrations and oxidative base damage in both supplemented Nr and supplemented Tg mice. The defence against ROS-associated damage provided by the dietary supplement has clearly demonstrated that a holistic approach provides far greater protection and support for the maintenance of normal cell functioning than supplementation with single or a small number of compounds. This diet could therefore have important applications for protecting individuals exposed to high levels of occupational radiation such as astronauts. This protective effect would also apply to individuals with genetic predisposition to elevated free radicals. Furthermore, protection of normal tissue during radiation therapy could be another important use for this dietary supplement. We conclude that this diet formulation is a safe and potent radioprotectant.

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