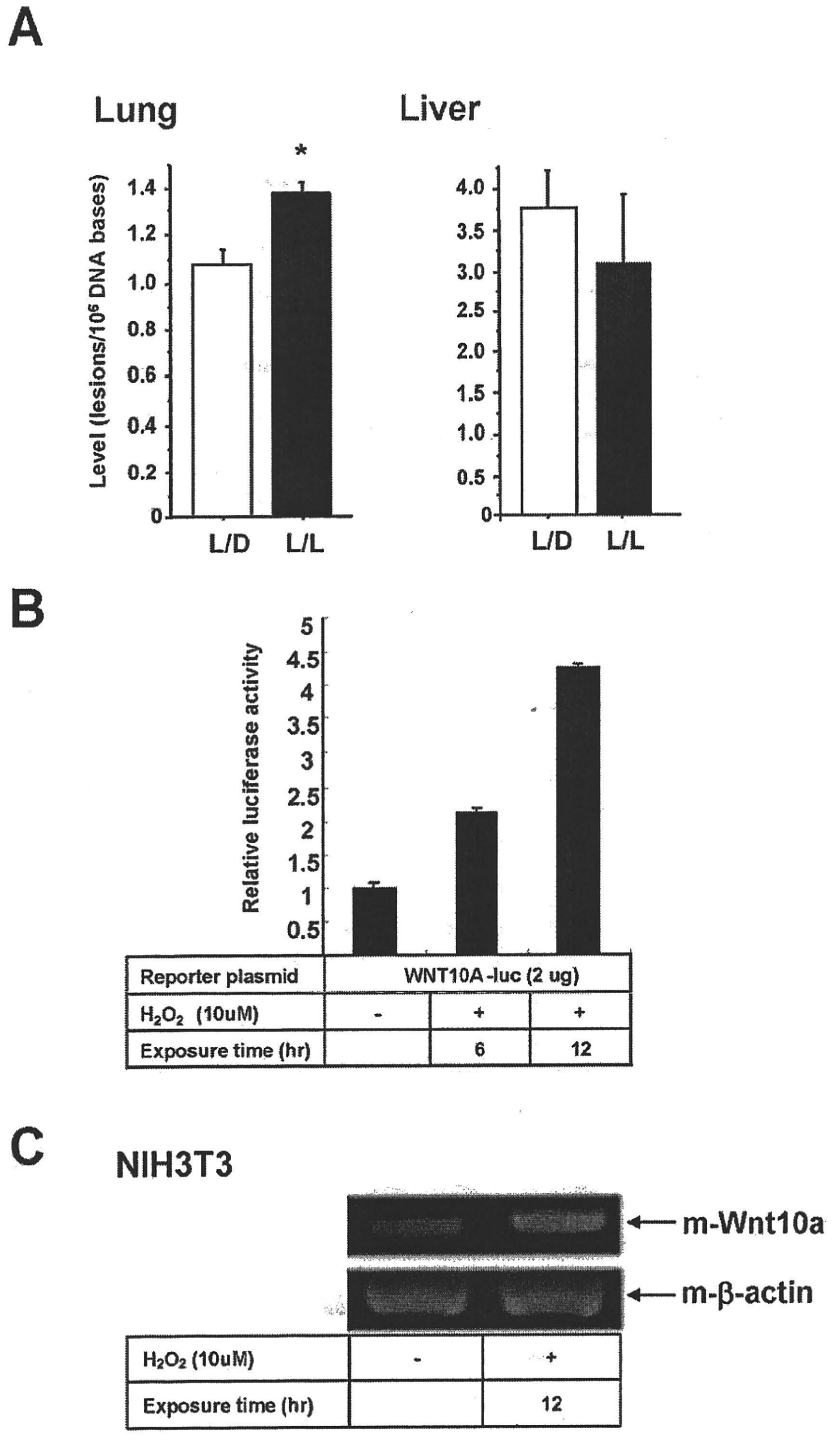

Figure 7. WNT10A is induced by oxidative stress. (A) 8-hydroxydeoxyguanosine levels are significantly increased in the lung tissues of L/L compared with L/D mice (*$P<0.05$). (B) Reporter assays. The promoter activity of the WNT10A gene was measured using a luciferase system after the addition of hydrogen peroxide. 42 hours after transfection (exposure time 6 hr) or 36 hours after transfection (exposure time 12 hr) of the reporter plasmid into PC3 cells, cells were treated with 10 μM of hydrogen peroxide. Luciferase activities were assayed after 48 hours of transfection. The results shown are normalized against protein concentrations measured using the Bradford method and are representative of at least three independent experiments. (C) Induction of mouse Wnt10a transcripts by oxidative stress. NIH3T3 cells were treated with or without H_2O_2 (10 μM) for 12 hours. Total RNAs were assayed by semi- quantitative RT-PCR. Mouse β-actin was used for internal control.
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cell line PC3 was kindly gifted by Dr M Nakagawa (Kagoshima University, Kagoshima, Japan) [34]. Although HeLa cell line was kindly gifted by Dr S Akiyama (Tokushima University, Tokushima, Japan) as human epidermoid cancer KB cell line [35], we carried out STR profiling at National Institute of Biomedical Innovation in Japan and revealed that KB cell line is same as Hela cell line. Mouse fibroblast NIH3T3 cell line was obtained from the Japanese Cancer Research Resources Bank (JCRB) [36]. HeLa cells and human prostate cancer PC3 cells were cultured in Eagle's minimal essential medium as described previously [37,38]. NIH3T3 cells were cultured in Dulbecco's modified Eagle's minimal essential medium. These mediums were purchased from Nissui Seiyaku (Tokyo, Japan) and contained 10% fetal bovine serum. Cell lines were maintained in a 5% CO_2 atmosphere at 37°C.

Anti-WNT10A antibody

A polyclonal antibody was raised against WNT10A by multiple immunization of a New Zealand white rabbit with synthetic peptides. The synthetic peptide sequences were RKLHRLQL DALQRGKGLSHGVPEHPALPC (aa 172–199) and CGGQL EPGPAGAPSPAPGAPGPRRRASPA (aa 307–334). This antibody was used for the Western blot and cell proliferation assays. For the cell proliferation assays, antibodies were purified from both control and WNT10A antisera using protein G columns (Mab Trap, Amersham Pharmacia Biotech).

Mouse studies

All protocols were approved by the Ethics Committee of Animal Care and Experimentation, University of Occupational and Environmental Health (admission number; AE-07039), and were performed according to the Institutional Guidelines for Animal Experiments and to Law (number 105) and Notification (number 6) of the Japanese government. All surgery was performed under anesthetization (mixture of ketamine 50 mg/kg and medetomidine 1 mg/kg), and all efforts were made to minimize suffering. Eight-week-old male nude mice (BALB/c nu/nu; Kyudo Co.) were used for subcutaneous xenografting. Mice were injected with 100 µl (1×10^6 cells) of Hela cells or PC3 cells suspension at two separate dorsal sites. The subcutaneous xenegrafting experiments were carried out four times for HeLa cells and twice for PC3 cells. Mice were randomly caged (5/cage) and subdivided into L/L and L/D groups. Tumor volume was measured using the two principal perpendicular diameters: $V = \text{length (mm)} \times (\text{width (mm)})^2 \times 1/2$.

Preparation of human tissue samples

Human normal skin, keloid tissue and cancer samples from different organ were examined in the Department of Pathology and Cell Biology at University of Occupational and Environmental Health in Kitakyushu, Japan. The diagnosis was re-evaluated and confirmed by at least three board-certified surgical pathologists who had examined formalin-fixed, paraffin-embedded tissue sections stained with haematoxylin and eosin (H&E) or other appropriate immunohistochemical stains.

Immunohistochemistry and histpathology

Formalin-fixed tumors (transplanted to mice or human cancer specimens), normal human dermal tissues and human keloid tissues were embedded in paraffin and sections were immunostained using anti-CD34 (1:50; Immunotech), anti-aSMA (1:150, DAKO), anti-mouse Type I collagen (1:250; AbD Serotec), anti-WNT10A (1:50, Sigma-Aldrich) and anti-cytokeratin CAM5.2 (1:10, Becton Dickinson) according to the manufacturer's instructions. The anti-WNT10A antibody recognizes both human and mouse WNT10A. 3,3′-Diaminobenzidine (DAB) or Vulcan fast red were used as chromogen. The necrotic area/tumor area ratio was evaluated using NanoZoomer Digital Pathology Virtual Slide Viewer software (Hamamatsu Photonic Co.). Masson trichrome staining is used for evaluating extracellular matrix. All procedures were approved by the ethics committee of the University of Occupational and Environmental Health.

DNA microarray analysis and RT-PCR

DNA microarray analysis was performed using 3-DGene (Toray Industries). All data is MIAME compliant and that raw data has been deposited in a MIAME compliant database (GSE23969). Only one tumor from each L/D and L/L group which represent the typical look of tumors size and color was used for RNA preparation in same experiment. Total RNA was isolated from tumors and cultured cells using QIAshredder and RNeasy-Mini kits (Qiagen). RT-PCR was performed using the Qiagen OneStep RT-PCR kit. The primers used in this study are listed in Table S2. Cycle number is 30 excluding some exceptions. The cycle number of these exceptions is listed in each figure legend. Human specificity of h-WNT10A primers is shown using NHDF cells (Figure 4A) and mouse specificity of m-Wnt10a primers is shown using NIH3T3 cells (Figure S2A). Specificity of human and/or mouse β-actin primers is shown using Hela cells and NIH3T3 cells (Figure S2B).

Plasmid construction

WNT10A cDNA was constructed by PCR using a superscript cDNA library (Invitrogen) (Table S2). The PCR product was cloned into the pGEM-T easy vector (Promega) and the full-length cDNA fragment was recloned into the pcDNA3.1 vector (Invitrogen). To prepare the reporter plasmid containing the promoter region of the human WNT10A gene, PCR of human genomic DNA was performed using the appropriate primers (listed in Table S2). The PCR product was then cloned into the pGL3-basic vector (Promega).

Cloning of stable transfectants

HeLa cells were transfected with pcDNA3.1-WNT10A using the Effectene reagent (Qiagen) and cultured with 500 µg/ml hygromycin for 15–20 days. The resulting colonies were isolated and the cellular expression levels of WNT10A in each clone analyzed by Western blotting with an anti-WNT10A antibody.

Western blotting analysis

Whole-cell lysates were prepared as previously described [38,39]. The 100 µg of whole-cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) microporous membranes (Millipore, Billerica, MA, USA) using a semi-dry blotter. The blotted membranes were treated with 5% (w/v) skimmed milk in 10 mM Tris, 150 mM NaCl and 0.2% (v/v) Tween 20, and incubated for 1 h at room temperature with the primary antibody. The following antibodies and dilutions were used: 1:1,000 dilution of anti-WNT10A and 1:5,000 dilution of anti-β-actin. The membranes were then incubated for 45 min at room temperature with a peroxidase-conjugated secondary antibody, visualized using an ECL kit (GE Healthcare Bio-Science, Buckinghamshire, England, UK). The detection was performed with LAS-4000 mini (FUJIFILM).

WNT10A siRNA knockdown

Twenty-five base-pair double-stranded RNA oligonucleotides were commercially generated (Invitrogen) (Table S3). siRNA transfections were performed according to the manufacturer's

instructions with modifications (Invitrogen) [38,40]. Stealth™ RNAi Negative control Duplexes (Cat. No. 12935-300; Invitrogen) was used as control siRNA. Whole cell extracts (100 μg) were prepared from fibroblasts 72 hrs after siRNA transfection and Western blotting was performed.

Conditioned Media (CM)

Stable transfectants were cultured in MEM containing 10% FBS until they formed confluent monolayers. The MEM was then replaced with either conditioned EBM (all growth factors and FBS are 0.1 fold compared to normal EBM) or conditioned FBM (insulin, FGF and FBS are 0.1 fold compared to normal FBM) for 24 hours, after which the medium was collected. The CM was then centrifuged and filtered to remove cells and debris. Control-CM was prepared from the culture medium of growing control-cl2 cells, and WNT10A-CM was prepared from the cultured medium of growing WNT10A-cl25 cells.

Cell Proliferation Assays

WNT10A-overexpressing cell lines and control cell lines were seeded in 12-well plates and counted every 12 hours. NHDF cells were seeded in 12-well plates and transfected with siRNA as described above. For the purposes of analysis, "0 hours" was taken to be 12 hours post transfection. The cells were harvested with trypsin and counted with a Coulter-type cell size analyzer (CDA-500, Sysmex). BrdU was incorporated using a cell proliferation ELISA kit (Roche Diagnostics).

Luciferase assay

Transient transfection and luciferase assays were performed as previously described [40]. Briefly, PC3 cells (1×10^5) were seeded into 12-well plates and, one day later, transfected with the WNT10A reporter plasmid using the Superfect reagent (Qiagen). Finally, the cells were incubated under normal culture conditions, or in the presence of 10 μmol/L (10 μM) H_2O_2. Forty-eight hours post-transfection, the cells were lysed with reporter lysis buffer (Promega) and luciferase activity was detected using a Picagene kit (Toyoinki). The results shown are normalized against protein concentrations measured using the Bradford method and are representative of at least three independent experiments.

Measurement of 8-hydroxydeoxyguanosine

The amount of 8-hydroxydeoxyguanosine (8-OH-dG) present in the cellular DNA was measured using a high performance liquid chromatography (HPLC)-electrochemical detector (ECD) system as previously described [41]. The final 8-OH-dG value was calculated as the number of 8-OH-dG residues/10^6 dG residues.

Statistical analysis

We compared continuous variables with repeated measure analysis of variance (ANOVA), and differences between groups were determined by Scheffe's test. Student *t* test was used for statistical analysis of the variables between the two groups. All error bars indicate standard deviation.

Supporting Information

Figure S1 Comparison of HeLa cell tumors in L/L and L/D mice. Eight representative tumors are shown.
(TIF)

Figure S2 Analysis of specificity of mouse Wnt10a primers, human β-actin primers and mouse β-actin primers. (A) Mouse Wnt10a primers amplified mouse Wnt10a transcripts derived from mouse fibroblast NIH3T3 cells, but human WNT10A primers did not. (B) Human β-actin primers amplified human β-actin transcripts derived from human Hela cells, but mouse β-actin did not. Mouse β-actin primers amplified mouse β-actin transcripts derived from mouse fibroblast NIH3T3 cells, but human β-actin primers did not. The cycle number is 40 for all RT-PCR.
(TIF)

Figure S3 Immunohistochemical analysis of WNT10A in control tumors and WNT10A-overexpressing tumors.
(TIF)

Figure S4 Immunohistochemical analysis of β-catenin in L/D and L/L tumors, and control tumors and WNT10A-overexpressing tumors.
(TIF)

Table S1 Genes differentially expressed between L/L tumor and L/D tumor samples. The list of selected genes with fold change marked >2.0 between L/L tumor and L/D tumor samples.
(DOC)

Table S2 Primers used for construction of reporter plasmid, protein expression plasmid and semi-quantitative RT-PCR.
(DOC)

Table S3 Double-stranded RNA 25-base pair oligonucleotides used for WNT10A kockdown analysis.
(DOC)

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Author Contributions

Conceived and designed the experiments: YY K. Kohno. Performed the experiments: YY HI KYW SS YS K. Kawai HK KM TS EK GH AK MA BH YW II SH K. Kohno. Analyzed the data: YY HI KYW SS YS K. Kohno. Contributed reagents/materials/analysis tools: YY K. Kohno. Wrote the paper: YY K. Kohno.

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Induction effect of coadministration of soybean isoflavones and sodium nitrite on DNA damage in mouse stomach

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ABSTRACT

We have already found that nitrite-treated isoflavones exhibit genotoxic activities toward *Salmonella typhimurium* TA 100 and 98 strains (submitted: nitrite-treated genistein). However, we have not demonstrated genotoxic activity induced by simultaneous treatment with isoflavones and $NaNO_2$ *in vivo*. In the present study, we examined whether coadministration of isoflavones (such as daidzein and genistein) and $NaNO_2$ induces DNA damage in the stomach of ICR male mice. Mice were coadministered with isoflavones (1 mg/kg body weight) and $NaNO_2$ (10 mg/kg body weight), and dissected to collect tissues at 1, 3, and 6 h after administration. We used comet assay combined with repair enzyme formamidopyrimidine-*N*-glycosylase (FPG) to detect FPG-sensitive sites. An HPLC–ECD system was employed to determine 8-oxo-2′-deoxyguanosine (8-oxodG) in the stomach. In addition, we observed leukocyte infiltration by histopathological investigation, and measured total superoxide dismutase (SOD) in the stomach. We confirmed that oxidative DNA damage in the stomach was significantly increased by coadministration. Total SOD activities were also significantly stimulated by coadministration. However, the induction of inflammation in the stomach was not found. These data suggest that coadministration of isoflavones and $NaNO_2$ can cause DNA damage in the stomach because of the formation of radicals.

1. Introduction

Soy isoflavones have been proposed to convey many health benefits, such as preventive effects against climacteric disorder (Drews et al., 2007; Cassidy et al., 2006), breast cancer (Magee and Rowland, 2004; Ravindranath et al., 2004), and dowager's hump (Syed and Khosla, 2005; Zhang et al., 2008). Therefore, soy isoflavones have been marketed as health supplements in recent years. We recognize that soy isoflavones are useful and safe materials. However, some scientists have recently suggested that soy isoflavones can induce cytotoxic and genotoxic effects *in vitro* (Tayama et al., 2008; Virgilio et al., 2004; Boos and Stopper, 2000; Snyder and Gillies, 2003) and *in vivo* (Misra et al., 2002). Consequently, it is important to evaluate the risk of soy isoflavones to humans.

We regularly consume nitrite and nitrate through vegetables, tap water, and food additives (Karlik et al., 1995). Sodium nitrite ($NaNO_2$) has been shown to have genotoxic activities *in vitro* (Balimandawa et al., 1994). On the other hand, it did not show carcinogenic activity *in vivo* (Maekawa et al., 1982; Inai et al., 1979; Anderson et al., 1985; Hawkes et al., 1992). However, some researchers have reported that mutagenic/carcinogenic nitrosamines are formed by the reaction of nitrite and secondary amines in foodstuffs under acidic conditions. $NaNO_2$ is a precursor of *N*-nitroso compounds with strong genotoxic and carcinogenic potencies (Lacoste et al., 2007; Walker, 1990; Paula and Carlos, 2006).

Several food components also show genotoxic and carcinogenic activities after nitrite treatment. It has been reported that nitrite-treated flavonoids showed genotoxicity *in vitro* (Rueff et al., 1995), and combined treatment with green tea catechins and $NaNO_2$ induced carcinogenesis in forestomach of rats (Kuroiwa et al., 2007). Miyauchi et al. reported that combined treatment with tert-butylhydroquinone, α-tocopherol, propyl gallate, and $NaNO_2$ promoted forestomach carcinogenicity in rats (Miyauchi et al., 2002). Ishii et al. demonstrated that coadministration of catechol and $NaNO_2$ induced rat forestomach damage and cell

Abbreviations: Daid, daidzein; Gen, genistein; FPG, formamidopyrimidine-*N*-glycosylase; NO, nitric oxide; ROS, reactive oxygen species; SOD, superoxide dismutase; $NaNO_2$, sodium nitrite.

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proliferation (Ishii et al., 2006). Matsumoto et al. revealed that simultaneous treatment with ascorbic acid (AsA) and $NaNO_2$ produced positive results in bacterial reverse mutation tests using *Escherichia coli* WP2*uvrA*/pKM101 and chromosomal aberration test using cultured Chinese hamster lung CHL/IU cells (Matsumoto et al., 2008). Ohsawa et al. also elucidated that coadministration of $NaNO_2$ and ascorbic acid (AsA) induced DNA damaging potency in mouse stomach (Ohsawa et al., 2003). Okazaki et al. reported that forestomach carcinogenesis was strongly enhanced by the combination of AsA and $NaNO_2$ (Okazaki et al., 2006). Kuroiwa et al. demonstrated that combined treatment with AsA and $NaNO_2$ can cause epithelial hyperplasia and papilloma development in rat esophagus (Kuroiwa et al., 2008). We also reported that nitrite-treated bisphenol A (BPA) (Masuda et al., 2005), 17β-estradiol (E2) (Masuda et al., 2006), and 5-hydroxytryptamine (5-HT) (Masuda et al., 2005) showed strong genotoxic activities. Therefore, evaluation of the genotoxic and carcinogenic activities of food-related materials, such as isoflavones, after nitrite treatment, is needed.

There have been a few reports published about the formation of nitrate-treated soy isoflavones (daidzein and genistein) *in vitro* (D'Alessandro et al., 2003; Boersma et al., 1999). However, there is no published data about the genotoxicity of nitrite-treated isoflavones. The purpose of present study was to evaluate the induction of genotoxic activity by the coadministration of soy isoflavones and $NaNO_2$ *in vivo*. In addition, we evaluated total superoxide dismutase (SOD) activity and histological change.

2. Material and methods

2.1. Chemicals

Daidzein and genistein were purchased from LKT Laboratories Inc. (St. Paul, MN, USA). Low-melting-point (LMP) agarose, normal-melting-point (NMP) agarose, dimethyl sulfoxide (DMSO), formamidopyrimidine-*N*-glycosylase (FPG), nuclease P1, alkaline phosphatase from *E. coli*, and ribonucleases T1 and A from Boehringer Mannheim were bought from Sigma–Aldrich (St. Louis, MO, USA). Ethidium bromide and polyethylene glycol (PEG, molecular weight 7300–9000) were obtained from Merck (Darmstadt, Germany). Other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan).

2.2. Animals and treatments

Ethics approval for the study was obtained from the Committee for Animal Experimentation of the University of Shizuoka. We obtained ICR male mice weighing 40 ± 2 g from SLC, Hamamatsu, Japan. Before the experiment, mice were housed for 1 week in an air-conditioned room with free access to CE-2 commercially available food pellets (Clea Japan, Tokyo, Japan). Five mice were assigned to each experimental group. Samples dissolved in saline were orally administered isoflavones at doses of 1 and 10 mg/kg body weight and $NaNO_2$ at 10 mg/kg body weight. For the FPG-modified comet assay and 8-oxodG analysis, 1, 3, and 6 h after the administration of chemicals, stomach was collected from each treated mouse.

2.3. Standard comet assay and FPG-modified comet assay

Comet assay is the sensitive method to evaluate DNA damaging potencies of chemicals. Encapsulated cells in a LMP agarose are lysed (pH 10), and applied to electrophoresis in alkaline (pH > 13) condition Followed by, the visual analysis is performed by calculating fluorescence intensity and length of comet-liked image of DNA strained with fluorescence pigment to detect the extent of DNA damage. This can be performed by automatically by imaging software.

Formamidopyrimidine-*N*-glycosylase (FPG) is a repair enzyme to recognize oxidative DNA base, such as 8-oxo-2′-deoxyguanosine (8-oxodG), Fapy dG, and Fapy dA formation. Therefore, the modified comet assay with FPG is the more sensitive method to detect the oxidative DNA base. This modified comet assay was used the method of Shimoi et al. (Nishio et al., 2007).

Stomach was opened and rinsed with PBS, and mucosa was scraped into 4 ml chilled homogenizing buffer (pH 7.5) containing 0.075 M NaCl and 0.024 M EDTA-2Na, and homogenized gently using a homogenizer on ice. To obtain nuclei, the homogenate was centrifuged at 700*g* for 10 min at 4 °C, and the precipitate was re-suspended in chilled homogenizing buffer at 1 g organ weight per milliliter. Samples were mixed with 75 μl of 1% low-melting-point (LMP) agarose. The mixture (75 μl) was layered on a 1% LMP agarose layer and covered with 75 μl of 1% LMP agarose. After preparation, the slide was immersed in lysing solution and refrigerated at 4 °C for 1 h. After lysis, the slide was washed (100 ml × 3 for 5 min) with FPG buffer (10 mM Tris, pH 7.5, 1 mM EDTA-2Na, 100 mM NaCl, 100 μg/ml bovine serum albumin). Treatment with FPG was carried out as follows. Cells were embedded in agarose overlaid with 80 μl of FPG (1 μg/ml) and then a cover-slip was added. The slide was incubated for 15 min at 37 °C in a humidified atmosphere. For the slide without FPG treatment, FPG buffer was used. The slide was then placed in alkaline electrophoresis buffer for 10 min to allow salt equilibration and further DNA unwinding. Electrophoresis was performed at 30 V and 300 mA for 15 min at 4 °C. The slide was then neutralized with 0.4 M Tris buffer (pH 7.5) for 10 min. The cells were stained with 50 μl of ethidium bromide (20 μg/ml). Comet images were analyzed using a fluorescence microscope (magnification 200×) equipped with CCD camera. One hundred cells were examined per mouse. Tail moment of DNA was measured using Comet Analyzer Youworks Bio Imaging Software. Differences between the averages of treated animals and control animals (treated with saline) were compared using Dunnett's test after one-way ANOVA. A *p*-value of less than 0.05 was considered to be statistically significant.

2.4. Measurement of nuclear 8-oxodG

Nuclear DNA was isolated as described previously (Kaneko et al., 2001). Each tissue (about 150 mg) was minced with a pair of scissors in ice-cold 0.85% sodium chloride. The minced tissue was homogenized in a glass Teflon homogenizer in 0.3 M sucrose. The homogenates were centrifuged to remove the cytosolic fraction containing mitochondria. Pellets were incubated at 37 °C with proteinase K and 1% SDS/1 mM EDTA (pH 8.0) under an argon atmosphere. The resulting solution was mixed with 7 M NaI and isopropyl alcohol. The pelleted DNA, which was obtained by centrifuging the mixture, was rinsed with 70% ethanol. Ribonucleases T1 and A were added to the crude DNA solution, and the mixture was incubated at 37 °C under an argon atmosphere. Following incubation, chloroform:isoamyl alcohol (24:1, v/v) was added and mixed, and the mixture was centrifuged. The aqueous phase was mixed with 13% PEG solution containing 1.6 M NaCl. The mixture was centrifuged and the DNA obtained was dissolved in water. DNA was hydrolyzed with nuclease P1 and alkaline phosphatase, and 8-oxodG was analyzed by HPLC with an electrochemical detection device (ESA Coulochem II 5200 and analytical cell model 5011, Bedford, Mass., USA). Differences between the averages of treated and untreated control animals were compared using the Mann–Whitney *U*-test. A *p*-value of less than 0.05 was considered to be statistically significant.

2.5. Histological examination

Stomach tissues were fixed in 10% formalin solution and embedded in paraffin. The samples were sliced at a thickness of 5 μm and stained with hematoxylin and eosin for histopathological examination.

2.6. Measurement of total SOD activity

Total SOD activities were measured using a kit (Dojin, Japan) according to the manufacturer's instructions. SOD concentrations were calculated from the linear part of the standard curve. Protein concentrations were evaluated by BCA protein assay (PIERCE, USA) using bovine serum albumin as standard. Differences between the averages of treated and untreated control animals (treated with saline) were compared using Dunnett's test after one-way ANOVA. A *p*-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Cellular DNA damaging potency of isoflavones by nitrite treatment in stomach after 3 h

Figs. 1 and 2 show the mean values of DNA tail moment of single administration (only saline, isoflavones, or $NaNO_2$) and coadministration after 3 h. We confirmed that images of cell death were absent in each administered group. Therefore, we judged that the observed images were not associated with apoptosis and necrosis.

Single administrations of 1 mg/kg body weight daidzein (FPG−: 0.631 ± 0.484, FPG+: 0.851 ± 0.739), genistein (FPG−: 0.809 ± 0.891, FPG+: 0.980 ± 0.831), and 10 mg/kg body weight $NaNO_2$ (FPG−: 0.820 ± 0.834, FPG+: 0.855 ± 0.653) did not significantly induce DNA damaging potency. However, coadministration of isoflavones and $NaNO_2$ significantly increased DNA damage with or without FPG treatment ($p < 0.01$). From data for cases with FPG treatment, it was confirmed that coadministration of daidzein and $NaNO_2$ synergistically induced oxidative DNA damage (4.57 ± 3.32).

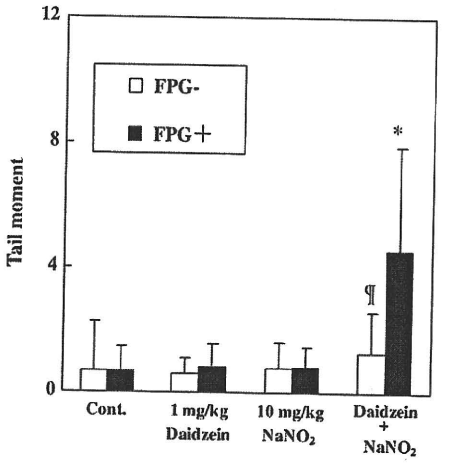

Fig. 1. DNA damage of mouse stomach at 3 h after coadministration of daidzein (1 mg/kg body weight) and $NaNO_2$ (10 mg/kg body weight). Fifty cells were counted per mouse. The mean values were obtained from 250 cells. Bars represent the SD values. □ showed results of comet assay without FPG (DNA strand breaks and alkali-labile sites). On the other hand, ■ showed results of comet assay with FPG (oxidative DNA base). $^{*}p < 0.01$ vs. control (FPG+), $^{\P}p < 0.01$ vs. control (FPG−).

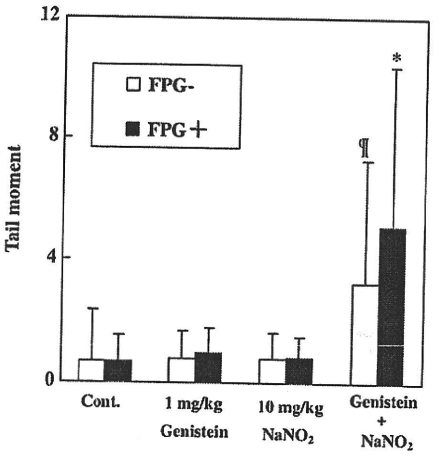

Fig. 2. DNA damage of mouse stomach at 3 h after coadministration of genistein (1 mg/kg body weight) and $NaNO_2$ (10 mg/kg body weight). Fifty cells were counted per mouse. The mean values were obtained from 250 cells. Bars represent the SD values. □ showed results of comet assay without FPG. On the other hand, ■ showed results of comet assay with FPG.$^{*}p < 0.01$ vs. control (FPG+), $^{\P}p < 0.01$ vs. control (FPG−).

Moreover, the group coadministered with gen and $NaNO_2$ also showed a synergistic increase in oxidative DNA damage (5.12 ± 5.27). From the results without FPG treatment, it was confirmed that coadministration of daidzein and $NaNO_2$ induced additive DNA damage (1.31 ± 1.31). Meanwhile, coadministration of genistein and $NaNO_2$ resulted in a synergistic increase in DNA damage (3.23 ± 4.09).

3.2. Determination of total SOD activity in stomach after 3 h

Total SOD activities in the stomachs of the single administration and coadministration groups after 3 h are shown in Figs. 3 and 4. Total SOD activities were significantly higher in the coadministration groups (daidzein + $NaNO_2$: 79.5 ± 9.45, genistein + $NaNO_2$: 72.2 ± 10.5) than in the single administration groups (e.g. Figs. 3 and 4).

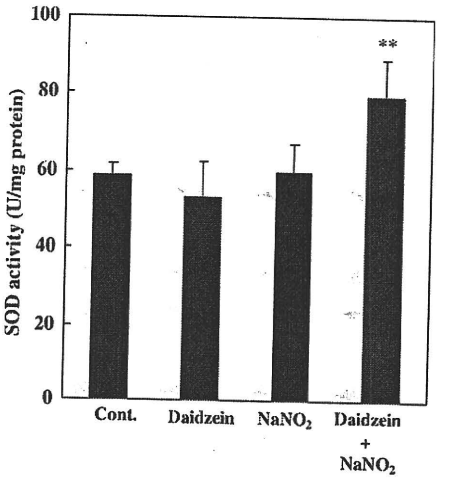

Fig. 3. Total SOD activity of mouse stomach at 3 h after coadministration of daidzein (1 mg/kg body weight) and $NaNO_2$ (10 mg/kg body weight). The mean values were obtained from five animals. Bars represent SD. $^{**}p < 0.01$ vs. control.

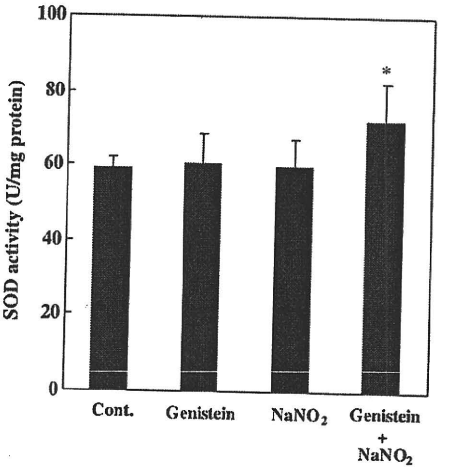

Fig. 4. Total SOD activity of mouse stomach at 3 h after coadministration of genistein (1 mg/kg body weight) and $NaNO_2$ (10 mg/kg body weight). The mean values were obtained from five animals. Bars represent SD. $^{*}p < 0.05$ vs. control.

3.3. Change in cellular DNA damaging potency of coadministration after 1, 3, and 6 h

Figs. 5 and 6 show the mean values of DNA tail moment by coadministration after 1, 3, and 6 h. The maximum value was confirmed after 1 h by coadministration of daidzein and $NaNO_2$ (8.47 ± 7.79). Meanwhile, coadministration of genistein and $NaNO_2$ showed a maximum value after 3 h (5.12 ± 5.27).

DNA damage was significantly increased at each time point after coadministration of isoflavones and $NaNO_2$ with or without FPG treatment ($p < 0.01$) (e.g. Figs. 5 and 6).

3.4. Histological observation of stomach

Single administration groups did not exhibit lymphocyte infiltration and other histologic changes (data not shown). No lymphocyte infiltration and other histological changes were observed at 1, 3 and 6 h after coadministration (e.g. Figs. 7 and 8).

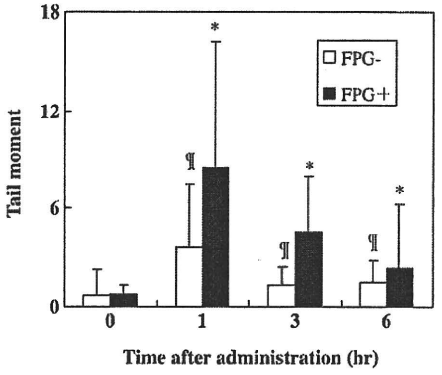

Fig. 5. Time courses (1, 3 and 6 h) of DNA damage in the stomach following coadministration with dadizein (1 mg/kg body weight) and $NaNO_2$ (10 mg/kg body weight). Fifty cells were counted per mouse. The mean values were obtained from 250 cells. Bars represent SD values. □ showed results of comet assay without FPG. On the other hand, ■ showed results of comet assay with FPG. *$p < 0.01$ vs. control (0 h: untreated group, FPG+), ¶$p < 0.01$ vs. control (0 h: untreated group, FPG−).

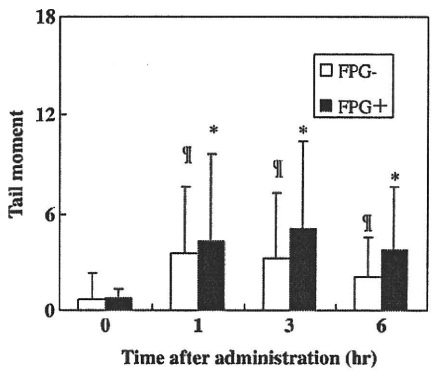

Fig. 6. Time courses (1, 3 and 6 h) of DNA damage in the stomach following coadministration with genistein (1 mg/kg body weight) and NaNO2 (10 mg/kg body weight). Fifty cells were counted per mouse. The mean values were obtained from 250 cells. Bars represent SD values. □ showed results of comet assay without FPG. On the other hand, ■ showed results of comet assay with FPG. *$p < 0.01$ vs. control (0 h: untreated group, FPG+), ¶$p < 0.01$ vs. control (0 h: untreated group, FPG−).

3.5. Effect of isoflavone dose on DNA damage induction at 3 h after coadministration

The mean values of DNA tail moment induced by coadministration of 1 mg/kg daidzein and 10 mg/kg $NaNO_2$ were 1.31 ± 1.31 (FPG−) and 4.57 ± 3.32 (FPG+). On the other hand, coadministration of 10 mg/kg daidzein and 10 mg/kg $NaNO_2$ gave values of 1.34 ± 1.54 (FPG−) and 1.30 ± 1.16 (FPG+).

The mean values of DNA tail moment induced by coadministration of 1 mg/kg genistein and 10 mg/kg $NaNO_2$ were 3.23 ± 4.09 (FPG−) and 5.12 ± 5.27 (FPG+). In addition, coadministration of 10 mg/kg genistein and 10 mg/kg $NaNO_2$ gave values of 0.804 ± 0.657 (FPG−) and 1.56 ± 1.51 (FPG+).

3.6. Concentration of 8-oxodG in stomach of rats treated with isoflavones and $NaNO_2$

The 8-oxodG concentrations in the mouse stomach at 1, 3, and 6 h after coadministration are shown in Figs. 9 and 10. It was confirmed that the value reached a maximum at 3 h after coadministration of daidzein and $NaNO_2$ (0.248 ± 0.0290). On the other hand, coadministration of genistein and $NaNO_2$ resulted in a maximum value being reached after 6 h (0.204 ± 0.0314). Groups coadministered with daidzein and $NaNO_2$ exhibited significantly increased concentrations of 8-oxodG after 3 and 6 h ($p < 0.01$). Groups coadministered with genistein and $NaNO_2$ exhibited a significantly increased concentration of 8-oxodG after 6 h ($p < 0.01$).

4. Discussion

The standard comet assay is a rapid and sensitive method for the detection of DNA strand breaks and alkali-labile sites in individual cells. These types of DNA damage are those initially induced by genotoxic chemicals. However, the standard comet assay cannot detect oxidative DNA base (such as the formation of 8-oxodG, Fapy dG, and Fapy dA). Therefore, we used the FPG-modified comet assay to detect oxidative DNA base in stomach cells (Arimoto-Kobayashi et al., 1997). Figs. 1 and 2 show the mean DNA tail moment values in the stomach at 3 h after coadministration of isoflavones and $NaNO_2$. No difference in the tail moment values with and without FPG treatment was observed between the single administration groups (only isoflavones or $NaNO_2$) and the control groups. However, coadministration of isoflavones and $NaNO_2$ significantly increased DNA damage with or without FPG treatment ($p < 0.01$).

Kikugawa and Kato have demonstrated that diazoquinone compounds, which showed strong genotoxicity, were formed by the reaction between phenol and nitrite under acidic conditions (Kikugawa and Kato, 1988; Kato et al., 1992). They found that DNA damage was induced by radicals formed in the reaction mixtures of phenol and nitrite. Some researchers have reported that combined treatment of antioxidants and $NaNO_2$ generates reactive oxygen species (ROS) and nitric oxide (NO) *in vitro* (Kuroiwa et al., 2007; Okazaki et al., 2006). We found that Total SOD activities were significantly increased by coadministration (Figs. 3 and 4). Studies have demonstrated that strand breaks and oxidative DNA damage are induced by ROS and NO (Paschalis-Thomas et al., 2001; Li et al., 2002). Blenda et al. identified nitrodaidzein and nitrogenistein formation in the reaction mixtures of $ONOO^-$ and isoflavones (Boersma et al., 1999). We also identified

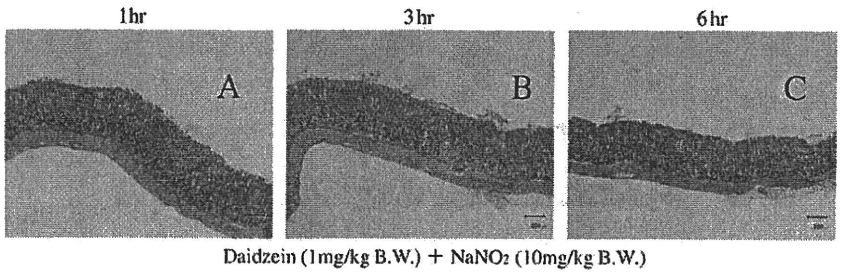

Fig. 7. Histopathological findings of coadministrateion of daidzein (1 mg/kg body weight) and $NaNO_2$ (10 mg/kg body weight). A, B and C showed result of 1, 3 and 6 h after coadministration, respectively.

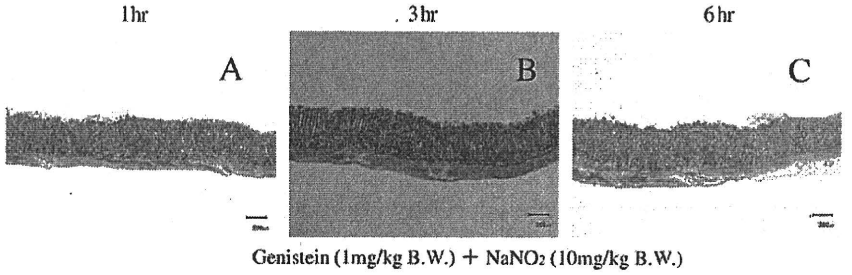

Fig. 8. Histopathological findings of coadministrateion of genistein (1 mg/kg body weight) and $NaNO_2$ (10 mg/kg body weight). A, B and C showed result of 1, 3 and 6 h after coadministration, respectively.

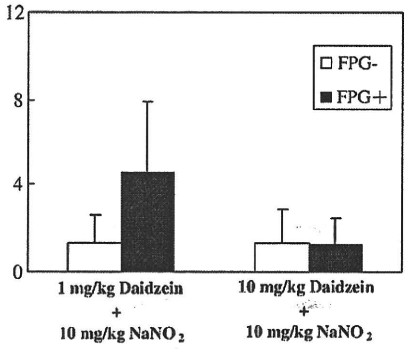

Fig. 9. DNA damage of mouse stomach at 3 h after coadministration of daidzein (1 mg/kg body weight) and $NaNO_2$ (10 mg/kg body weight). Effect of daidzein doses on DNA damage of mouse stomach at 3 h after coadministration of daidzein (1 and 10 mg/kg body weight) and $NaNO_2$ (10 mg/kg body weight). Bars represent SD values. □ showed results of comet assay without FPG (FPG−). On the other hand, ■ showed results of comet assay with FPG (FPG+).

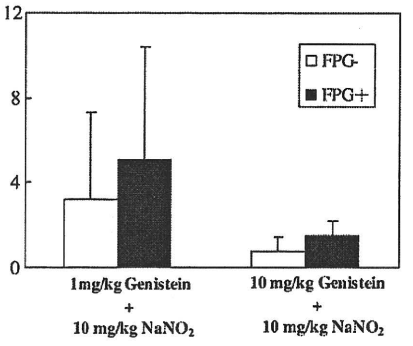

Fig. 10. Effect of genistein doses on DNA damage of mouse stomach at 3 h after coadministration of genistein (1 and 10 mg/kg body weight)and $NaNO_2$ (10 mg/kg body weight). Fifty cells were counted per mouse. The mean values were obtained from 250 cells. Bars represent SD values. □ showed results of comet assay without FPG (FPG−). On the other hand, ■ showed results of comet assay with FPG (FPG+).

2-nitro-daidzein and 2-nitro-genistein in the reaction mixtures of nitrite and isoflavones *in vitro* [data not shown]. The genotoxicity of nitro compounds is generally expressed by nitrate reductase. There are several kinds of reductase, such as NADPH-cytochrome c reductase (Heimbrook and Sartorelli, 1986), xanthine oxidase, DT-diaphorase (Eranster and Navazio, 1958; Ernster, 1967), and other enzymes. Nitro compounds show genotoxic activity through two metabolic pathways (Edwards, 1977). As a result, hydroxyl radicals and hydroxyl amino groups are produced and induce oxidative DNA damage and DNA adducts formation. Therefore, it

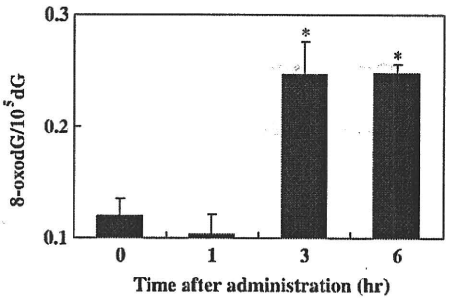

Fig. 11. Concentration of 8-oxodG in mouse stomach at 1, 3 and 6 h after coadministration of daidzein (1 mg/kg body weight) and $NaNO_2$ (10 mg/kg body weight). The mean values were obtained from five animals. Bars represent SD. $^*p < 0.01$ vs. control (0 h: untreated group).

is necessary to evaluate the genotoxic potencies of these nitro compounds.

Figs. 5 and 6 show the induction times of DNA damage after coadministration of $NaNO_2$ and isoflavones. The highest tail moment values were exhibited at 1 h after coadministration of daidzein and $NaNO_2$ compared with other daidzein groups (Fig. 5). On the other hand, the maximum tail moment value in genistein groups was confirmed at 6 h after coadministration of genistein and $NaNO_2$ (Fig. 6). The tail moment value in the daidzein-treated group was greater than that in the genistein-treated group. Chen et al. have found that the radical scavenging activity of daidzein was lower than that of genistein (Chen et al., 2005). As shown in Figs. 9 and 10, coadministered isoflavones and $NaNO_2$ exhibited DNA damaging potencies at a low concentration (1 mg/kg body weight) that were more pronounced than those at a high concentration (10 mg/kg body weight) of isoflavones. From these results, non-reacted isoflavones might be remained in the stomach, and have possibility for showing radical scavenging activities.

8-oxodG is well known as a biomarker of carcinogenicity (Loft and Poulsen, 1996) and oxidative DNA damage (Valavanidis et al., 2009; Shibutani et al., 1991). If 8-oxodG remains unrepaired, it can cause high levels of G:C to T:A transversion mutations (Valavanidis et al., 2009). In order to verify whether DNA damage was increased by 8-oxodG accumulation, we determined the 8-oxodG concentration in the mouse stomach tissue using the HPLC–ECD system. Figs. 11 and 12 show 8-oxodG concentrations in stomach tissue induced by coadministration. It was confirmed that 8-oxodG concentrations peaked at 3 h after coadministration of daidzein and $NaNO_2$ ($p < 0.05$). On the other hand, the peak for the genistein group was reached after 6 h ($p < 0.05$). From these results, the increase in FPG-treated DNA damage was shown to be associated with 8-oxodG production.

In order to clarify the formation mechanism of FPG-sensitive sites, we looked for leukocyte infiltration in stomach. Inflammation

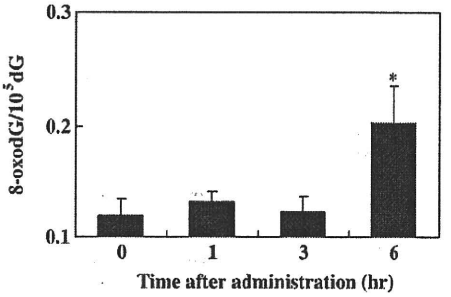

Fig. 12. Concentration of 8-oxodG in mouse stomach at 1, 3 and 6 h after coadministration of genistein (1 mg/kg body weight) and $NaNO_2$ (10 mg/kg body weight). The mean values were obtained from five animals. Bars represent SD. *$p < 0.01$ vs. control (0 h: untreated group).

was not observed in the stomach tissue upon histopathological examination (Figs. 7 and 8). These data suggested that coadministration of both compounds might induce genotoxicity in the stomach by several chemical reactions and metabolic responses *in vivo*.

The general population consumes approximately "12.5–35 mg of $NaNO_2$" and "16.4 mg of daidzein and 30.1 mg of genistein"/day through foodstuffs and water (Archer, 2002; Xia et al., 2003; Kimura et al., 1998; Arai et al., 2000). In our experiment, isoflavones and nitrite intakes were approximately 2–5 or 14–40 times greater than the actual daily intake, respectively. Therefore, simultaneous intake of isoflavones and $NaNO_2$ may not be a risk factor for humans. However, it is possible to induce DNA damage by administration of isoflavones in cases of high concentrations of NO such as in those with a gastric ulcer (Rachmilewitz et al., 1994), and may be a risk factor.

In conclusion, it is thought that DNA damage induced by coadministration of isoflavones and $NaNO_2$ in the mouse stomach results from the induction of ROS and several other factors. Other food constituents might also show similar behaviors and genotoxic activities in the stomach by treatment with nitrite. Therefore, evaluation of the risks of coadministration of other commonly eaten food constituents and nitrite is needed

Conflict of interest

The authors declare that there are no conflict of interest.

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Leaf Extract of *Wasabia japonica* Relieved Oxidative Stress Induced by *Helicobacter pylori* Infection and Stress Loading in Mongolian Gerbils

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Infection with *Helicobacter pylori* (*H. pylori*) can induce gastric disorders, and though its presence cannot explain disease pathogenesis and does not have associations with other factors, it is well known that *H. pylori* infection causes stomach inflammation following oxidative stress. We examined the suppressive effects of a leaf extract of *Wasabia japonica* on *H. pylori* infection and on stress loading in Mongolian gerbils. Following oral administration of wasabi extract of 50 and 200 mg/kg B.W./d for 10 d, the animals were exposed to restraint stress for 90 and 270 min. As for the results, the level of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in the stomach and oxidative DNA damage in peripheral erythrocytes at 270 min significantly increased. That elevation was significantly suppressed by the addition of the leaf extract. We concluded that the simultaneous loading of *H. pylori* infection and physical stress loading might induce oxidative DNA damage additively, while a leaf extract attenuated this DNA damage in the stomach as well as the peripheral erythrocytes.

Key words: *Helicobacter pylori*; Mongolian gerbils; 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG); oxidative DNA damage; *Wasabia japonica*

Helicobacter pylori (*H. pylori*), which infects over half of all people in the world, is one of the most widespread human pathogens in diseases such as chronic gastritis and gastric and duodenal ulcers.[1,2] In 1994, the organism was classified into group I, carcinogenic to humans, by the World Health Organization/International Agency for Research on Cancer (WHO/IARC).[3] Although triple therapy using two antibiotics (amoxicillin and clarithromycin) and a proton pump inhibitor is widely employed in the treatment of *H. pylori*, antibiotic resistance to clarithromycin leads to treatment failure, especially in Asian countries.[4,5]

Adhesion of *H. pylori* to gastric epithelial cells is recognized as one of the essential steps in the development of gastritis, which leads to injection of the definitive virulence factor, cytotoxin-associated antigen A (CagA), through type-IV secretion systems.[6,7] Several reports have suggested that *H. pylori* inoculates CagA into gastric epithelial cells inducing phosphorylations of MEK, Src, and SHP-2, thereby promoting the production of IL-8, IL-1β, and tumor necrosis factor-α (TNF-α).[8] These cytokines play many crucial roles in *H. pylori*-associated gastritis through recruitment, activation, and infiltration of neutrophils into the sites of infection as well as chronic inflammation and gastric injury.[9–11] Reactive oxygen and nitrogen species generated by activated inflammatory cells upon infection can contribute to carcinogenesis through the formation of DNA base lesions, such as 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), a marker of oxidative DNA damage, resulting from G:C-to-T:A transversion.[12] In addition, 8-oxodG is remarkably increased in the gastric epithelium of patients infected with *H. pylori*.[13]

Since the development of *H. pylori*-associated diseases is influenced by a complicated cross talk among the bacteria, the infected host, and the environmental situation, the presence of *H. pylori* itself does not explain fully pathogenesis.[14–18] This is reflected by the fact that many people do not develop *H. pylori* associated diseases, and that *H. pylori*-negative gastric ulcer patients are often found. Hence other factors, such as stress, diet, smoking, sanitation, and host genetic background might contribute to the pathogenesis of *H. pylori* associated diseases. Especially with respect to these factors, psychological stress has been found to trigger many diseases.[19–24] A study done immediately after the great Hanshin Earthquake in 1995 in Kobe,

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Abbreviations: CFU, colony forming units; Fpg, formamidopyrimidine DNA glycosylase; IL, interleukin; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine

Japan, found that the recurrence rate of gastric ulcers in patients infected with *H. pylori* was much higher than that in patients in whom *H. pylori* had been eradicated.[25,26] Hence we hypothesized that a synergic relationship between *H. pylori* infection and psychological stress on ulcer formation might exist.

Wasabi (*Wasabia japonica*) is used as a Japanese traditional spice to avoid both food poisoning and odor. Allyl isothiocyanate is a major pungent component of wasabi root, and is known to have strong anti-microbial activity.[27,28] Recently, other compounds such as polyphenols in wasabi leaves have been identified that exhibit anti-oxidative and anri-*H. pylori* activities.[28–31]

It is widely accepted that the Mongolian gerbil is good model for *H. pylori* infection, because *H. pylori* can easily be inoculated as compared with mice, rats, and others.[32,33] In this study, we examined the effects of a leaf extract of *Wasabia japonica* (Wasabi extract) on oxidative DNA damage induced by *H. pylori* infection and on stress loading in Mongolian gerbils.

Materials and Methods

Animals. Male Mongolian gerbils (13 weeks old, MGS/Sea) infected with *H. pylori* (ATCC43504) were purchased from Seac Yoshitomi (Fukuoka, Japan).[32,33] Thirteen week old male Mongolian gerbils (MON/Jms/Gbs) were purchased from Japan SLC (Shizuoka, Japan). The animals were housed in cages at a temperature 23 ± 2 °C and humidity 55 ± 5% under a 12 h light and dark cycle, and were given Oriental MF® sterilized with γ-rays from Oriental Yeast (Tokyo) and water *ad libitum* throughout the experiment. The animals were handled according to the guidelines of the Committee for Ethics in Animal Experimentation of the University of Shizuoka.

Leaf extract of Wasabia japonica. Leaves of *Wasabia japonica* were kindly provided by Tamaru-ya Honten (Shizuoka, Japan), and extracts were prepared by a method described previously.[29,30] Briefly, fresh leaves (9.0 kg) were extracted with 20 liters MeOH, 3 times at room temperature. After concentration of the solvent with a rotary evaporator *in vacuo*, the concentrate (273.2 g) was suspended in water and treated successively with hexane, EtOAc, and *n*-BuOH to divided it into hexane-, EtOAc-, *n*-BuOH- and water-soluble layers. In this study, a mixture of EtOAc and *n*-BuOH-soluble layers, which exhibited antioxidative activity as previously described,[29,30] suspended in distilled water was used as Wasabi extract.

Experimental methods. Following a pre-feeding period of 1 week, infected and no-infected Mongolian gerbils were divided into four experimental groups by Wasabi extract doses: untreated, 10, 50, and 200 mg/kg B.W./d. The Wasabi extract was administered orally following a 6-h fast every day continuously for 10 d. After the dosing period, the animals of all experimental groups, following a 24-h fast, were exposed to restraint stress for 0, 90, or 270 min. After treatment, the animals were sacrificed, and then the stomach and whole blood were obtained immediately. Then the stomach was opened along the greater curvature, and the intragastric contents were removed gently. To count colony forming units (CFU), half of each stomach was cut finely, homogenized in 7 ml of sterilized saline, followed by serial dilution with the same saline. Aliquots of the diluted homogenate (0.1 ml) were inoculated onto the *Helicobacter* agar plate (Nissui Pharmaceutical, Tokyo). The plates were incubated at 37 °C under microaerophilic conditions for 5 d, and then the colonies were counted to detect the CFU. The degrees of gastric mucosal erosion and hemorrhage were determined by scoring the following parameters: gastric mucosal erosion (0, normal; 1, edematous; 2, erosion; 3, multiple erosion; 4, hemorrhage erosion and/or ulcers larger than 1 mm in diameter); hemorrhage (0, no bleeding; 1, one small bleeding spot; 2, multiple small bleeding spots; 3, one bleeding area; 4, multiple bleeding area).

Table 1. Experimental Groups

H. pylori	Wasabi extract (mg/kg B.W./d)	Restraint time (min)		
		0	90	270
−	0	Group 1	Group 5	Group 8
	10	Group 2	—	—
	50	Group 3	Group 6	Group 9
	100	Group 4	Group 7	Group 10
+	0	Group H1	Group H5	Group H8
	10	Group H2	—	—
	50	Group H3	Group H6	Group H9
	100	Group H4	Group H7	Group H10

Quantification of the level of 8-oxodG. Nuclear DNA isolation was carried out as previously described.[34] Each stomach was homogenized in an ice-cold 0.3 M Sucrose solution. The crude pellets were incubated with proteinase K and 1% SDS/1 mM EDTA (pH 8.0) at 37 °C for 90 min. The solution was mixed with 7 M NaI and isopropyl alcohol, and left to stand at −20 °C for 10 min. The pellet DNA, was centrifuged and rinsed with 70% ethanol. Ribonucleases T_1 and A were added to the withdrawn crude DNA, and the reaction mixture was incubated at 37 °C for 1 h. Then chloroform: isoamyl alcohol (24:1, *v/v*) was added, and the resulting aqueous phase was mixed with 13% PEG solution *w/v* containing 1.6 M NaCl. The DNA obtained was dissolved in water, and hydrolyzed with nuclease P_1 at 37 °C for 30 min, and then with alkaline phosphatase at 37 °C for 1 h. The filtrated DNA was applied to a HPLC (LC-10 pump, Shimadzu, Kyoto, Japan) equipped with a Symmetry C18 column (particle size, 3.5 mm; 4.6 × 100 mm; Waters, Milford, MA). The mobile phase was 12.5 mM citrate buffer (pH 5.1) containing 6% methanol, and the flow rate was 0.8 ml/min. 8-oxodG was measured by electrochemical detection (ECD; ESA Coulochem II 5200, Bedford, MA) using an analytical cell model 5011 (Detector I, 150 mV; Detector II, 350 mV). Oxidative damage to DNA was expressed as the molar ratio of 8-oxodG to 10^5 2-deoxyguanosine (dG). The amount of dG was calculated from the absorption at 260 nm in the same sample as measured with a UV detector.

Comet assay to detect Fpg-sensitive site. Comet assay applied to detect formamidopyrimidine DNA glycosylase (Fpg)-sensitive sites was carried out basically as previously described, with some modification.[35,36] Fpg has *N*-glycosylase and AP-lyase activities and repairs oxidative DNA damage by efficiently removing formamidopyrimidine lesions and 8-oxodG residues from DNA.[37] The slide preparation was based on our previous studies.[35,36] The slides were immersed in lysis solution (2.5 M NaCl, 0.2 M NaOH, 0.1 M EDTA, 0.01 M Tris base, 1% salcosinate, 10% DMSO and 1% Triton X-100, (pH 10)) at 4 °C for 1 h. Treatment with Fpg (Sigma-Aldrich, St. Louis, MO) was carried out as follows: the cells, embedded in agarose, were overlaid with Fpg (1 μg/ml) or Fpg buffer (0.1 M NaCl, 1 mM EDTA, 0.01 M Tris base pH 7.5 and 100 mg/l BSA) at 37 °C for 15 min. The slides were placed in alkali electrophoresis buffer (0.3 M NaOH and 1 mM EDTA). Following electrophoresis, they were washed with neutralizing Tris buffer (0.4 M Tris base pH 7.5) and stained with ethidium bromide solution. Comets in each slide were analyzed using a CCD camera and the Comet Analyzer (YOU WORKS, Tokyo). DNA strand breaks and Fpg-sensitive sites in the DNA of peripheral blood cells were represented by tail moment for 50 comets from one blood sample.

Statistical analysis. Data were expressed as mean ± SD or SE. Statistical significance at the same restraint stress time was evaluated by Tukey's multiple comparison test after one-way ANOVA. Differences were considered significant at $p < 0.05$.

Results

Improvement in lesions of the stomach induced by H. pylori *infection and/or restraint stress by the leaf extract of* Wasabia japonica

In the present study, administration of wasabi extract did not affect food intake, water intake, or body weight (data not shown). The effects of wasabi extract on

stomach weight and CFU are shown in Table 2. After administration of wasabi extract for 10 d, the average stomach weight markedly increased, by 2.0–fold, in group H1 (*H. pylori* infection) as compared with group 1 (no infection). The elevation was significantly less in groups H2 and H3 (10 and 50 mg/kg B.W./d during the experiment), although in group H3 (100 mg/kg) was not effective. On the other hand, the wasabi extract did not cause a reduction in *H. pylori* colonization. The macroscopic findings for gastric mucosa of the animals are shown in Table 3. The gastric mucosal erosion score significantly increased in group H1 (15-fold, *H. pylori* infection) and group 8 (9.0-fold, no infection, 270 min stress loading) as compared with group 1 (no infection). A decrease in the erosion score was observed only in group H9 (*H. pylori* infection, 270 min stress, 50 mg/kg) as compared to that of group H8 (*H. pylori* infection, 270 min stress). The hemorrhage score significantly increased in group H1 (*H. pylori* infection) and group 8 (270 min stress loading) as compared to group 1 (no infection). The score for group H9 (*H. pylori* infection, 270 min stress, 50 mg/kg) showed a 47% decrease as compared with group H8 (*H. pylori* infection, 270 min stress).

Table 2. Suppressive Effects of Leaf Extract of *Wasabia japonica* on Stomach Weight and CFU with and without *H. pylori* Infection

	H. pylori	Wasabi extract (mg/kg B.W./d)	Restraint time (min) 0
Stomach weight (g/100 g B.W.)	−	0	0.87 ± 0.03^{c}
		10	0.88 ± 0.07^{c}
		50	0.90 ± 0.02^{c}
		100	0.88 ± 0.06^{c}
	+	0	1.70 ± 0.07^{a}
		10	1.52 ± 0.06^{b}
		50	1.54 ± 0.05^{b}
		100	$1.61 \pm 0.07^{a,b}$
CFU (log of CFU/Stomach)	+	0	5.03 ± 0.35
		10	5.31 ± 0.37
		50	4.98 ± 0.50
		100	5.04 ± 0.67

Values are expressed as means ± SD. Statistical significance at the same restraint stress time was evaluated using Tukey's multiple comparison test after one-way ANOVA. Means without a common letter differ, $p < 0.05$. CFU, Colony forming unit.

Suppressive effects of leaf extract of Wasabia japonica *on the level of 8-oxodG in the stomach*

The concentrations of 8-oxodG in the stomach are shown in Table 4. Compared with group 1 (no infection), group H1 (*H. pylori* infection), and group 8 (270 min stress loading), they showed 2.4 and 2.7-fold increases in the level of 8-oxodG. Group H3 (*H. pylori* infection, 50 mg/kg) exhibited a 34% decrease in 8-oxodG the level as compared with group H1 (*H. pylori* infection). 50 and 100 mg/kg Wasabi extract (groups 9 and 10) decreased the level of 8-oxodG by 32–41% as compared with group 8 (no infection, 270 min stress). Group H8 (*H. pylori* infection, 270 min stress) showed a significant 1.4-fold increase as compared with group H1 (*H. pylori* infection), but the leaf extract decreased the levels of 8-oxodG by 41–48% (groups H9 and 10).

Suppressive effects of the leaf extract of the Wasabia japonica *on oxidative DNA damage in whole blood*

The levels of the DNA strand break and the Fpg-sensitive site of the whole blood are shown in Table 5. Oxidative DNA damage in group H1 (*H. pylori* infection) showed a 4.6-fold increase as compared with group 1 (no infection), with statistical significance, but the leaf extract decreased by 27% the oxidative DNA damage (group H3 and H4). Oxidative damage significantly increased in group H8 (*H. pylori* infection, 270 min stress) as compared with group H1 (*H. pylori* infection). Furthermore, oxidative damage showed a 1.3-fold increase under *H. pylori* infection and 270 min stress loading (group H8) as compared with group H1 (*H. pylori* infection), but the leaf extract suppressed the damage by 26–33% (groups H9 and H10).

Discussion

Previous reports have indicated the influence of *H. pylori* infection on the development of stress-induced gastric mucosal injury in animal models and humans.[26,38] Despite exhibiting the close relationship between *H. pylori* infection and stress, as described

Table 3. Suppressive Effects of Leaf Extract of *Wasabia japonica* on Scores of Gastric Mucosal Erosion and Hemorrhage with and without *H. pylori* Infection

	H. pylori	Wasabi extract (mg/kg B.W./d)	Restraint time (min) 0	90	270	Two-way ANOVA
Gastric mucosal erosion	−	0	0.2 ± 0.4^{b}	0.8 ± 0.8^{b}	1.8 ± 0.4^{b}	R, W
		50	0.0 ± 0.0^{b}	1.2 ± 0.8^{b}	2.2 ± 1.1^{b}	
		100	0.8 ± 0.8^{b}	3.0 ± 0.7^{a}	2.8 ± 1.1^{b}	
	+	0	3.0 ± 0.0^{a}	3.6 ± 0.5^{a}	4.0 ± 0.0^{a}	W, R × W
		50	2.2 ± 0.4^{a}	2.6 ± 0.9^{a}	2.0 ± 0.0^{b}	
		100	3.2 ± 0.4^{a}	3.2 ± 0.4^{a}	3.4 ± 0.5^{a}	
Hemorrhage	−	0	0.0 ± 0.0^{b}	0.8 ± 0.8^{b}	1.4 ± 1.5	R
		50	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	1.4 ± 1.5	
		100	0.0 ± 0.0^{b}	0.2 ± 0.4^{b}	1.4 ± 1.9	
	+	0	3.0 ± 1.0^{a}	3.0 ± 1.4^{a}	3.2 ± 0.4	W
		50	3.0 ± 0.0^{a}	$2.2 \pm 1.6^{a,b}$	1.7 ± 0.8	
		100	3.0 ± 0.7^{a}	3.8 ± 0.4^{a}	2.8 ± 0.8	

Values are expressed as means ± SD. Two-way ANOVA was separately done with and without *H. pylori*. Statistical significance at the same restraint stress time was evaluated by Tukey's multiple comparison test after one-way ANOVA. Means without a common letter differ, $p < 0.05$. R, restraint time; W, Wasabi extract.

Table 4. Effects of Leaf Extract of *Wasabia japonica* on Amounts of 8-oxodG in Gastric Mucosa with and without *H. pylori* Infection

	H. pylori	Wasabi extract (mg/kg B.W./d)	Restraint time (min) 0	90	270	Two-way ANOVA
8oxodG/10^5dG	−	0	0.329 ± 0.038^{c}	0.424 ± 0.171^{b}	0.880 ± 0.142^{b}	R, W, R × W
		50	0.329 ± 0.020^{c}	0.333 ± 0.019^{b}	0.595 ± 0.157^{c}	
		100	0.429 ± 0.058^{c}	0.496 ± 0.102^{b}	0.509 ± 0.148^{c}	
	+	0	0.807 ± 0.051^{a}	0.967 ± 0.053^{a}	1.148 ± 0.020^{a}	R, W, R × W
		50	$0.535 \pm 0.051^{b,c}$	0.519 ± 0.031^{b}	$0.676 \pm 0.028^{b,c}$	
		100	$0.589 \pm 0.038^{a,b}$	0.666 ± 0.021^{b}	0.599 ± 0.091^{c}	

Values are expressed as means ± SD. Two-way ANOVA was separately done with and without *H. pylori*. Statistical significance at the same restraint stress time was evaluated by Tukey's multiple comparison test after one-way ANOVA. Means without a common letter differ, $p < 0.05$. R, restraint time; W, Wasabi extract.

Table 5. Effects of Leaf Extract of *Wasabia japonica* on Fpg-Sensitive Sites in DNA of Whole Blood with and without *H. pylori* Infection

	H. pylori	Wasabi extract (mg/kg B.W./d)	Restraint time (min) 0	90	270	Two-way ANOVA
Fpg (−)	−	0	$1.02 \pm 0.06^{a,b}$	0.86 ± 0.05^{b}	$1.45 \pm 0.12^{a,b}$	R, W, R × W
		50	0.70 ± 0.05^{b}	0.85 ± 0.05^{b}	0.88 ± 0.05^{c}	
		100	0.70 ± 0.04^{b}	0.70 ± 0.04^{b}	$1.16 \pm 0.07^{b,c}$	
	+	0	1.46 ± 0.20^{a}	1.38 ± 0.10^{a}	1.57 ± 0.16^{a}	R
		50	1.38 ± 0.16^{a}	1.78 ± 0.09^{a}	1.77 ± 0.11^{a}	
		100	1.39 ± 0.14^{a}	1.47 ± 0.10^{a}	$1.52 \pm 0.09^{a,b}$	
Fpg (+)	−	0	1.40 ± 0.08^{c}	1.63 ± 0.11^{c}	2.98 ± 0.21^{c}	R, W, R × W
		50	0.97 ± 0.06^{c}	1.30 ± 0.11^{c}	1.85 ± 0.15^{c}	
		100	1.27 ± 0.09^{c}	1.46 ± 0.14^{c}	1.94 ± 0.16^{c}	
	+	0	6.38 ± 0.47^{a}	6.90 ± 0.49^{a}	8.18 ± 0.63^{a}	R, W
		50	4.68 ± 0.35^{b}	5.45 ± 0.41^{b}	6.01 ± 0.40^{b}	
		100	4.58 ± 0.35^{b}	5.30 ± 0.37^{b}	5.44 ± 0.29^{b}	

Fifty cells were used per Mongolian gerbil. Mean values were obtained for 250 cells. The values are expressed as means ± SEM. Two-way ANOVA was separately done with and without *H. pylori*. Statistical significance at the same restraint stress time was evaluated by Tukey's multiple comparison test after one-way ANOVA. Means without a common letter differ, $p < 0.05$. R, restraint time; W, Wasabi extract.

above, few trials for prevention, especially in phytochemicals, has been yet to be reported. In this study, development of edema, erosions, ulcers, and hemorrhage spots in the gastric mucosa were observed after infection for several months (Table 2), but the mechanism and causal factor contributing to aggravation of gastric injury induced by physiological stress in a presence of *H. pylori* remain unknown. We suggested that oxidative stress was responsible for *H. pylori* and stress-associated gastric injury, since treatment by an antioxidant potently prevented *H. pylori* and stress-induced oxidative DNA damage.

In this study, we found no effect on *H. pylori* colonization under treatment with wasabi extract for 10 d. Adhesion of *H. pylori* to gastric epithelial cells is one of the initial steps in gastric inflammation. Recent studies have provided evidence that chronic inflammation that follows oxidative stress caused by *H. pylori* infection plays a critical role in the development of gastric cancer.[39] Ohshima *et al.* reported that 8-oxodG and 8-nitroguanine as markers of DNA damage were stored in patients infected with *H. pylori*.[13] Although in gastritis and gastric ulcer patients infected with *H. pylori* NADPH oxidase and inducible nitric oxide synthease (iNOS) expression as an effect of infiltrating inflammatory cells has been observed, eradication of *H. pylori* attributed to decrease these cells invasion and iNOS expression in the gastric mucosa.[40,41] In the present study, wasabi extract did not effect *H. pylori* colonization, but oxidative DNA damage was decreased in the stomach and whole blood (Tables 4 and 5). In this context, a previous report suggested that antioxidant α-tocopherol protects against gastric injury, although the presence of *H. pylori* caused significant deterioration of stress-induced gastric mucosal lesions as a result of increasing oxidative stress.[42] In sum, antioxidant treatment decreased the risk of gastric injury due to oxidative stress.

Our previous study indicated that wasabi extract showed anti-oxidative and anti-*H. pylori* activities *in vitro* and stronger activity than the root extract.[31] In this study, we found wasabi extract to decrease the level of oxidative stress caused by both *H. pylori* infection and stress loading in Mongolian gerbils. Although major components of the root are isothiocyanates (allyl, 6-methylsulfinylhexyl, and other isothiocyanates), isothiocyanates in the leaf accounted for one-third as compared with the root.[28] These data indicate that other components were associated with these activities. In this context, recently Hosoya *et al.* reported that wasabi extract consisted of flavonoids and its glycosides, such as apigenine, luteoline, and isovitexin (the major component).[29,30] Isovitexin is one of the antioxidants involved in natural products such as rice hulls and is known for various biological activities, for example, anti-oxidative and anti-inflammatory effects.[43,44] These effects contributed to the scavenging superoxide anions, inhibited NO production, and freed of transcriptional control of cyclooxygenase (COX)-2, a mediator of inflammation. On the other hand, we have reported that

a leaf extract of *Wasabia japonica* inhibited the urease activity of *H. pylori*.[28] Urease accounts for 5–10% of bacterial whole protein and is expressed in most if not all *H. pylori* strains.[45] This enzyme catalyzes the hydrolysis of urea into ammonia and carbon dioxide, and its most important role is to protect the bacteria from the acidic conditions of the stomach by neutralization.[46] Therefore, *H. pylori* urease is considered to be essential for bacterial colonization. Furthermore, ammonia produced by urease of *H. pylori* reacts with hypochlorous acid (HOCl) to generate monochrolamine (NH_2Cl), stronger in DNA damage potency than HOCl.[47,48] In other words, the leaf extract partly suppresses not only the stability of *H. pylori* colonization inhibiting urease activity, but also oxidative DNA damage.

Eradication therapy against *H. pylori* is fairly successful in many cases, although sometimes treatment failure occurs due to an antibiotic resistant strain.[4,5] Therefore it is important to seek a non-antibiotic therapy that is not only highly effective but also non-harmful to humans, and to examine the suppression of chronic gastritis in high-risk areas including Japan. In conclusion, our data suggest that it is likely to be able to suppress oxidative DNA damage derived from reactive oxygen and nitrogen species induced by *H. pylori* infection and stress loading by treatment with antioxidants.

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Body iron store as a predictor of oxidative DNA damage in healthy men and women

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While iron plays an important role in many cellular functions, excess iron storage induces DNA damage by generating hydroxyl radicals and thus promotes carcinogenesis. However, it remains unclear whether body iron levels that are commonly observed in a general population are related to oxidative DNA damage. We examined the association between serum ferritin concentrations and levels of urinary 8-hydroxydeoxyguanosine (8-OHdG), a biomarker of systemic oxidative DNA damage and repair, in 528 Japanese men and women aged 21–67 years. Men had much higher ferritin levels than in women, and the levels were significantly greater in women aged 50 years or older than in women aged less than 50 years. Urinary 8-OHdG concentrations were significantly and positively associated with serum ferritin levels in all the subgroups. The Spearman rank correlation coefficients were 0.47, 0.76, and 0.73 for men overall, women aged less than 50 years, and women aged 50 years or older, respectively. These associations were materially unchanged after adjustment for potential confounding variables. In men, a more pronounced association was observed in nonsmokers than in smokers. Our results suggest body iron storage is a strong determinant of levels of systemic oxidative DNA damage in a healthy population. (*Cancer Sci* 2010; 101: 517–522)

Iron plays an important role in cellular metabolism and aerobic respiration. In healthy adults, 1 to 2 mg of dietary iron is absorbed a day, and body iron is distributed between blood (~3000 mg, mostly as hemoglobin), liver (~1000 mg, mostly as ferritin), skeletal muscle (~300 mg), and macrophages (~600 mg).[1] Besides, iron generates hydroxyl radicals according to the Fenton reaction *in vivo*,[2] and thus has been hypothesized to promote carcinogenesis through lipid peroxidation and oxidative DNA and protein damage.[3] In experimental animals,[4] excess intake of heme iron induces the formation of radicals and the occurrence of colon cancer. In humans, high dietary intake of heme iron[5,6] and blood measurements of iron[7–10] have been shown to be associated with an increased risk of cancer. More recently, a randomized control trial found that phlebotomy, accompanied by a considerable reduction in serum ferritin levels, significantly decreased risk of cancer in men with a peripheral arterial disease.[11] Such evidence suggests that cancer risk may vary according to body iron status even at levels commonly observed among the general population who do not have iron metabolic disorders. However, epidemiologic evidence regarding iron and cancer is far from consistent[12,13] and the finding from the above-mentioned trial should be interpreted cautiously because cancer was not the primary outcome.[14] Investigations linking body iron to biomarkers of carcinogenesis may provide data to support or refute whether iron level currently admitted as normal influences cancer risk.

Urinary 8-hydroxydeoxyguanosine (8-OHdG) is a reliable biomarker of systemic oxidative DNA damage.[2] Further, epidemiologic studies have shown that urinary 8-OHdG concentrations can predict cancer risk.[15–17] However, few studies have been performed to quantitate 8-OHdG levels in association with body iron status. Nakano *et al.* reported a positive correlation between serum ferritin concentrations and urinary 8-OHdG levels in 2507 healthy men and women.[18] However, they did not control for smoking and body mass index, factors known to be associated with 8-OHdG levels.[19,20] In a small study of 48 mild dyslipidemic men, Tuomainen *et al.* demonstrated a linear, positive relationship between serum ferritin and urinary 8-OHdG with adjustment for smoking, body mass index, and physical activity.[21] To further explore this issue, the present study examined the association between serum ferritin concentrations, a marker of body iron storage[22] and urinary 8-OHdG levels in healthy men and women while adjusting for potential confounding factors.

Materials and Methods

Study participants. In 2006, a health survey was conducted among employees of two municipal offices in north-eastern Kyushu, Japan.[23] At the time of routine health check-up, all full-time workers ($n = 601$) except those on long sick-leave or maternity-leave were invited; of these, 547 subjects (323 men and 224 women aged 21–67 years) participated (response rate, 91%). Prior to the examination, participants completed a questionnaire on lifestyle including smoking, alcohol drinking, diet, and exercise, which was then checked by research staff for completeness and, where necessary, clarified by asking the subject. Participants were also asked to donate blood and urine specimens. We excluded 13 subjects with missing information on 8-OHdG and ferritin concentrations, body mass index, and smoking status. Furthermore, those who reported they had cancer (one with thyroid cancer and two with breast cancer) or other diseases that affect serum ferritin levels (one with anemia and two with chronic liver disorder) were also excluded. Finally, 528 subjects (313 men and 215 women) remained for the present analyses. The ethics committee of the International Medical Center of Japan approved the protocol of the study, and written informed consent was obtained from each participant.

Measurement of urinary 8-OHdG. Urinary 8-OHdG and creatinine were determined by a method previously described.[24] In short, a human urine sample was mixed with the same volume of a dilution solution containing the ribonucleoside marker 8-hydroxyguanosine. A 20-μL aliquot of the diluted urine sample

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was injected into HPLC-1 (MCI GEL CA08F, 7 μm, 1.5 × 120 mm; elution, 2% acetonitrile in 0.3 mM sulfuric acid, 50 μL/min, 65°C), via the guard column (1.5 × 40 mm), and the chromatograms were recorded by a Gilson UV detector (UV/VIS-155 with 0.2 mm light path cell). Creatinine was detected at 245 nm. The 8-OHdG fraction was collected, depending on the relative elution position from the peak of the added marker, 8-hydroxyguanosine, and was automatically injected into the HPLC-2 column. The 8-OHdG fraction was fractionated by the HPLC-2 column (Capcell Pak C18, Shiseido, Tokyo, Japan; 5 μm, 4.6 × 250 mm; elution, 10 mM sodium phosphate buffer [pH 6.7] containing 5% methanol and an antiseptic Reagent MB [100 μL/L], 1 mL/min, 40°C). The 8-OHdG was detected by a Coulochem II EC detector (ESA, Chelmsford, MA, USA) with a guard cell (5020) and an analytical cell (5011) (applied voltage: guard cell, 350 mV; E1, 170 mV; E2, 300 mV). The accuracy of the measurement, estimated from the recovery of an added 8-OHdG standard, was 90–98%. When the same urine sample was analyzed three times, the variation of the data was within 7%. 8-OHdG levels were adjusted for urinary creatinine levels before statistical analysis.

Measurement of serum ferritin. From each individual, 9 mL of venous blood was drawn in a vacuum blood collection tube and carried to our laboratory in a cooled box. Blood was centrifuged for 15 min and the serum separated was stored in a maximum of six tubes (0.5 mL each) at −20°C until analysis. Serum ferritin concentrations were measured by chemiluminescence immunoassay on the Bayer ADVIA Centaur at an external laboratory (Mitsubishi Chemical Medicine, Tokyo, Japan).

Other variables. Body height was measured to the nearest 0.1 cm with the subject standing without shoes. Body weight in light clothes was measured to the nearest 0.1 kg. Body mass index (BMI) was calculated as the body weight (kg) divided by the square of body height (m). Smoking status and alcohol intake were self-reported in the lifestyle questionnaire. Participants were asked about weekly hours of leisure-time physical activity engaged in for each of the four activities: strolling or walking; mild exercise; moderate intensity exercise; strong intensity exercise. Weekly minutes for walking or cycling while commuting to and from the work were also ascertained. Average metabolic equivalent task (MET) values were assigned to each level of activity according to the intensity of exercise, and total MET-hours per week was estimated by summing all of the values for each participant. Dietary habit for the preceding month was assessed with a brief self-administered diet history questionnaire.[25] Intakes of iron, vitamin C, and vitamin E were estimated by an ad hoc computer algorithm, and their energy-adjusted values (per 1000 kcal) were used for analysis. Blood hemoglobin was measured by sodium lauryl sulfate–hemoglobin method, serum iron was determined by colorimetric assay, and red blood cells were counted by automated blood counting machine at an external laboratory.

Statistical analysis. Median and inter-quartile range of serum ferritin, urinary 8-OHdG, and blood hemoglobin concentrations were calculated according to age (<35, 35–49, or ≥50 years), smoking status (nonsmoker, quitter, smoking 1–19 cigarettes/day, or smoking ≥20 cigarettes/day), BMI (tertile), ethanol consumption (0, 0.1–19.9, 20–39.9, or ≥40 g/day), physical activity (0, 0.1–4.9, 5–9.9, or ≥10 MET-h/week), vitamin C (tertile), and vitamin E (tertile), and the difference between groups was assessed using the Wilcoxon two-sample test. The Spearman rank correlation coefficient was calculated to assess the association between serum ferritin and urinary 8-OHdG concentrations. In women, because serum ferritin concentrations considerably increase after menopause,[26] analyses were done separately for those aged less than 50 years, and 50 years or older, with reference to data regarding the mean age of menopause in Japanese women (48.3 years old).[27]

Both ferritin and 8-OHdG concentrations were log-transformed for the following parametric analyses. The geometric mean and its 95% confidence interval of urinary 8-OHdG concentrations were calculated for each tertile of the serum ferritin levels for the three groups: men, women aged less than 50 years, and women aged 50 years or older. To control the effects of potential confounding variables, we performed three types of analysis. In Model 1, we adjusted for age (continuous), smoking status (nonsmoker or smoker), and BMI (continuous). In Model 2, we additionally adjusted for hemoglobin levels (continuous). In Model 3, we adjusted for alcohol consumption (0, 0.1–19.9, 20–39.9, or ≥40 g/day), physical activity (0, 0.1–4.9, 5–9.9, or ≥10 MET-h/week), vitamin C intake (tertile), and vitamin E intake (tertile) in addition to the covariates in Model 1. Trend association was evaluated by assigning 1–3 to the lowest through highest tertile categories of ferritin concentrations. Because smoking is a known, consistent determinant of urinary 8-OHdG concentrations,[28] analysis was repeated by smoking status in men. Statistical tests were two-sided and regarded as statistically significant at P-value <0.05. Analysis was done with STATA SE version 10.0 (Lakeway Drive College Station, TX, USA).

Results

Table 1 presents medians of urinary 8-OHdG and serum ferritin concentrations according to age, smoking, and BMI for women and men. There was no significant difference in 8-OHdG concentration between women and men (2.95 *vs* 3.10 μg/g creatinine, $P = 0.45$), although women showed a greater variation of 8-OHdG concentrations than did men. In women, those aged 50 years or older had significantly higher 8-OHdG levels than those aged less than 50 years (3.35 *vs* 2.90, $P = 0.043$). Median serum ferritin concentration markedly differed among the three groups ($P < 0.001$): 24.9, 51.2, and 130 ng/mL for women under 50 years, women aged 50 years or older, and men, respectively. In men, smokers had significantly higher 8-OHdG ($P < 0.001$) and ferritin concentrations ($P = 0.042$) than nonsmokers. Blood hemoglobin levels did not appreciably differ according to demographic and lifestyle factors except smoking; heavy smokers showed a higher mean of hemoglobin levels than nonsmokers in men. In both women and men, ferritin concentrations tended to increase with BMI. In men, 8-OHdG levels decreased as BMI increased (P for trend = 0.01) but tended to increase with increasing intake of vitamin C. In women, both serum ferritin and 8-OHdG levels were significantly higher in the highest category of vitamin C intake or physical activity than in the lowest category of the respective variable.

Serum ferritin concentrations were significantly and positively correlated with urinary 8-OHdG concentrations in both women and men (Fig. 1), with the Spearman rank correlation coefficient being 0.47, 0.76, and 0.73 for men, women aged less than 50 years, and women aged 50 years or older, respectively. In men, the coefficient was 0.52, 0.52, 0.45, and 0.31 for nonsmokers, quitters, 1–19 cigarettes/day, and 20 or more cigarettes/day, respectively. Meanwhile, regression coefficients of log-transformed ferritin (ng/mL) on log-transformed 8-OHdG (μg/g creatinine) were 0.748, 0.737, and 0.546, for women under 50 years, women aged 50 years or older, and men, respectively.

As Table 2 shows, the geometric mean of urinary 8-OHdG concentrations increased steadily as serum ferritin levels increased in all the three groups (P for trend <0.001), and this association was materially unchanged after adjustment of potential confounders. Of all the subgroups divided by sex, age, and serum ferritin levels, the highest unadjusted geometric mean of 8-OHdG concentrations was recorded in the highest tertile of ferritin among women aged 50 years or older (4.87 μg/g creatinine), whereas the lowest mean was observed in the lowest

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Table 1. Description of study participants (n = 528)

	n	Urinary 8-OHdG concentrations (μg/g creatinine)	Serum ferritin concentrations (ng/mL)	n	Blood hemoglobin levels (g/dL)†
Women (n = 215)					
Age (years)					
<35	72	2.84 (2.17–3.85)	24.4 (12.3–44.3)	72	13.2 (12.5–14.0)
35–49	82	3.09 (2.04–4.03)	25.5 (12.5–54.8)	82	13.2 (12.7–13.6)
≥50	61	3.35 (2.37–4.84)	51.2 (20.8–119.0)**	61	13.3 (12.8–14.1)
Smoking					
Nonsmoker	208	2.99 (2.20–4.20)	28.9 (14.0–56.9)	208	13.2 (12.7–13.8)
Quitter	3	2.75 (2.58–2.77)	24.0 (11.8–57.5)	3	13.1 (12.7–14.4)
Current smoker	4	2.76 (1.76–5.60)	24.3 (9.1–82.3)	4	14.3 (13.4–15.2)
BMI (kg/m^2)					
<18.5	46	2.64 (2.10–3.43)	22.5 (11.6–46.6)	46	13.1 (12.5–13.9)
18.5–21.9	106	3.13 (2.32–4.22)	29.1 (16.4–54.9)	106	13.1 (12.5–13.6)
≥22	63	3.05 (2.15–4.42)	35.5 (13.8–93.7)	63	13.5 (12.9–14.1)
Alcohol (ethanol consumption, g/day)					
0	69	2.91 (2.12–4.42)	29.3 (10.9–57.3)	69	13.1 (12.4–14.0)
0.1–19.9	131	2.95 (2.20–4.00)	27.9 (13.9–56.4)	131	13.2 (12.7–13.7)
20–39.9	15	3.07 (2.83–3.95)	32.7 (19.7–129.0)	15	13.6 (12.8–14.0)
Physical activity involved in leisure time exercise and commuting (MET-h/week)					
0	110	2.90 (2.20–4.07)	29.1 (13.9–52.7)	110	13.2 (12.7–13.9)
0.1–4.9	64	3.13 (2.17–4.02)	29.1 (14.3–68.7)	64	13.2 (12.7–13.8)
5–9.9	23	2.82 (2.26–3.95)	19.8 (10.0–43.9)	23	13.0 (12.1–13.8)
≥10	17	3.16 (2.76–4.59)	67.2 (20.8–115.0)*	17	13.4 (12.8–13.6)
Vitamin C consumption (mg/1000 kcal)‡					
<62	71	2.89 (2.39–3.80)	29.3 (13.8–52.7)	71	13.1 (12.4–13.6)
62–83.9	70	2.74 (1.89–4.06)	21.2 (8.9–44.0)	70	13.1 (12.5–13.7)
≥84	72	3.22 (2.31–4.40)	40.8 (21.2–101.1)**	72	13.4 (12.9–14.0)
Vitamin E consumption (mg/1000 kcal)					
<4.13	72	2.84 (2.06–3.80)	27.6 (12.2–52.1)	72	13.1 (12.4–14.0)
4.13–4.949	71	3.10 (2.15–4.18)	29.3 (14.7–57.3)	71	13.2 (12.7–13.8)
≥4.95	70	3.09 (2.29–4.26)	29.8 (13.9–79.4)	70	13.2 (12.7–13.8)
Men (n = 313)					
Age (years)					
<35	76	3.19 (2.55–3.86)	130.5 (78.4–191.0)	57	15.5 (15.1–16.1)
35–49	111	3.33 (2.41–4.28)	139.0 (90.2–232.0)	111	15.6 (15.1–16.2)
≥50	126	3.05 (2.43–3.72)	124.0 (75.7–196.0)	126	15.3 (14.8–16.1)
Smoking (cigarettes/day)					
Nonsmoker	113	2.92 (2.24–3.92)	120.0 (71.0–205.0)	102	15.4 (14.9–16.0)
Quitter	62	3.00 (2.37–3.60)	124.5 (83.3–181.0)	59	15.4 (14.9–15.9)
1–19	43	3.30 (2.79–4.27)*	128.0 (74.6–188.0)	38	15.4 (14.9–16.2)
≥20	95	3.38 (2.71–4.19)**	153.0 (93.0–249.0)*	95	15.7 (15.0–16.3)*
BMI(kg/m^2)					
<22	110	3.36 (2.63–4.12)	113.0 (79.2–184.0)	97	15.5 (14.9–16.1)
22–24.9	102	3.16 (2.43–3.96)	135.0 (78.0–198.0)	100	15.3 (14.9–16.0)
≥25	101	2.97 (2.35–3.77)*	156.0 (84.3–261.0)*	97	15.6 (14.9–16.2)
Alcohol (ethanol consumption, g/day)					
0	44	2.95 (2.33–3.78)	112.0 (65.5–183.0)	42	15.5 (14.9–16.1)
0.1–19.9	166	3.09 (2.52–4.14)	135.0 (78.4–195.0)	154	15.5 (14.9–16.1)
20–39.9	71	3.29 (2.43–3.96)	134.0 (98.1–249.0)*	67	15.4 (14.9–16.3)
≥40	32	3.24 (2.47–3.77)	135.0 (92.9–233.0)*	31	15.7 (15.3–16.3)
Physical activity involved in leisure time exercise and commuting (MET-h/week)					
0	108	3.32 (2.47–3.99)	131.5 (85.3–210.0)	101	15.5 (15.0–16.2)
0.1–4.9	82	2.89 (2.41–4.16)	143.5 (83.9–206.0)	79	15.6 (14.9–16.2)
5–9.9	42	3.01 (2.48–3.54)	146.0 (78.1–280.0)	40	15.6 (14.8–16.3)
≥10	79	3.19 (2.29–4.14)	120.0 (76.0–186.0)	72	15.3 (14.8–16.1)
Vitamin C consumption (mg/1000 kcal)‡					
<41	100	3.06 (2.40–3.67)	121.0 (83.5–189.0)	93	15.5 (14.9–16.1)
41–59.9	102	3.00 (2.37–3.90)	135.0 (88.4–211.0)	99	15.4 (14.9–16.3)
≥60	108	3.32 (2.60–4.18)*	134.0 (76.4–214.0)	99	15.5 (15.0–16.1)
Vitamin E consumption (mg/1000 kcal)					
<3.42	102	3.18 (2.42–3.90)	125.0 (83.7–212.0)	97	15.6 (14.9–16.2)
3.42–4.119	103	3.05 (2.43–4.06)	127.0 (83.6–187.0)	94	15.5 (15.0–16.2)
≥4.12	105	3.13 (2.50–3.99)	139.0 (76.0–224.0)	100	15.5 (14.9–16.0)

Values are median (inter-quartile range) unless otherwise stated. *P < 0.05, **P < 0.01 as compared with those in the lowest tertile (age, BMI, alcohol, physical activity, vitamin C and E) or with nonsmokers (smoking). †Blood hemoglobin has 19 missing values for men less than 35 years old. ‡Vitamin C and vitamin E have two missing values for women and three missing values in men. 8-OHdG, 8-hydroxydeoxyguanosine; BMI, body mass index; MET, metabolic equivalent task.