Electronic Supplementary Information (ESI)

Comparison of *in vivo* and *in vitro* antioxidative parameters for eleven food factors

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Experimental details

Cell line

143B human osteosarcoma cells (thymidine kinase [TK]-deficient)[S1] were supplied by Dr. Douglas C. Wallace. The cells were cultured in high-glucose (4.5 g L^{-1}) Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Bio Source International, Camarillo, CA), 110 μg mL^{-1} pyruvate, and 50 μg mL^{-1} uridine at 37 °C with 5% CO_2.

X-ray irradiation

143B human osteosarcoma cells in dishes were irradiated (18.8 Gy) at room temperature using an X-ray unit HITACHI MBR-1505R at a dose rate of 1.3 Gy min^{-1} (120 kVp, 4.2 mA, 0.5 mm Al filter).
The food factors (24 μM) were added to the culture medium right after 18.8 Gy irradiation.

Relative levels of mitochondrial reactive oxygen species (ROS)

2-[6-(4'-Hydroxy)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (HPF) (Daiichi Pharmaceutical Co., Tokyo, Japan), a new fluorescence dye for selectively detecting hydroxyl radical (•OH), which was recently developed by our group,[S2] was used in the present study. •OH is highly reactive oxygen species (hROS). The dynamic range of fluorescence augmentation should be wide, because both molar absorption and quantum efficiency values are greatly increased upon *O*-deacylation. Although HPF alone fluoresces only to a small extent, it selectively and dose-dependently yields a strongly fluorescent compound, fluorescein, upon reaction with hROS, but not other ROS. Glass-bottomed (35-mm) dishes (MatTek Corp., Ashland, MA) with monolayers were prepared for staining with HPF. Two hours after irradiation, the cell culture medium was replaced with a modified Hanks' balanced salt solution (HBSS) containing 10 mM HEPES, 1.0 mM $MgCl_2$, 2.0 mM $CaCl_2$, and 2.7 mM glucose adjusted to pH 7.3 ± 0.05. Then, 10 μM HPF was added to the cells before incubation for 15 min at 37 °C. Bioimages of HPF were obtained using a CSU-10 confocal laser scanning unit (Yokogawa Electric Co., Tokyo, Japan) coupled to an IX90 inverted microscope with a UPlanAPO X20 objective lens (Olympus Optical Co., Tokyo, Japan) and a C5810-01 color chilled 3CCD camera (Hamamatsu Photonics, Hamamatsu, Japan). HPF was excited at 488 nm and the emissions were filtered using a 515-nm barrier filter. The intensity of the laser beam, the exposure time of the 3CCD camera, and the gain of the amplifier were held at 500 μW, 1.0 sec, and 18 decibels, respectively, to allow quantitative comparisons of the relative fluorescence intensity of the cells between groups. Cells were chosen on a random basis and scanned at more than three points for analysis. Average fluorescence intensity per cell was determined using IPLab Spectrum version 3.0 software (Scanalystics Inc., Fairfax, VA) with some modification of the program by one of the authors (H.J.M). The hROS-suppression indexes in Table 1 were calculated by (fluorescent intensity with α-tocopherol)/(fluorescent intensity with food factor).

Immunofluorescent staining for 4-hydroxy-2-nonenal (HNE)

Glass-bottom (35-mm) dishes (MatTek Corp.) with monolayers were prepared for immunofluorescent staining with a monoclonal antibody specifically directed against proteins modified by the major membrane lipid peroxidation product HNE (Majima et al., 2002).[S3] This monoclonal antibody (NOF Corp., Tokyo, Japan) was raised by immunizing mice with an HNE modified keyhole limpet hemocyanin (HNE-KLH) conjugate.[S4] Cells were fixed with 4% formaldehyde/phosphate buffer saline (PBS) at room temperature for 30 min, rinsed twice with PBS, and membranes were permeabilized by incubation in 95% ethanol with 5% acetic acid for 10 min. After being washed twice with PBS, the cells were incubated for 30 min in a blocking serum (1% bovine serum albumin in PBS), and for 1 h in anti-HNE mouse monoclonal antibody at a dilution of 1:200. The cells were rinsed twice with 0.1% bovine serum albumin in PBS, and reincubated with Alexa Fluor 488 goat anti-mouse IgG (H+L) conjugate (Molecular Probes, Eugene, OR) for 1 h at room temperature. Image acquisition and analysis procedures were similar to those used for HPF staining, except that the exposure time of the 3CCD camera was 4 s. The anti-lipid peroxidation indexes in Table 1 were calculated by (fluorescent intensity with α-tocopherol)/(fluorescent intensity with food factor).

Microscopic assessment of nuclear chromatin condensation and fragmentation

Cells grown on glass-bottom (35-mm) dishes (MatTek Corp.) were stained with the fluorescent dye Hoechst 33342 (Molecular Probes). Seventy-two hours after irradiation, the cells were fixed for 30 min in a solution containing 4% formaldehyde in PBS and then incubated in PBS with 1 mg mL^{-1} of the dye for 30 min. The cells were washed twice with PBS and fluorescence was visualized using an IX90 inverted microscope with a UPlanAPO 20× objective lens (Olympus Optical Co.). The dye was excited at 340 nm and emission was filtered with a 510-nm barrier filter. Photographs of microscopic fields were taken using a C5810-01 color chilled 3CCD camera (Hamamatsu Photonics). More than 300 cells per culture dish were counted and counts were made in three separate cultures per irradiation. Analyses were performed without knowledge of the irradiation history of the culture dishes, by random examinations. The percentage of apoptotic cells (apoptosis frequency) in each culture dish was determined. The anti-apoptotic indexes in Table 1 were calculated by (apoptosis frequency with α-tocopherol)/(apoptosis frequency with food factor).

Statistical analysis

Statistical analysis was performed by an analysis of variance (ANOVA), followed by Scheffe's F tests. A *P* value of less than 0.05 was considered statistically significant. Data were presented as the mean ± S.E. Calculations were performed with Excel, on a Power Macintosh G4 (Apple Computer, Inc., Cupertino, CA).

ESR measurements

Hypoxanthine (HPX) was purchased from Sigma. Xanthine oxidase (XOD) was commercially obtained from Nacalai Tesque, Inc. Japan. 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Dojindo. PBS (0.1 M, pH 7.4) was treated with Chelex 100 resin. Superoxide anion ($O_2^{\bullet -}$) was generated

using the HPX (0.5 mM)/XOD (0.1 U mL^{-1}) system in the presence and absence of different concentrations of the food factors and the apparent yield of $O_2^{\bullet-}$ was determined by electron spin resonance (ESR) using DMPO (100 mM) as spin trap. The ESR measurements were carried out on a JEOL RE-1X spectrometer (X-band) with 100 kHz field modulation. ESR spectra were recorded at room temperature in a JEOL flat quartz cell.

Kinetic measurements

Since radical species may readily react with molecular oxygen (O_2), the reactions were carried out under strictly deaerated conditions. The rates of the scavenging reactions of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) by the food factors in deaerated EtOH were determined by monitoring the absorbance change at 516 nm due to DPPH$^{\bullet}$ (ε = 9.88×10^3 M^{-1} cm^{-1}) using a stopped-flow technique on a UNISOKU RSP-1000-02NM spectrophotometer at 298 K. A continuous flow of Ar gas was bubbled through an EtOH solution (3.0 mL) containing DPPH$^{\bullet}$ (6.7×10^{-5} M) or an antioxidant (1.1×10^{-3}–4.4×10^{-3} M) in a reservoir of the spectrophotometer for 7 min. The pseudo-first-order rate constants (k_{obs}) were determined by a least-squares curve fit using an Apple Macintosh personal computer. The first-order plots of $\ln(A - A_\infty)$ vs time (A and A_∞ are denoted as the absorbance at the reaction time and the final absorbance, respectively) were linear until three or more half-lives with the correlation coefficient $\rho > 0.999$.

Electrochemical measurements

The cyclic voltammetry (CV) and second-harmonic alternating current voltammetry (SHACV)[S5] measurements were performed on an ALS-630A electrochemical analyzer in deaerated EtOH containing 0.10 M Bu_4NClO_4 as a supporting electrolyte. The Pt working electrode (BAS) was polished with BAS polishing alumina suspension and rinced with methanol before use. The counter electrode was a platinum wire. The measured potentails were recorded with respect to an $Ag/AgNO_3$ (0.01 M) reference electrode.

Theoretical calculations

Density functional theory (DFT) calculations were performed with Gaussian 09 (Revision A.02, Gaussian, Inc.).[S6] The calculations were performed on a 32-processor QuantumCube^{TM}. The values of ionization potentials (IP) were obtained from the energy difference between neutral and one-electron oxidized molecules calculated at the B3LYP/6-31G(d) level of theory.[S7]

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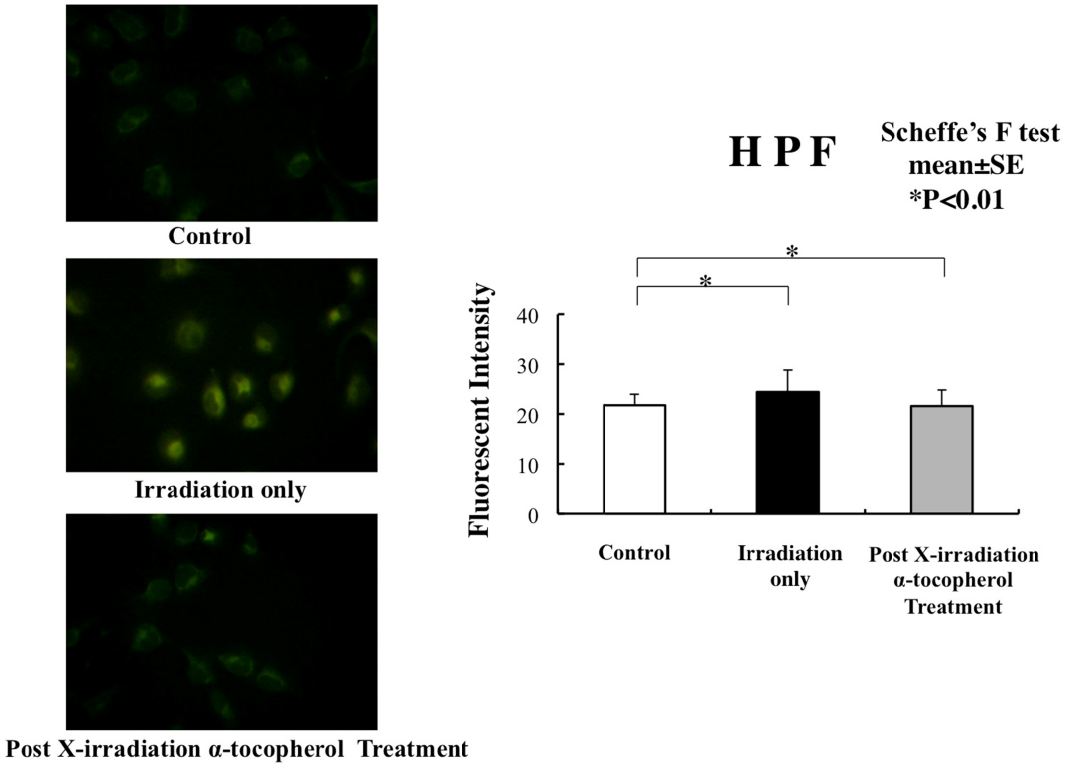

Fig. S1. Mitochondrial hROS generation detected by using HPF fluorescent dye. Two hours after irradiation, the cell culture medium was replaced with a modified Hanks' balanced salt solution (HBSS). The cells were then loaded with 10 μM HPF for incubation for 15 min at 37 °C. The fluorescence intensity of irradiation only cells significantly increased compared with that of no irradiated cells. In post X-irradiation α-tocopherol (24 μM) treatment cells, a significant decrease in intensity was observed. Data are shown as the mean ± S.E. by analysis of Scheffe's F test. *$P < 0.01$.

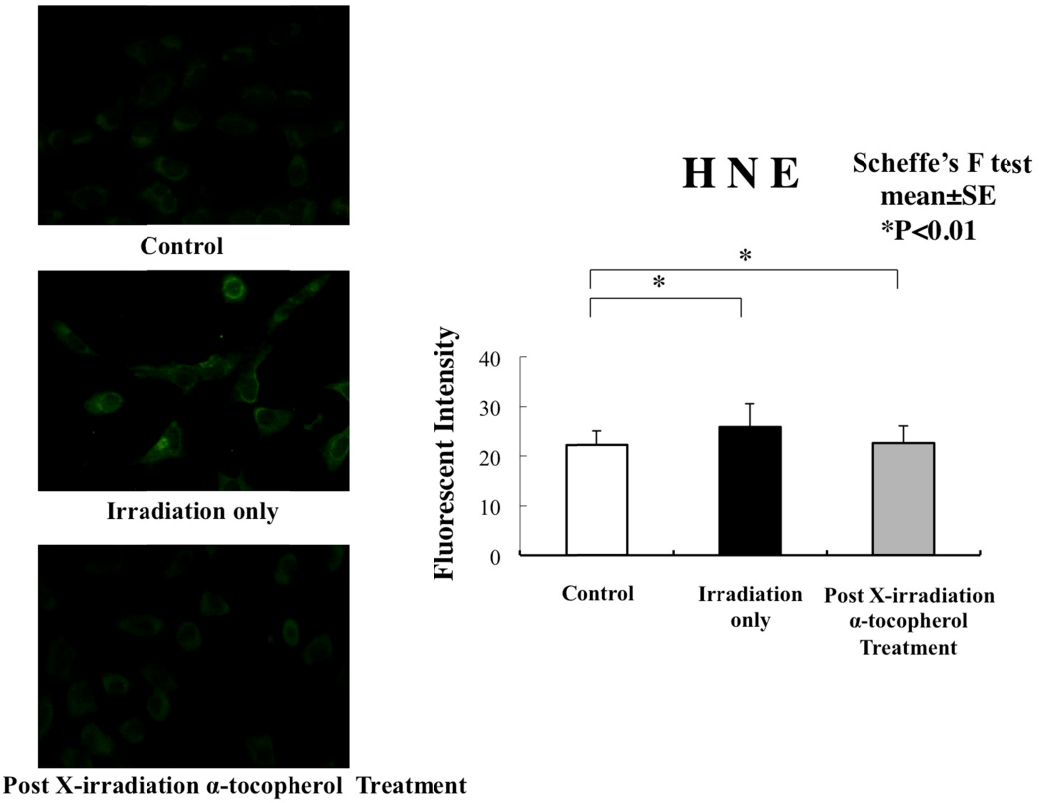

Fig. S2. Intracellular HNE generation (lipid peroxidation). Two hours after irradiation, the cells were permeabilized by incubation in 95% ethanol with 5% acetic acid. After washing, the cells were incubated in a blocking serum and then with anti-HNE mouse monoclonal antibody. Representative images of immunofluorescence staining and the fluorescence intensity of non-irradiation control cells, irradiation only cells, and post X-irradiation α-tocopherol (24 μM) treatment cells after 18.8 Gy irradiation are shown. Note that the generation of intracellular HNE was significantly suppressed in post X-irradiation α-tocopherol treatment cells. Data are shown as the mean ± S.E. by analysis of Scheffe's F test. $*P < 0.01$.

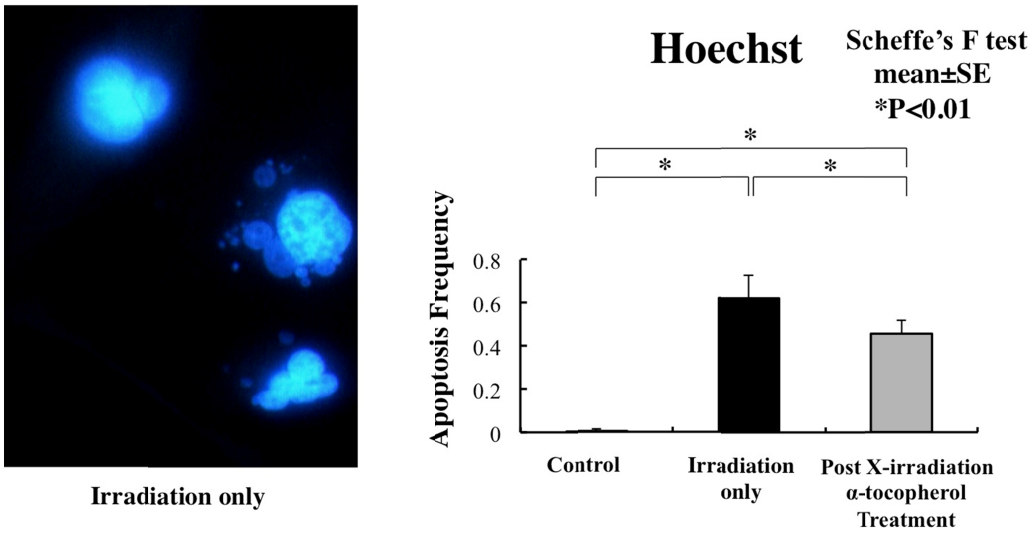

Fig. S3. Apoptosis. Seventy-two hours after irradiation, cells were fixed for 30 min and then incubated in PBS with 1 mg/mL of Hoechst 33342 stain for 30 min. The percentage of apoptotic cells in each culture dish was determined. The apoptotic frequency of non-irradiated control cells, irradiation only cells, and post X-irradiation α-tocopherol (24 μM) treatment cells after 18.8 Gy irradiation are shown. Post X-irradiation α-tocopherol treatment cells were more resistant to irradiation. Data are shown as the mean ± S.E. by analysis of Scheffe's F test. *$P < 0.01$.