Original Article

Preparation of an experimental mouse model lacking selenium-dependent glutathione peroxidase activities by feeding a selenium-deficient diet

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Relatively young (4-week-old) selenium deficient (SeD) mice, which lack the activity of selenium-dependent glutathione peroxidase (GSH-Px) isomers, were prepared using torula yeast-based SeD diet. Mice were fed the torula yeast-based SeD diet and ultra-pure water. Several different timings for starting the SeD diet were assessed. The weekly time course of liver comprehensive GSH-Px activity after weaning was monitored. Protein expression levels of GPx1 and 4 in the liver were measured by Western blot analysis. Gene expression levels of GPx1, 2, 3, 4, and 7 in the liver were measured by quantitative real-time PCR. Apoptotic activity of thymocytes after hydrogen peroxide (H_2O_2) exposure was compared. Thirty-day survival rates after whole-body X-ray irradiation were estimated. Pre-birth or right-after-birth starting of the SeD diet in dams was unable to lead to creation of SeD mice due to neonatal death. This suggests that Se is necessary for normal birth and healthy growing of mouse pups. Starting the mother on the SeD diet from 2 weeks after giving birth (SeD-trial-2w group) resulted in a usable SeD mouse model. The liver GSH-Px activity of the SeD-trial-2w group was almost none from 4 week olds, but the mice survived for more than 63 weeks. Protein and gene expression of GPx1 was suppressed in the SeD-trial-2w group, but that of GPx4 was not. The thymocytes of the SeD-trial-2w group were sensitive to H_2O_2-induced apoptosis. The SeD-trial-2w group was sensitive to whole-body X-ray irradiation compared with control mice. The SeD-trial-2w model may be a useful animal model for H_2O_2/hydroperoxide-induced oxidative stress.

***Key Words*: glutathione peroxidase, hydrogen peroxide, selenium-deficiency, radio-sensitivity, mouse**

Selenium (Se), which is an essential trace element, has important roles in the biological redox regulation system as the catalytic center of several isozymes of the glutathione peroxidase (GSH-Px or occasionally abbreviated as GPx) family.[1] GSH-Px family enzymes can reduce hydrogen peroxide (H_2O_2) to water at the expense of the reduced form glutathione (GSH). There are four major Se-dependent GSH-Px isozymes, which contain selenocysteine as the active center, in mammalian tissues, which are classical GSH-Px isozyme (GPx1), gastrointestinal isozyme (GPx2), plasma isozyme (GPx3), and phospholipid hydroperoxide isozyme (PHGPx or GPx4).[2] PHGPx, which is a membrane specific subclass of GSH-Px, can reduce phospholipid hydroperoxide to the corresponding alcohol in membrane.[3] Four other GSH-Px members, GPx5, 6, 7, and 8, were recently reported in mammals. GPx6 is a Se-dependent isozyme, whereas GPx5, 7, and 8, which contain cysteine as their active center, are non-Se-dependent isozymes.[4] Higher peroxidase activity is observed by Se-dependent isozymes, but non-Se-dependent isozymes exhibit relatively low peroxidase activity.[4] GPx7 is another type of PHGPx, also known as NPGPx.[5]

A Se-deficient (SeD) rat model, which was prepared by feeding a torula yeast-based SeD diet, was proposed as an oxidative stress model.[6] In this model, pregnant Wistar rats on the 15th day of pregnancy were fed the SeD diet. Newly born rats were kept with their own mother for four weeks until weaning. The young weaned rats were then fed the SeD diet until the experiments. This SeD rat preparation method leads to the creation of 4-week-old weaned rats with Se deficiency and almost no detectable GSH-Px activity.[7]

Se deficiency causes malfunction of Se-dependent GSH-Px isozymes, i.e., GPx1–4 and 6, and can cause oxidative stress due to increased H_2O_2.[8] However, the oxidative stress in Se deficiency is not lethal to rats, in contrast to the marked inactivation of comprehensive GSH-Px. It was previously reported that the SeD model rats can survive more than 50 weeks, although they have almost no GSH-Px throughout their life.[7] In addition, the SeD rats were fertile when mated (unpublished data). Inactivity of GSH-Px itself can not immediately cause oxidative stress. However, H_2O_2 levels can be easily increased in biological conditions lacking GSH-Px activity and H_2O_2 levels in SeD rats may increase due to additional oxidative stress.

On the other hand, GPx4 knockout[9,10] was embryonic lethal in mice. Furthermore, seleno-cysteine tRNA gene knockout caused early embryonic lethality in mice.[11] These results suggested that Se is essential in mice during fetal development. Indeed, most SeD mouse models reported were prepared by starting the SeD diet after weaning or later.[12–15] By such post-weaning preparation, only relatively older (>8 weeks old) SeD or Se-insufficient model mice are available.

In this study, relatively young SeD model mice were prepared using a torula yeast-based SeD diet. Several different timings for starting the SeD diet were assessed and the weekly time course of comprehensive GSH-Px activity after starting the SeD diet was monitored. Sensitivity of the SeD model mice to X-ray- or H_2O_2/hydroperoxide-induced oxidative stresses was investigated.

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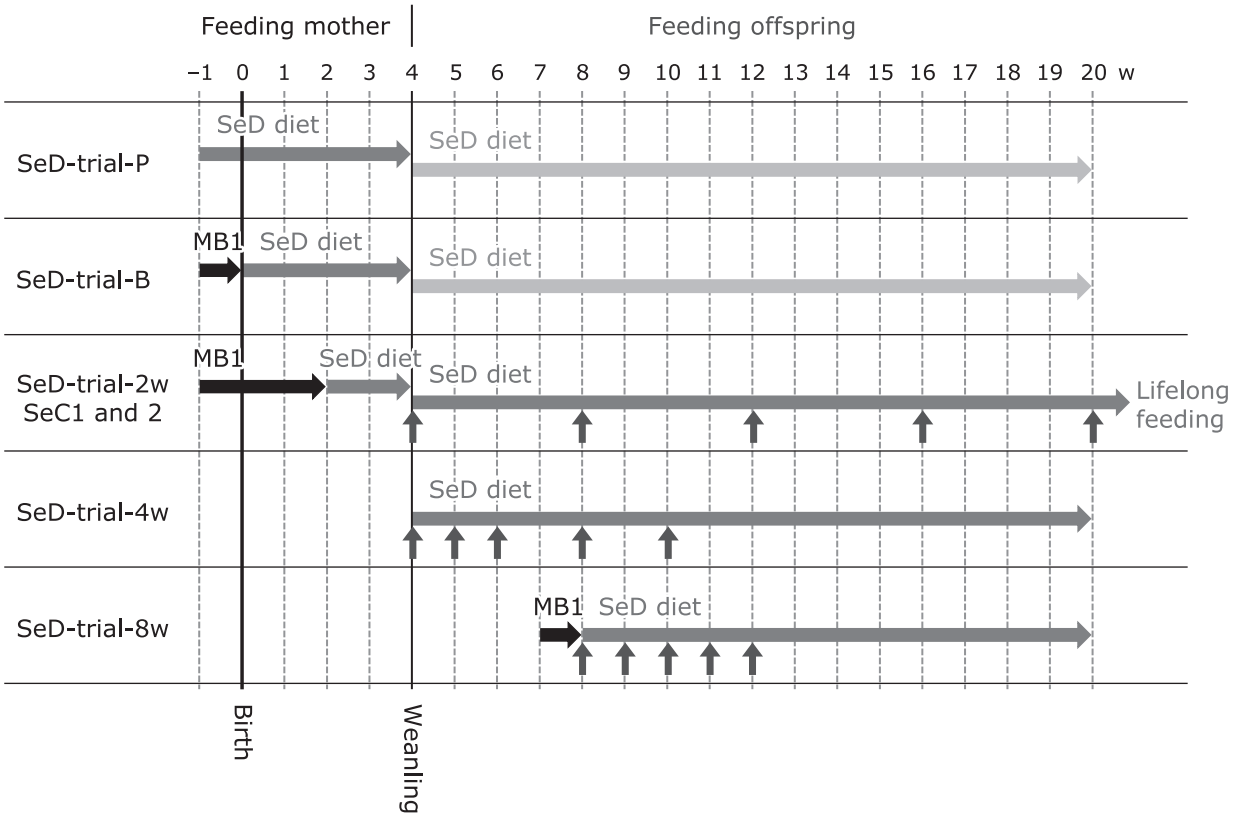

Fig. 1. Schematic drawing of the feeding period with the SeD diet and the timing for tissue sampling. Horizontal black arrows indicate the feeding periods with the MB-1 diet. Horizontal dark gray arrows indicate implemented feeding periods with the SeD diet. Horizontal light gray arrows indicate default feeding periods with the SeD diet, which was stopped due to death of the pups. Upward arrows indicate the timing of tissue sampling for the measurement of comprehensive GSH-Px activity. Ultra-pure water (Milli-Q water) was given as drinking water except to the SeC1 and 2 groups, which received Se-containing drinking water (0.64 or 0.32 mg/L of Se, respectively). Five male and female SeD-trial-2w mice were each continued to be fed for lifetime observation.

Materials and Methods

Chemicals. Seleno-L-mechionin and sodium selenite was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Preparation of animal models. The feeding schemes described below are summarized in Fig. 1. SeD-trial-P: C3H/HeSlc mice on the 15th day of pregnancy were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and then fed a torula yeast-based SeD diet (F2SeDD, Oriental Yeast Co., Ltd., Tokyo, Japan) and ultra-pure water (Milli-Q water). Newborn mice were caged with their mothers.

SeD-trial-B: C3H/HeSlc mice on the 15th day of pregnancy were purchased, and then fed a normal diet (MB-1, Funabashi Farm Co., Funabashi, Japan) and ultra-pure water. The mother mice were fed the SeD diet from 1 day after giving birth. Newborn mice were caged with their mothers.

SeD-trial-2w: C3H/HeSlc mice on the 15th day of pregnancy were purchased, and then fed a normal diet (MB-1) and ultra-pure water. The mother mice were fed the SeD diet from 2 weeks after giving birth. Newborn mice were caged with their mothers until weaning at 4 weeks old. After weaning, the young mice were fed the SeD diet and ultra-pure water until the experiments.

SeD-trial-4w: Female and male just-weaned (4 weeks old) C3H/HeSlc mice were purchased, and then fed the SeD diet and ultra-pure water until the experiments.

SeD-trial-8w: Female and male 8-week-old C3H/HeSlc mice were purchased, and then fed the SeD diet and ultra-pure water until the experiments.

Se-control (SeC): C3H/HeSlc mice on the 15th day of pregnancy were purchased, and housed under identical conditions to the SeD-trial-2w group, except that Se-containing drinking water was supplied. For the SeC1 group, 1.22 mg of seleno-L-mechionin and 0.26 mg of sodium selenite, at a molar ratio of 8:2, was added to 1 L of drinking water (0.64 mg/L of Se). For the SeC2 group, half the amount of Se of the SeC1 group, i.e., 2-times diluted Se-containing drinking water (0.32 mg/L of Se) was supplied.

Normal-control (NrmC): C3H/HeSlc mice 1 week younger than the corresponding age were purchased, and used for experiments after 1-week habituation. NrmC mice were fed a normal diet (MB-1) and Milli-Q water until the experiments.

SeD rats were prepared as reported previously[4] to assess reproducibility. Wistar rats on the 15th day of pregnancy were purchased from Japan SLC, Inc., and then fed the SeD diet and ultra-pure water. Newborn rats were caged with their mothers until weaning at 4 weeks old. After weaning, the young rats were fed the SeD diet and ultra-pure water until experiments.

The animal experiments were carried out in compliance with the Guidelines for Animal Care and Use of the National Institute of Radiological Sciences (NIRS), and approved by The Institutional Animal Care and Use Committee of NIRS.

Sample preparation for measurement of comprehensive GSH-Px activity, protein expression, and gene expression of GSH-Px isozymes. The experimental animals were anesthetized by breathing 2% isoflurane in air (700–1,000 L/min). Blood was drawn from the right atrial appendage and the entire body was perfused with ice-cooled saline (0.9% NaCl). The liver was removed. After the perfused solution was removed by filter paper,

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the liver was weighed. The liver sample was frozen in liquid nitrogen and stored at –80°C until measurement of comprehensive GSH-Px activity, protein expression, and gene expression of a particular GSH-Px isozyme.

Measurement of comprehensive GSH-Px activity. The tissues/organs were homogenized with a 4-fold volume of physiological saline (0.9% NaCl) (×5 homogenate). The tissue homogenate was again diluted in saline (1.15% KCl) to 1/125–1/1,250. Comprehensive GSH-Px activity in the tissue homogenate was measured based on the method described by Paglia and Valentine[16] with some modifications. Two-hundred microliters of PBS (pH 7.4), 50 µl of 20 mM NaN_3, 50 µl of 40 mM glutathione, 50 µl of 20 U/ml glutathione reductase, and 50 µl of 4 mM NADPH were added in a microtube. An aliquot (500 µl) of the diluted liver homogenate was added into the reaction mixture. The reaction was started by adding 100 µl of 1.0 mM H_2O_2. The time course of the absorption at 340 nm (NADPH) of the reaction mixture was measured. The GSH-Px activity was calculated from the slope of the plot of absorption over time. Consumption of 1 µmol of NADPH per minute was converted as 1 U of GSH-Px activity. The GSH-Px activity was standardized by the protein concentration and expressed as U/mg protein. The protein concentration of the liver homogenate was measured using the method described by Lowry *et al.*[17] with some modifications.

Protein expression levels of GSH-Px isozymes. Protein expression levels of GSH-Px subclass isozymes (GPx1 and 4) were measured by Western blot analysis. Mouse livers were lysed in RIPA buffer containing 1× protease inhibitor cocktail (Nakalai Tesque, Kyoto, Japan). Protein concentrations were quantified by the Protein Assay Kit (Bio-Rad, CA) and equalized. Lysates were mixed with 4× sample buffer (200 mM Tris-HCl, pH 7.5; 50% glycerin; 4% SDS; 0.04% bromophenol blue, and 50 mM DTT). Samples were incubated at 95°C for 5 min and then cooled. Proteins were size-fractionated by SDS-PAGE using Mini-Protean TGX gel Any KD (Bio-Rad). Proteins were transferred onto a nitrocellulose membrane (GE Healthcare, UK) by semi-dry blotting (ATTO). Immunodetection of proteins was achieved by specific primary antibodies: Gpx1 (AF2798, 1 µg/ml, R&D), Gpx4 (MAB5457, 0.5 µg/ml, R&D), and Actb (C4, 1:200 dilution, Santa Cruz), and secondary HRP-conjugated anti-goat IgG antibody (1:5,000 dilution, proteintech) or anti-mouse IgG antibody (1:5,000 dilution, Promega). Clarity Western ECL Blotting Substrates (Bio-Rad) were used for quantification.

Gene expression levels of GSH-Px isozymes. Gene expression levels of GSH-Px subclass isozymes (GPx1, 2, 3, 4, and 7) were measured for female NrmC and SeD-trial-2w groups. Total RNA was isolated from the livers using Direct-zol RT (Zymo Research, CA). Isolated total RNA was reverse-transcribed using the PrimeScript RT-PCR Kit with random N6 primers (Takara Bio, Shiga, Japan). Quantitative Real-time PCR (RT-PCR) was performed using the Thunderbird SYBR qPCR mix (Toyobo Co, Osaka, Japan) and the CFX Maestro (Bio-Rad). The housekeeping gene Hprt1 was selected as an internal standard. Gene-specific primers used for quantitative RT-PCR are listed in Table 1.

Sensitivity of the thymus cells to X-ray irradiation or H_2O_2 exposure. A male or female mouse from the NrmC or SeD-trial-2w model group was anesthetized by breathing 2% isoflurane in air. The mouse was euthanized by bleeding from the underarm. The thymus was removed and squeezed using tweezers to obtain thymocytes. The thymocytes were placed in cell culture medium (RPMI-1640 Medium, Sigma) supplemented with 10% fetal bovine serum (FBS) and grained into disaggregated cells. The thymocytes were irradiated by 2 Gy of X-rays at a dose rate of 1.0 Gy/min, or were treated with 50, 100, or 500 µM H_2O_2 for 5 min. The H_2O_2-treated cells were washed once with Dulbecco's phosphate-buffered saline (PBS, Sigma), but X-ray-treated cells were not. Cells were next incubated in the same culture medium as above for 4 h at 37°C in 5% CO_2 atmosphere. After incubation, the size and number of thymocytes were measured by a flow cytometer (FACSCalibur; Becton, Dickinson and Company, Franklin Lakes, NJ). The ratio of shrunken thymocytes was estimated as the ratio of apoptotic thymocytes.

Table 1. Gene-specific primers used for quantitative RT-PCR

Gene	Primer sequence
Gpx1-F	5'-GGGACCTCGTGGACTGGTGGTGCT
Gpx1-R	5'-CCCGCCACCAGGTCGGACGTACT
Gpx2-F	5'-CGCCTGGTAGTTCTCGGCTTCCCTT
Gpx2-R	5'-GGGCTGGTACCCACCCCCAGGT
Gpx3-F	5'-GACCAGGTGGGGGCTTTGTGCCTAAT
Gpx3-R	5'-AGAGGCGGCCAGGTGAGCCCAG
Gpx4-F	5'-GTCCAGCCCAAGGGCAGGGGCAT
Gpx4-R	5'-GCGCTTCACCACGCAGCCGTTCT
Gpx7-F	5'-TTGCCCGCCGCACCTACAGTGTCT
Gpx7-R	5'-GGGGTCCCATGCTCCCACCACCTTT
Hprt1-F	5'-CAACGGGGGACATAAAAGTTATTGGTGGA
Hprt1-R	5'-TGCAACCTTAACCATTTTGGGGCTGT

Thirty-day survival rate after X-ray irradiation. Conscious mice were placed in a special acryl box for whole-body irradiation and irradiated with 5.6 Gy of X-rays. Male and female NrmC and SeD-trial-2w model mice were irradiated. Each group consisted of 10 mice. X-ray irradiation was performed with PANTAK 320S (Shimadzu, Kyoto, Japan). The effective energy was 80 keV under the following conditions: X-ray tube voltage was 200 kV, X-ray tube current was 20 mA, and the thickness and materials of the pre-filter were 0.5-mm copper and 0.5-mm aluminum. The dose rate of X-ray irradiation was 0.54 Gy/min when the distance between the X-ray tube and the sample was 50 cm. Survival curves after X-ray irradiation of mouse groups were observed.

Statistical Test. Significant differences were estimated by alternative Student's or Welch's *t* tests. The test suitable for the data was automatically selected according to variance. Significance was considered at $p<0.05$.

Results and Discussion

The number of confirmed live births was 5.0 ± 0.8 ($n = 4$) and 4.2 ± 1.1 ($n = 5$) for SeD-trial-P and -B mothers, respectively. The neonatal mice in both SeD-trial-P and -B groups were visually smaller than normal pups and lacked active wiggling. The mammary pads of mothers in both the SeD-trial-P and -B groups were smaller than those in the control group during the lactation period. The newborn pups survived less than 21 days in the SeD-trial-P group and 13 days in the SeD-trial-B group. However, SeD rats were prepared according to the method reported previously,[6] i.e., starting the SeD diet from the 15th day of pregnancy. The rat pups survived until they were used for measuring liver GSH-Px activity at 8 weeks old even though they had no GSH-Px activity (data not shown). In addition, the SeD rats had a normal life span, which was more than 50 weeks.[7] This suggests that Se is essential for mice to give birth and grow healthily. The reason may be similar to that for the embryonic lethality of PHGPx knockout[9,10] and seleno-cysteine tRNA gene knockout.[11]

For the first trial to prepare a SeD mouse model, the C3H/HeSlc mouse strain was selected. This mouse strain was used in our previous animal experiments to estimate the biological effects of artificial oxidative stresses such as hyperthermia, X-rays, and/or heavy-ion (carbon-ion) beam irradiation.[18–24] High linear energy transfer (LET) beams, such as heavy-ion beams, yield more H_2O_2 than low LET photon radiation such as X-rays and/or gamma-rays.[25] High LET beams can create H_2O_2 independently of oxygen, but low LET radiation creates H_2O_2 mainly in an oxygen-dependent manner.[26] Therefore, high LET beams can yield H_2O_2

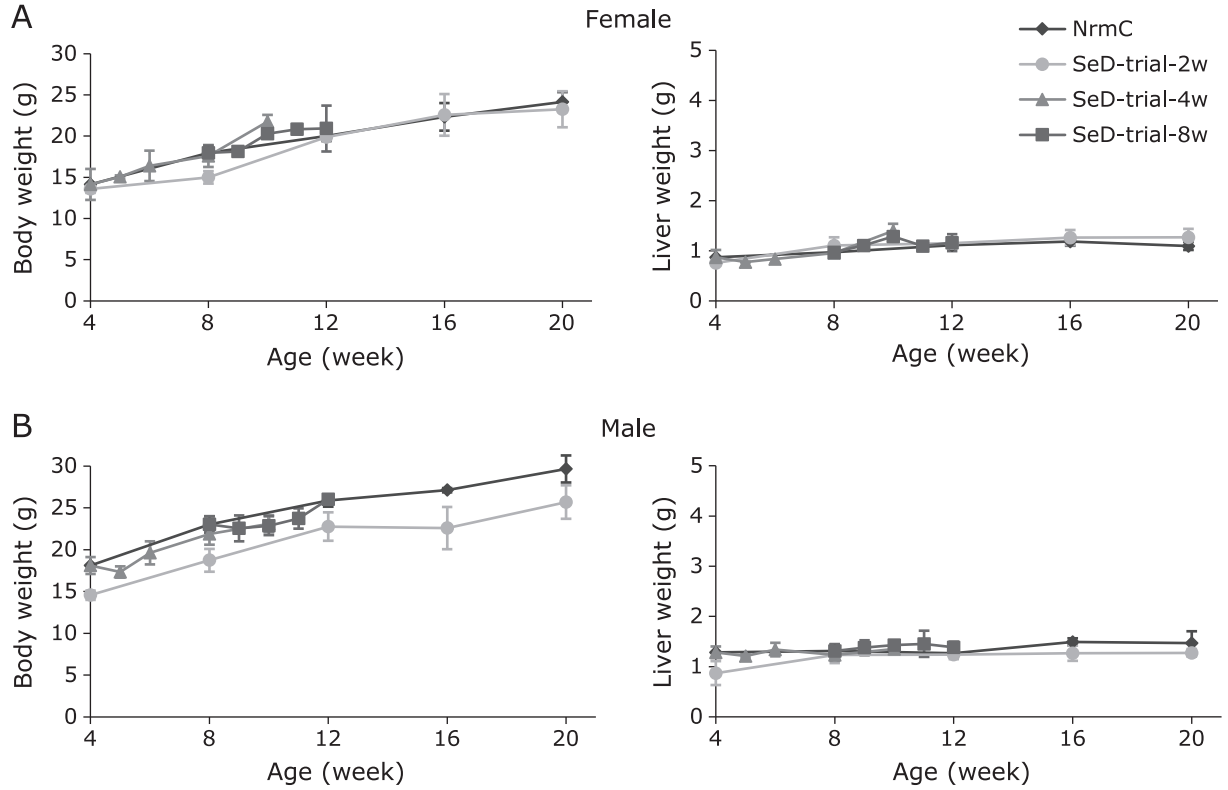

Fig. 2. Time courses of body and liver weights of the NrmC and SeD model mice. Body and liver weights of the (A) female and (B) male mice used for assessing GSH-Px activity were measured just before the experiments. The mice were fasted one night before the experiment. Diamonds, circles, triangles, and squares indicate values of NrmC, SeD-trial-2w, SeD-trial-4w, and SeD-trial-8w groups, respectively. The marks and error bars indicate the average value ± SD of 3 mice.

even under low-oxygen conditions such as *in vivo*. The Se-deficient mouse model may be suitable for estimating the effects of radiation-induced H_2O_2 and/or lipid peroxidation.

The time course of body and liver weights of SeD mice models is shown in Fig. 2. The values in the SeD-trial-4w group at 4 weeks old and the SeD-trial-8w group at 8 weeks old were similar to control values. There was no significant difference in liver weight among groups at 8 weeks old. Percentage ratios of the liver weight to the body weight at 8 weeks old were 7.45 ± 1.47, 5.18 ± 0.45, and 5.41 ± 0.36% for female SeD-trial-2w, SeD-trial-4w, and SeD-trial-8w groups, respectively. The livers of female SeD-trial-2w mice were swollen, although not significantly. Liver swelling in the SeD model rats has been reported.[27,28]

To confirm Se deficiency, GSH-Px activity in the liver was measured. GSH-Px activity in the liver at several ages is shown in Fig. 3. The SeD-trial-8w group (squares) exhibited a rapid decrease in GSH-Px activity during the 4-week period of the experiment; however, they still had a relatively high GSH-Px activity level at 12 weeks old. The SeD-trial-4w group (triangle) also exhibited a rapid decrease in GSH-Px activity during the 4-week period of the experiment; however, they still had slight GSH-Px activity at 8 weeks old. The SeD-trial-4w group had no GSH-Px activity at 10 weeks old. The SeD-trial-2w group (circles) had almost no GSH-Px activity from 4 weeks old and throughout their life. Starting the SeD diet in older mice makes it take longer for the GSH-Px activity to become undetectable. However, the SeD-trial-2w preparation method proposed in this study yielded young (from 4 weeks old) SeD model mice. The SeD-trial-2w mice survived for more than 63 weeks with life-long feeding of the SeD diet and Milli-Q water.

A comparison of GSH-Px activity in the livers of NrmC, SeD-trial-2w, SeC1, and SeC2 groups at 8 weeks old is shown in Fig. 4. The SeD-trial-2w group had no GSH-Px activity, as shown in Fig. 3. The tissue GSH-Px activity of female and male SeC1 mice, which were fed the SeD diet and 0.64 mg/L of Se in drinking water, recovered to 66% and 80% of that in female and male NrmC mice, respectively. The female and male SeC2 mice, which were fed the SeD diet and 0.32 mg/L of Se in drinking water, exhibited 62% and 52% of the GSH-Px activity observed in female and male NrmC mice, respectively.

The tissue Se content in the mouse models used in this study was not measured. Tissue GSH-Px activity was reported to be a good indicator of tissue Se content.[29] Matsumoto, *et al.*[7,30,31] previously confirmed that feeding of a Se-deficient diet may lead to limited or no Se in most tissues, and that it disturbs GSH-Px activity in a rat model. Relatively low Se content can maintain nearly normal GSH-Px activity,[30] whereas low GSH-Px activity is not observed when Se content is maintained. The purpose of this study was to create an animal model lacking GSH-Px activity, and the observed near lack of GSH-Px activity confirmed Se-deficiency.

A comparison of protein expression levels of GPx1 and GPx4 in the liver is shown in Fig. 5. The protein expression level of GPx1 was suppressed in both male and female SeD-trial-2w groups. However, protein expression levels of GPx4 were not affected by Se deficiency. Next, the gene expression of subclasses of GSH-Px was analyzed.

A comparison of gene expression levels of GSH-Px subclasses in the liver is shown in Fig. 6. Female NrmC and SeD-trial-2w groups were compared. GSH-Px gene expression was observed in both NrmC and SeD-trial-2w groups, whereas GSH-Px activity was almost absent in SeD model mice. The expression levels of mRNA were in order of GPx1 > GPx4 > GPx7 > GPx3. GPx2 was not measured in NrmC and SeD-trial-2w groups. Therefore, the

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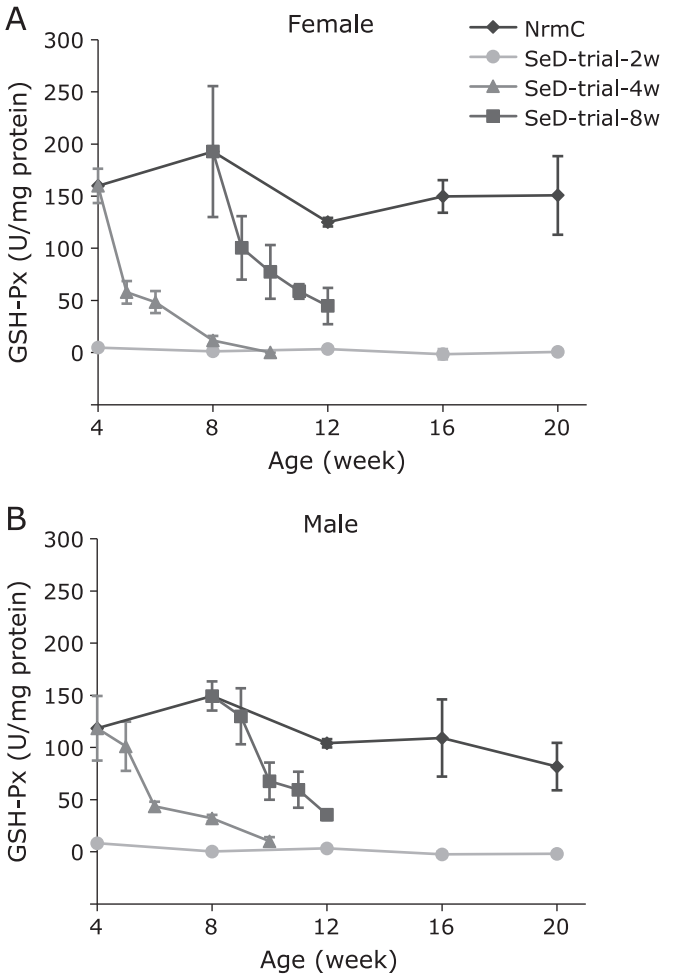

Fig. 3. Time courses of GSH-Px activity in the liver of the NrmC and SeD model mice. Liver GSH-Px activity was assessed for (A) female and (B) male mice of each group. Diamonds, circles, triangles, and squares indicate values of NrmC, SeD-trial-2w, SeD-trial-4w, and SeD-trial-8w groups, respectively. The marks and error bars indicate the average value ± SD of 3 mice.

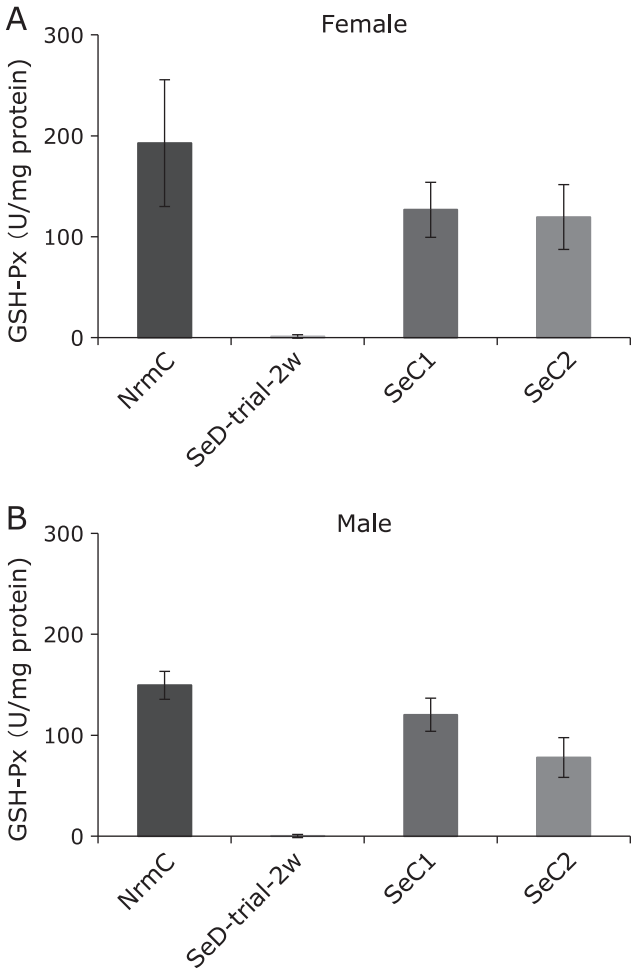

Fig. 4. GSH-Px activity in the livers of the NrmC, SeD-trial-2w, SeC1, and SeC2 groups assessed at 8 weeks old. Comparison of the GSH-Px activity of (A) female and (B) male mice. The columns and error bars indicate the average value ± SD of 3 mice.

GSH-Px activity shown in Fig. 3 and 4 mainly reflected GPx1. Se deficiency did not affect the gene expression level of GPx3, 4, or 7, although the GPx1 gene expression level significantly decreased in SeD mice. This suggests a role of Se in GPx1 mRNA transcription or protein stability. Although the GPx1 gene expression level was lower in SeD mice, protein synthesis was retained. However, the protein expression level of GPx1 in SeD mice was reduced more than the reduction in the gene expression level (Fig. 5). Se deficiency, i.e., exhaustion of selenium-containing amino acids, may more greatly affect protein expression. These results suggest that the elimination of GSH-Px activity in SeD groups was due to the lack of Se.

Suppression of GPx1 protein expression was observed in all male and female SeD-trial-2w mice subjected to the experiment. Then, gene expression levels in the same identical liver samples of the female SeD-trial-2w and NrmC mice were measured (Fig. 6). Reduced GPx1 gene expression levels were noted in the SeD-trial-2w group, which was almost one-quarter of the NrmC group. The lower protein expression of GPx1 in the SeD-trial-2w group may be due to the down-regulation of gene expression of GPx1 and Se-deficiency may slightly affect its regulation. The detailed mechanisms of the suppression of GPx1 gene expression should be investigated in the future, but it is another focus.

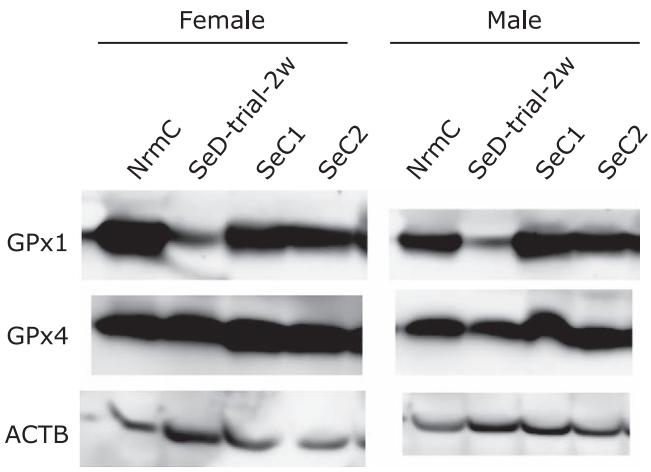

Fig. 5. GPx1 and GPx4 protein expression in the liver. Western blotting was used to analyze the expression of GPx1 and GPx4 protein in the liver of the NrmC, SeD-trial-2w, SeC1, and SeC2 groups. Beta-actin (ACTB) served as a loading control. The Western blotting images are representative from 3 independent sets.

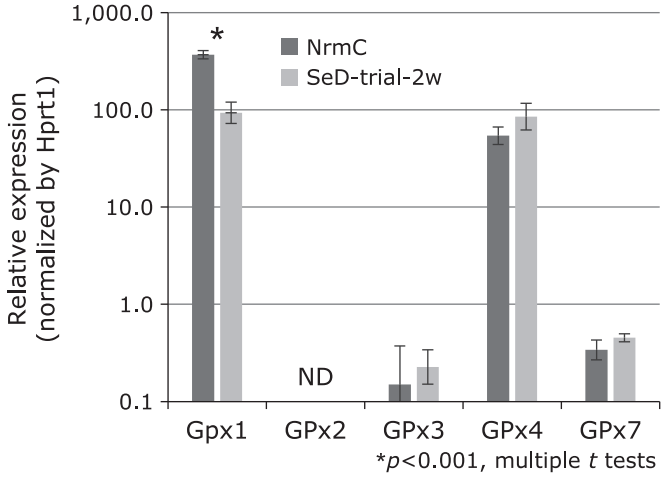

Fig. 6. Gene expression levels of GPx1, 2, 3, 4, and 7 in the liver. Quantitative RT-PCR was performed to evaluate gene expression of the GPx family. The data are presented as a logarithmic plot of the 2-ΔCt values and normalized by Hprt1 mRNA levels. The data are presented as the average value ± SD of 3 mice from each group. ND, not detected, the signal was below the sensitivity limit of the assay.

Suppressed protein expression of GPx4 was observed in two (one male and one female) SeD-trial-2w mice (data not shown), but the remaining mice had similar levels to those in other groups, as shown in Fig. 5. As the GPx4 protein level was inherently lower than that of GPx1, relatively low suppression of GPx4 protein levels may be difficult to observe beyond inter-individual variation. However, slight up-regulation of GPx4 gene expression in the SeD-trial-2w group was observed (Fig. 6), which was 1.57-times higher than that in the NrmC group, but not significant. Slightly up-regulated GPx4 gene expression in the SeD-trial-2w was noted when another housekeeping gene was used as a standard. The up-regulation of anti-oxidative enzymes under oxidative stress may be a natural feedback response of living organisms.

Markedly low selenium/selenocysteine levels may be related to the lower protein expression levels of GSH-Px isozymes, but they are not due to simple interruption of protein synthesis. Indeed, detectable levels of proteins were synthesized using an alternate amino acid instead of selenocysteine, as described below.

The antibody used in the Western blot experiment recognizes a downstream region from the position of selenocysteine; therefore, an alternate amino acid must be inserted into the synthesized protein. Selenocysteine is encoded by the stop codon, UGA. Targeted insertion of cysteine at the UGA selenocysteine codon of thioredoxin reductase 1 was previously reported.[32] Furthermore,[33] both GPx1 and GPx4 had significant levels of cysteine in place of selenocysteine following treatment with Geneticin, an antibiotic, but no detectable amounts of tryptophan, which is also encoded by the UGA codon in some cases. Cysteine may be the major alternative to selenocysteine. Analysis of the detailed mechanism of this misreading/extemporaneous-reading is underway.

The Se content in this Se-deficient diet reported in the previous paper was low (0.017 mg/kg), being one-fiftieth of that of the normal diet (CE-2, CLEA Japan, Inc., Tokyo) but "not zero".[34] Low Se levels were maintained in some organs, such as the kidneys and spleen, in the case of the Se-deficient rat model, whereas no Se was detectable in the liver.[7]

Loss of the GPx1 gene has limited or no effects in mice,[35] whereas the loss of the GPx4 gene is embryonic lethal.[9,10] Administration of excess vitamin E to the culture media rescued GPx4 knock-out mouse embryonic cell death.[36] The Se-deficient diet contains 2.6-times more vitamin E (102 mg/kg) than the normal

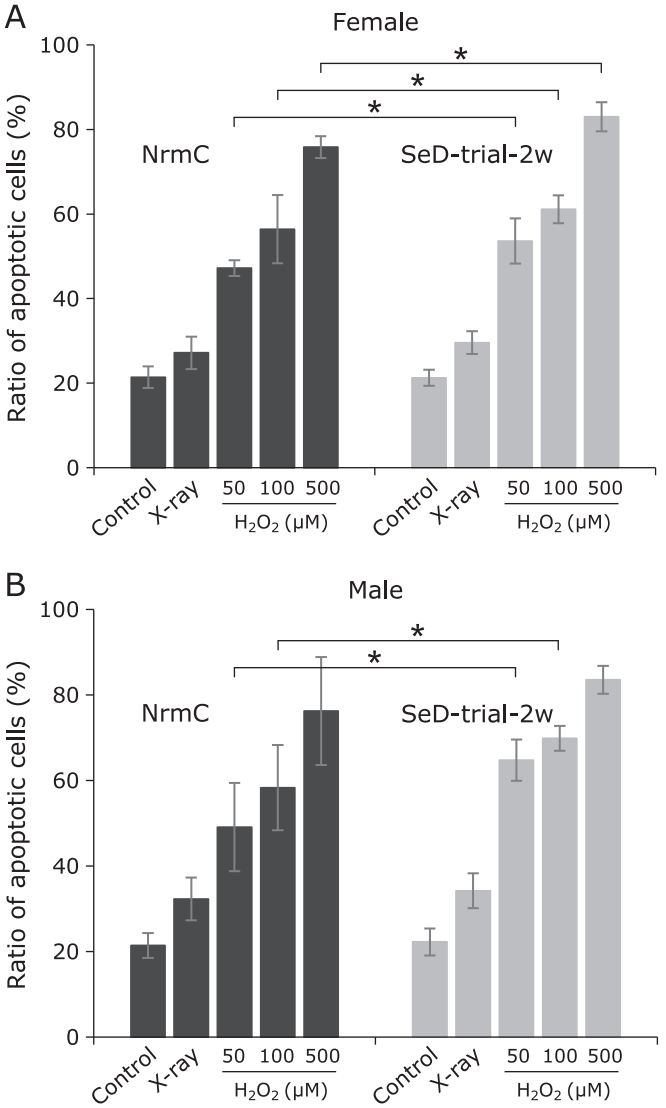

Fig. 7. A comparison of the sensitivity of thymocytes to oxidative stress. Comparison of NrmC (left panel) and SeD-trial-2w (right panel) model mice. (A) Female and (B) male groups were tested in the same manner. The columns and error bars indicate the average value ± SD of at least 3 experiments; each experiment was triplicated.

diet (39 mg/kg), but this was unable to rescue the death of SeD-trial-P and -B pups during infancy. As GPx4 gene expression is inherently lower than that of GPx1 (as shown in Fig. 6), higher vitamin E content in the Se-deficient diet may be able to save adult mice from death even if they have almost no GSH-Px activity.

The gene and protein expression of GPx4 may be regulated by feedback from pathological/physiological pathways at that age and by alternative functions such as between selenocysteine and cysteine.

A comparison of the ratio of apoptotic thymocytes after X-ray irradiation or H_2O_2 exposure is shown in Fig. 7. Regardless of the presence of selenium (left and right panels) and gender (Fig. 7A or B), the ratio of apoptotic cells was around 20% for the control (no test) cells. The ratio of apoptotic cells was increased by X-ray irradiation and/or H_2O_2 exposure. At 2-Gy X-ray irradiation, no significant difference was observed between thymocytes from NrmC and SeD-trial-2w model mice. Concentrations of 50, 100, or 500 μM H_2O_2 led to significantly higher levels of

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apoptosis in SeD-trial-2w model mice than in those in NrmC model mice, excluding 500 μM H_2O_2 in male mice thymocytes, which exhibited no significant difference. X-ray irradiation was reported to cause only 0.26 μM/Gy of H_2O_2 in water.[26] As H_2O_2 generation caused by low LET radiation, such as X-rays, requires oxygen consumption, the X-ray-induced H_2O_2 generation in cells or *in vivo* tissue may be much lower. Therefore, the main reactive oxygen species (ROS) causing oxidative stress during the X-ray irradiation in the thymocytes in this experiment was probably hydroxyl radical (˙OH). Downregulation of selenium may make the mice thymocytes sensitive to the oxidative stress caused by H_2O_2.

Thirty-day survival curves after whole-body X-ray irradiation are shown in Fig. 8. Half of the male and female NrmC mice survived for 30 days after X-ray irradiation, whereas all of the SeD-trial-2w mice died by 15 days after irradiation. SeD-trial-2w models demonstrated radio-sensitivity compared with NrmC mice. The effects of X-ray irradiation on the survival of SeD mice were marked even though the effects of X-rays on the SeD mouse thymocytes were limited, as shown in Fig. 7. Delayed superoxide generation after X-ray irradiation *in vivo* is expected to be due to mitochondrial dysfunction.[37] Mitochondria oxidatively injured by initial ROS during X-ray irradiation can generate excess superoxide continuously for several days after X-ray irradiation. Excess mitochondrial superoxide may yield excess H_2O_2 via the physiological role of SOD.

In this study, healthy female control mice exhibited higher GSH-Px activity in the liver than males at all ages examined (Fig. 3). Several previous studies reported sex differences in GSH-Px activity in the tissues of experimental animals. For example, GSH-Px activity in liver cytosol of 4–29-month-old Fischer 344 rats were higher in females.[38] Liver GSH-Px activities in SD rats were higher in females at both 7 and 12 weeks of age.[39] GSH-Px activity was higher in the liver mitochondria from female rats than in those from male rats.[40] In humans, higher GSH-Px activity in females than in males has been reported.[41,42] In general, be it experimental animals[43,44] or humans,[45,46] females store more body fat than males at the same age. Female mammals may require greater antioxidative protection for fat/lipid molecules stored in their body. GSH-Px family enzymes, especially both PHGPx isozymes GPx4 and 7, are likely important for preventing lipid oxidation. Our study demonstrated the large effects of Se-deficiency, i.e., malfunction of GSH-Px, in female mice (Fig. 8).

SeD mice may be a useful animal model to investigate the effects of H_2O_2 on radiobiological responses in detail. H_2O_2 is a relatively stable molecule compared with other ROS, but it easily accumulates and can move relatively long distances. In addition, H_2O_2 can react with metal ions, such as Cu^+ and/or Fe^{++}, to make ˙OH, which is known as a Fenton reaction. The reaction of $O_2^{\bullet-}$ and H_2O_2 can also yield ˙OH. The ˙OH again plays the essential oxidant. The direct role of H_2O_2 in the effects of radiation remains unclear, but it must be considered when discussing radiobiological effects.

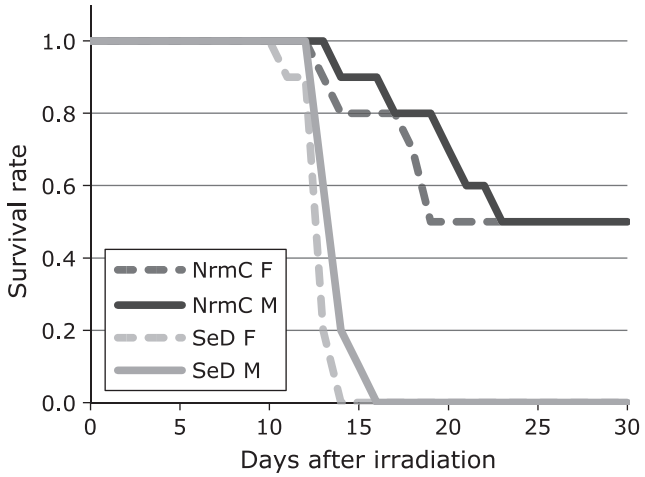

Fig. 8. Comparison of the 30-day survival rate after 5.6-Gy X-ray irradiation. Male and female NrmC and SeD-trial-2w groups were irradiated. Each group consisted of 10 mice.

In conclusion, the same procedure that was reported for a SeD rat model,[6–8,27,28] i.e., the SeD-trial-P group in this study, was unable to be used to make SeD model mice due to neonatal death. Starting the mother mice on the SeD diet from 2 weeks after giving birth, i.e., the SeD-trial-2w group, yielded relatively young SeD model mice. Liver GSH-Px activity in the SeD-trial-2w group was almost absent at 4 weeks old. Supplying 0.64 or 0.32 mg/L of Se in drinking water to the SeD-trial-2w group, i.e., SeC1 and SeC2 groups, restored GSH-Px activity. Protein expression of GPx1 was suppressed in the SeD-trial-2w model mice, but that of GPx4 was not. Although Se deficiency did not affect the gene expression of GPx4, the GPx1 gene expression level was significantly suppressed in the SeD-trial-2w model mice. These mice were also radio-sensitive compared with the NrmC group mice. These SeD-trial-2w model mice may represent a H_2O_2/hydroperoxide-induced oxidative stress model for future experiments.

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Conflict of Interest

No potential conflicts of interest were disclosed.

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