Mechanisms of oxidative stress-induced in vivo mutagenicity by potassium bromate and nitrofurantoin

Takuma Tsuchiya1, 2, Aki Kijima1, Yuji Ishii1, Shinji Takasu1, Yuh Yokoo1, Akiyoshi Nishikawa1, Tokuma Yanai2, and Takashi Umemura1, 3*

1 Division of Pathology, National Institute of Health Sciences, 3-25-26 Tonomachi, Kawasaki-ku, Kawasaki-shi, Kanagawa 210-9501, Japan
2 Pathogenetic Veterinary Science, United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu-shi, Gifu 501-1193, Japan
3 Department of Animal Nursing, Yamazaki Gakuen University, 4-7-2 Minamiosawa, Hachioji-shi, Tokyo 192-0364, Japan

Abstract: Oxidative stress is well known as a key factor of chemical carcinogenesis. However, the actual role of oxidative stress in carcinogenesis, such as oxidative stress-related in vivo mutagenicity, remains unclear. It has been reported that 8-hydroxydeoxyguanosine (8-OHdG), an oxidized DNA lesion, might contribute to chemical carcinogenesis. Potassium bromate (KBrO3) and nitrofurantoin (NFT) are known as renal carcinogens in rats. Our previous studies showed an increase in mutant frequencies accompanied by an increased level of 8-OHdG in the kidneys of rodents following KBrO3 or NFT exposure. Furthermore, KBrO3 and NFT induced different types of gene mutations. Thus, in the present study, we performed reporter gene mutation assays and 8-OHdG measurements following KBrO3 or NFT exposure using Nrf2-proficient and Nrf2-deficient mice to clarify the relationship between KBrO3- or NFT-induced oxidative stress and subsequent genotoxicity. Administration of 1,500 ppm of KBrO3 in drinking water resulted in an increase in deletion mutations accompanied by an increase in 8-OHdG level, and administration of 2,500 ppm of NFT in diet induced an increase in guanine base substitution mutations without elevation of the 8-OHdG level in Nrf2-deficient mice. These results demonstrated that the formation of 8-OHdG, which resulted from the oxidizing potential of KBrO3, was directly involved in the increase in deletion mutations, although factors related to oxidative stress other than 8-OHdG might be crucial for NFT-induced guanine base substitution mutations. The present study provides new insight into oxidative stress-related in vivo mutagenicity. (DOI: 10.1293/tox.2018-0024; J Toxicol Pathol 2018; 31: 179–188)

Key words: bromates, nitrofurantoin, NF-E2-related factor 2, DNA damage, mutagens, kidney

Introduction

The formation of reactive oxygen species (ROS) is considered one of the key factors in chemical carcinogenesis. However, the actual role of oxidative stress remains unclear. Some reports suggest that ROS play an important role in the promotion of chemical carcinogenesis by stimulating the proliferation of initiated cells1–3, while others demonstrate that ROS might be an initiator by forming oxidized DNA lesions. 4– 6, 8-Hydroxydeoxyguanosine (8-OHdG) is the most abundant oxidized DNA lesion among the many oxidized nucleosides known and is fairly stable. 7 Repair of 8-OHdG is carried out by the base excision repair enzymes. In humans, OGG1, MUTYH, and MTH1 repair 8-OHdG and contribute to the protection of genomic DNA from oxidative stress8. The remaining 8-OHdG is considered to cause G:C to T:A transversions by mispairing with adenine and 8-OHdG9, 10.

Potassium bromate (KBrO3) induces renal cell tumor formation in F344 rats and has been classified as a genotoxic carcinogen because of positive mutagenicity in the Ames11, chromosome aberration 12, and micronucleus tests13. Studies demonstrating induction of 8-OHdG by KBrO3 in vitro and in vivo suggest that 8-OHdG plays a key role in KBrO3 mutagenesis and carcinogenesis14–17. It was reported that KBrO3 produces bromine radicals, which oxidize guanine bases18. Additionally, our previous study using a two-stage rat renal carcinogenesis model clarified the in vivo mutagenicity and initiation following induction of an oxidized DNA lesion in the kidneys of rats administered KBrO319 and showed that high amounts of 8-OHdG resulted in several types of mutations, including deletion mutations, in addition to G:C to T:A transversions19. The antimicrobial compound nitrofurantoin (NFT) is also known as a renal carcinogen in rats and is prohibited for veterinary use in Japan20. The reduction of the nitro group of NFT induces oxidative stress,
which exerts antibacterial activity21–23. Moreover, the involvement of oxidative stress is suspected in NFT-induced carcinogenesis. In fact, our recent study showed increased levels of 8-OHdG and gpt mutant frequencies (MFs) with guanine base substitution mutations, including G:C to T:A transversions, in the kidneys of gpt delta rats treated with NFT24. Nonetheless, the relationship between the formation of 8-OHdG and several types of mutations, including deletion mutations and G:C to T:A transversions, remains unclear.

One of the redox-sensitive transcription factors, nuclear factor erythroid 2-related factor 2 (NRF2), regulates cellular responses to oxidative stress by transactivation of antioxidant-related enzymes, such as NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HO1), and glutathione S-transferase (GST)25, 26. Because of the function of NRF2, Nrf2-deficient mice show higher sensitivity to various toxicants that induce oxidative stress27–31; therefore, these mice are quite suitable for investigation of the involvement of oxidative stress in chemical-induced genotoxicity and carcinogenesis.

In the present study, Nrf2-proficient and Nrf2-deficient mice were exposed to KBrO3 in drinking water or NFT in diet for 4 and 13 weeks and then subjected to reporter gene mutation assays32, 33 and measurement of 8-OHdG levels in the kidney DNA to clarify the relationship between the formation of 8-OHdG and several types of mutations. In addition, the study aimed to elucidate the detailed mechanism of oxidative stress involvement in KBrO3- or NFT-induced renal carcinogenesis.

Materials and Methods

Chemicals

Potassium bromate (MW 167, CAS No. 7758-01-2) and NFT (C8H3N4O6, MW 238.2, CAS No. 67-20-9) were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Sigma-Aldrich (St. Louis, MO, USA), respectively.

Animals, diet, and housing conditions

The study protocol was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences. Nrf2-deficient mice with the C57BL/6J background established by Itoh et al.34 were crossed with gpt delta mice with the C57BL/6J background (Japan SLC, Shizuoka, Japan). Nrf2−/− gpt delta mice and Nrf2+/− gpt delta mice were then obtained from the F1 generation and genotyped by polymerase chain reaction (PCR) with DNA taken from the tail of each mouse. All mice were housed in polycarbonate cages (3 to 5 mice per cage) with hardwood chips for bedding in a conventional animal facility maintained at a controlled temperature (23 ± 2°C) and humidity (55 ± 5%), with 12 air changes per hour and a 12-h light/dark cycle. Mice were given free access to CRF-1 basal diet (Charles River Laboratories Japan, Kanagawa, Japan) and tap water.

Experimental design

Six-week-old male mice of each genotype were divided into six groups (four to eight mice per group). KBrO3 was dissolved in distilled water at concentration of 1,500 ppm, and the prepared water was given to the animals ad libitum for 4 or 13 weeks. NFT was mixed in CRF-1 basal diet at concentration of 2,500 ppm, and the prepared diet was given to the animals ad libitum for 4 or 13 weeks. Mice of the control group were given distilled water and CRF-1 basal diet. Dose levels of KBrO3 and NFT were selected as each maximum dose that could be administered to mice for 13 weeks based on a report of intestinal carcinogenesis in Nrf2−/− mice27, a subacute toxicity study of KBrO335, and a toxicology and carcinogenesis study of NFT conducted by the National Toxicology Program36. In the present study, the 4- and 13-week administration groups were set for the objective of detection of early changes and subsequent changes in 8-OHdG levels and in vivo mutagenicity induced by KBrO3 or NFT, respectively. Body weights were measured every week. Animals were killed by exsanguination under isoflurane (Mylan Inc., Tokyo, Japan) anesthesia. At necropsy, the bilateral kidneys were collected, and their weights were measured. The kidneys-to-body weight ratios (relative weights) were calculated as grams organ weight/grams body weight. A portion of the kidney tissues was frozen with liquid nitrogen and stored at −80°C for use in the in vivo mutation assay and 8-OHdG measurement. Another portion was homogenized in ISOGEN (Nippon Gene, Tokyo, Japan) and stored at −80°C until used for isolation of total RNA.

In vivo mutation assays

6-Thioguanine (6-TG) and Spi– selections were performed using the methods described by Nohmi et al.32. Briefly, genomic DNA was extracted from the kidneys of animals in each group using a RecoverEase DNA Isolation Kit (Agilent Technologies, Santa Clara, CA, USA), and lambda EGI0 DNA (48 kb) was rescued as phages by in vitrowhich is used in packaging using Transpack Packaging Extract (Agilent Technologies). For 6-TG selection, packaged phages were incubated with Escherichia coli YG6020, which expresses Cre recombinase, and converted to plasmids carrying genes encoding glutamic–pyruvate transaminase and chloramphenicol acetyltransferase. Infected cells were mixed with molten soft agar and poured onto agar plates containing chloramphenicol and 6-TG. In order to determine the total number of rescued plasmids, infected cells were also poured on plates containing chloramphenicol without 6-TG. The plates were then incubated at 37°C for selection of 6-TG-resistant colonies, and the gpt MF was calculated by dividing the number of gpt mutants after clonal correction by the number of rescued phages. Gpt mutations were characterized by amplifying a 739-bp DNA fragment containing the 456-bp coding region of the gpt gene32 and sequencing the PCR products with an Applied Biosystems 3730xl DNA Analyzer (Life Technologies, Carlsbad, CA, USA). For Spi selection, packaged phages were incubated with E. coli XL-1 Blue MRA for survival titration and E. coli XL-1
Blue MRA P2 for mutant selection. Infected cells were mixed with molten lambda-trypticase agar and poured onto lambda-trypticase agar plates. The next day, plaques (Spi– candidates) were punched out with sterilized glass pipettes, and the agar plugs were suspended in SM buffer. The Spi– phenotype was confirmed by spotting the suspensions on three types of plates on which XL-1 Blue MRA, XL-1 Blue MRA P2, or WL95 P2 strain was spread with soft agar. Spi– mutants, which manifested as clear plaques, were counted on every plate.

**Measurement of 8-OHdG**

Three animals in each group were selected randomly, and kidneys of those animals were used for the measurement of 8-OHdG. Renal DNA of $Nrf2^{-/-} gpt$ delta mice and $Nrf2^{+/+} gpt$ delta mice was extracted and digested as described previously. Briefly, nuclear DNA was extracted with a DNA Extractor WB Kit (Wako Pure Chemical Industries). For further prevention of artifactual oxidation in the cell lysis step, deferoxamine mesylate (Sigma-Aldrich) was added to the lysis buffer. The DNA was digested to deoxynucleotides by treatment with nuclease P1 and alkaline phosphatase, using an 8-OHdG Assay Preparation Reagent Set (Wako Pure Chemical Industries). The levels of 8-OHdG (8-OHdG/10^5 dG) were measured by high-performance liquid chromatography with an electrochemical detection system (Coulochem II, ESA, Bedford, MA, USA) as previously reported.

**RNA isolation and quantitative real-time PCR for evaluation of mRNA expression**

Total RNA was extracted using ISOGEN according to the manufacturer’s instructions. cDNA of total RNA was obtained using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies).

All PCR reactions were performed with an Applied Biosystems 7900HT FAST Real-Time PCR System with primers for mouse $Nqo1$ (coding NAD(P)H:quinone oxidoreductase 1) obtained from TaqMan® Gene Expression Assays and TaqMan® Rodent GAPDH Control Reagents. The expression levels of the target gene were calculated by the relative standard curve method and were determined as ratios to Gapdh levels. Data are presented as fold-change values of treated samples relative to controls.

**Statistical analysis**

The significance of differences in the results for body weights, kidney weights, mRNA expression levels, 8-OHdG levels, $gpt$ and Spi– MFs, and $gpt$– and Spi– mutation spectra were analyzed by Student’s t-test depending on the homogeneity. P values < 0.05 were considered significant.

**Results**

**Body and kidney weights**

Body and kidney weights of $Nrf2$-proficient and $Nrf2$-deficient mice treated with KBrO₃ or NFT for 4 or 13 weeks are summarized in Fig. 1 and Table 1. For both genotypes and time points, no significant change was observed in body and kidney weights of treated and respective control animals.

**Quantitative real-time PCR for evaluation of Nqo1 mRNA expression**

Expression levels of $Nqo1$ in the kidneys are shown in Fig. 2. In $Nrf2^{+/+}$ mice, the expression level of $Nqo1$ was significantly increased by 4 or 13 weeks of exposure to KBrO₃ ($P<0.01$) and 13 weeks of exposure to NFT ($P<0.05$). Four weeks of exposure to NFT resulted in a tendency toward increased expression of $Nqo1$ in $Nrf2^{+/+}$ mice. In $Nrf2^{-/-}$ mice, increased $Nqo1$ expression was not induced by KBrO₃ or NFT treatment at either time point. The $Nqo1$ expression levels of control, KBrO₃-treated, and NFT-treated $Nrf2^{-/-}$ mice were significantly lower ($P<0.01$) than those of the corresponding $Nrf2^{+/+}$ mice at both time points.

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**Fig. 1.** Growth curves for $Nrf2^{+/+}$ or $Nrf2^{-/-}$ mice treated with KBrO₃ or NFT for 4 weeks (A) or 13 weeks (B). For both genotypes, there were no significant differences in body weight between treated and untreated mice at either time point.
Measurement of 8-OHdG in kidney DNA

The results of 8-OHdG measurement in the kidneys are shown in Fig. 3. At both time points, KBrO₃ treatment significantly increased the level of 8-OHdG in the kidneys of both genotypes, and the degrees of 8-OHdG increase were as follows: 4 weeks $Nr{f_2}^{+/+}$, × 2.8; 4 weeks $Nr{f_2}^{-/-}$, × 2.8.

Table 1. Final Body and Kidney Weights of Male $Nr{f_2}^{+/+}$ or $Nr{f_2}^{-/-}$ gpt Delta Mice Treated with KBrO₃ or NFT for 4 or 13 Weeks

|                  | $Nr{f_2}^{+/+}$        | $Nr{f_2}^{-/-}$        |
|------------------|------------------------|------------------------|
|                  | Control 1,500 ppm KBrO₃ | 2,500 ppm NFT          | Control 1,500 ppm KBrO₃ | 2,500 ppm NFT |
|                  |                        |                        |                        |               |
| 4 weeks          |                        |                        |                        |               |
| No. of animals   | 4                      | 5                      | 4                      | 5             |
| Final body weights (g) | 27.43 ± 2.59 b | 25.70 ± 2.35        | 23.56 ± 2.15        | 26.40 ± 2.18  |
| Kidneys (g)      | 0.33 ± 0.03            | 0.32 ± 0.04           | 0.33 ± 0.04           | 0.32 ± 0.03   |
| Kidneys (g%)a    | 1.22 ± 0.11            | 1.26 ± 0.09           | 1.38 ± 0.06           | 1.20 ± 0.10   |
|                  |                        |                        |                        |               |
| 13 weeks         |                        |                        |                        |               |
| No. of animals   | 8                      | 8                      | 8                      | 8             |
| Final body weights (g) | 33.53 ± 3.45 | 29.18 ± 2.45        | 28.28 ± 1.67        | 30.05 ± 2.38  |
| Kidneys (g)      | 0.35 ± 0.03            | 0.34 ± 0.03           | 0.38 ± 0.05           | 0.35 ± 0.05   |
| Kidneys (g%)a    | 1.06 ± 0.08            | 1.16 ± 0.08           | 1.35 ± 0.12           | 1.18 ± 0.15   |

*Kidneys-to-body weight ratios (relative weights) are given as grams organ weight/grams body weight. bMean ± SD.

Fig. 2. Changes in mRNA levels of the $Nr{f_2}$-target gene $Nqo1$ in the kidneys of $Nr{f_2}^{+/+}$ or $Nr{f_2}^{-/-}$ mice treated with KBrO₃ or NFT for 4 weeks (A) or 13 weeks (B). Values are means ± SD. *Significantly different ($P<0.05$) from the respective control group. **Significantly different ($P<0.01$) from the respective control group. ##Significantly different ($P<0.01$) from the respective $Nr{f_2}^{+/+}$ animals.

Fig. 3. 8-OHdG levels in the kidneys of $Nr{f_2}^{+/+}$ or $Nr{f_2}^{-/-}$ mice treated with KBrO₃ or NFT for 4 weeks (A) or 13 weeks (B). Values are means ± SD. **Significantly different ($P<0.01$) from the respective control group. ††Significantly different ($P<0.01$) from the respective control group.
× 3.6; 13 weeks Nrf2+/+, × 2.1; 13 weeks Nrf2−/−, × 3.3 (vs. respective control). On the other hand, NFT treatment did not increase the level of 8-OHdG in the kidneys of either genotype at either time point. There was no significant difference in 8-OHdG level between the Nrf2-proficient and Nrf2-deficient mice of each treatment group.

**In vivo mutation assay of kidneys**

The results of the gpt assay of the kidneys of Nrf2-proficient and Nrf2-deficient mice treated with KBrO₃ or NFT are shown in Tables 2 to 5. At both time points, KBrO₃-treated mice showed a significant increase or tendency toward increase in gpt MFs compared with those in the respective control groups (Table 2). The degrees of increase in gpt MFs caused by 13 weeks of treatment with KBrO₃ were as follows: Nrf2+/+, × 2.2; Nrf2−/−, × 4.4 (vs. respective control; Table 2). Specific MFs of deletion mutations were increased in the spectrum analysis of gpt mutants in KBrO₃-treated mice (Table 3 and 4). The frequencies of deletion mutations of more than two base pairs were increased by 13

**Table 2. Gpt MFs in the Kidneys of Nrf2+/+ or Nrf2−/− gpt Delta Mice Treated with KBrO₃ or NFT for 4 or 13 Weeks**

| Treatment period | Genotype  | Treatment | No. of animals | MF# |
|------------------|-----------|-----------|----------------|-----|
| 4 weeks          | Nrf2+/+   | Control   | 4              | 0.36 ± 0.09 |
|                  |           | 1,500 ppm KBrO₃ | 5              | 0.70 ± 0.19* |
|                  |           | 2,500 ppm NFT  | 5              | 0.55 ± 0.27 |
|                  | Nrf2−/−   | Control   | 4              | 0.33 ± 0.08  |
|                  |           | 1,500 ppm KBrO₃ | 4              | 0.59 ± 0.15  |
|                  |           | 2,500 ppm NFT  | 5              | 0.55 ± 0.06  |
| 13 weeks         | Nrf2+/+   | Control   | 8              | 0.69 ± 0.43  |
|                  |           | 1,500 ppm KBrO₃ | 8              | 1.57 ± 0.67** |
|                  | Nrf2−/−   | Control   | 8              | 0.48 ± 0.18  |
|                  |           | 1,500 ppm KBrO₃ | 8              | 1.86 ± 0.78†† |
|                  |           | 2,500 ppm NFT  | 8              | 1.04 ± 0.24** |

*P<0.05 vs. respective control group; **P<0.01 vs. respective control group. ††Mean ± SD. Gpt assays were performed in three divided groups: the 4-week treatment group, 13-week treatment of KBrO₃ group, and the 13-week treatment of NFT group.

**Table 3. Mutation Spectra of gpt Mutant Colonies in the Kidneys of Nrf2+/+ or Nrf2−/− gpt Delta Mice Treated with KBrO₃ or NFT for 4 Weeks**

| Genotype  | Control  | 1,500 ppm KBrO₃ | 2,500 ppm NFT |
|-----------|----------|----------------|--------------|
| Transversions |
| G:C to T:A | 7 (18.4) | 16 (18.6) | 16 (21.6) |
| G:C to C:G | 2 (5.3) | 2 (2.3) | 7 (9.5) |
| A:T to T:A | 2 (5.3) | 10 (11.6) | 2 (2.7) |
| A:T to C:G | 0 | 2 (2.3) | 4 (5.4) |
| Deletions |
| Single bpa | 5 (13.2) | 16 (18.6) | 26 (35.1) |
| Over 2 bpb | 2 (5.3) | 7 (8.1) | 3 (4.1) |
| Insertions |
| Single bpa | 2 (2.6) | 7 (8.1) | 0 |
| Complex | 2 (5.3) | 3 (3.5) | 2 (2.7) |
| Total | 38 | 86 | 74 |

| Genotype  | Control  | 1,500 ppm KBrO₃ | 2,500 ppm NFT |
|-----------|----------|----------------|--------------|
| Transversions |
| G:C to T:A | 7 (15.9) | 6 (10.2) | 19 (25.7) |
| G:C to C:G | 2 (4.5) | 3 (5.1) | 5 (6.8) |
| A:T to T:A | 0 | 7 (11.9) | 6 (8.1) |
| A:T to C:G | 2 (4.5) | 1 (1.7) | 2 (2.7) |
| Deletions |
| Single bpa | 6 (13.6) | 19 (32.2) | 11 (14.9) |
| Over 2 bpb | 1 (2.3) | 5 (8.5) | 1 (1.4) |
| Insertions |
| Single bpa | 1 (2.3) | 1 (1.7) | 1 (1.4) |
| Complex | 3 (6.8) | 2 (2.7) | 1 (1.4) |
| Total | 44 | 59 | 74 |

*P<0.05 vs. respective control group; ††P<0.01 vs. respective control group. aSingle base pair deletion mutation; bDeletion mutation of more than two base pairs.
weeks of treatment with KBrO₃ in both genotypes (Table 4). Furthermore, in both genotypes, gpt MFs were increased by 13 weeks of treatment with NFT, despite no change at 4 weeks (Table 2). The degrees of increase in gpt MFs caused by 13 weeks of treatment with NFT were as follows: Nrf2⁺/⁺, × 2.1; Nrf2⁻/⁻, × 3.3 (vs. respective control; Table 2). In both genotypes, guanine base substitution mutations, including G:C to T:A or G:C to C:G transversion mutations, were increased by 13 weeks of treatment with NFT (Table 5).

The results of the Spi⁻ assay of the kidneys of Nrf2-proficient and Nrf2-deficient mice treated with KBrO₃ or NFT are shown in Tables 6 to 8. At both time points, KBrO₃-treated mice showed a significant increase or tendency toward increase in Spi⁻ MFs compared with those in the respective control groups (Table 6). The degrees of increase in Spi⁻ MFs caused by 13 weeks of treatment with KBrO₃ were as follows: Nrf2⁺/⁺, × 3.0; Nrf2⁻/⁻, × 4.1 (vs. respective control; Table 6). In the spectrum analysis of Spi⁻ mutants

### Table 4. Mutation Spectra of gpt Mutant Colonies in the Kidneys of Nrf2⁺/⁺ or Nrf2⁻/⁻ gpt Delta Mice Treated with KBrO₃ for 13 Weeks

| Nrf2⁺/⁺ | Nrf2⁻/⁻ |
|---------|---------|
| Control | 1,500 ppm KBrO₃ | Control | 1,500 ppm KBrO₃ |
| Number (%) | Mutation frequencies (10⁻⁵) | Number (%) | Mutation frequencies (10⁻⁵) | Number (%) | Mutation frequencies (10⁻⁵) | Number (%) | Mutation frequencies (10⁻⁵) |
| Base substitution |
| Transversions |
| G:C to T:A | 15 (25.9) | 0.18 ± 0.16 | 20 (10.7) | 0.16 ± 0.11 | 13 (28.9) | 0.13 ± 0.06 | 15 (8.2) | 0.15 ± 0.11 |
| G:C to C:G | 2 (3.4) | 0.03 ± 0.06 | 5 (2.7) | 0.04 ± 05 | 1 (2.2) | 0.01 ± 0.02 | 2 (1.1) | 0.02 ± 0.03 |
| A:T to T:A | 0 | 0 | 11 (5.9) | 0.10 ± 0.11* | 3 (6.7) | 0.03 ± 0.03 | 28 (15.4) | 0.27 ± 0.16† |
| A:T to C:G | 0 | 0 | 10 (5.3) | 0.07 ± 0.06** | 0 | 0 | 1 (0.5) | 0.01 ± 0.03 |
| Deletion |
| Single bp | 11 (19.0) | 0.12 ± 0.11 | 69 (36.9) | 0.60 ± 0.34** | 3 (6.7) | 0.03 ± 0.03 | 62 (34.1) | 0.65 ± 0.41†† |
| Over 2 bp | 2 (3.4) | 0.03 ± 0.05 | 20 (10.7) | 0.17 ± 0.07** | 1 (2.2) | 0.01 ± 0.03 | 29 (15.9) | 0.30 ± 0.13†† |
| Insertion | 4 (6.9) | 0.05 ± 0.08 | 7 (3.7) | 0.06 ± 0.09 | 3 (6.7) | 0.04 ± 0.03 | 4 (2.2) | 0.05 ± 0.06 |
| Total | 58 | 0.69 | 187 | 1.57 | 45 | 0.48 | 182 | 1.86 |

*P<0.05 vs. respective control group; **P<0.01 vs. respective control group; †P<0.05 vs. respective control group; ††P<0.01 vs. respective control group. aSingle base pair deletion mutation; bDeletion mutation of more than two base pairs.

### Table 5. Mutation Spectra of gpt Mutant Colonies in the Kidneys of Nrf2⁺/⁺ or Nrf2⁻/⁻ gpt Delta Mice Treated with NFT for 13 Weeks

| Nrf2⁺/⁺ | Nrf2⁻/⁻ |
|---------|---------|
| Control | 2,500 ppm NFT | Control | 2,500 ppm NFT |
| Number (%) | Mutation frequencies (10⁻⁵) | Number (%) | Mutation frequencies (10⁻⁵) | Number (%) | Mutation frequencies (10⁻⁵) | Number (%) | Mutation frequencies (10⁻⁵) |
| Base substitution |
| Transversions |
| G:C to T:A | 9 (22.0) | 0.08 ± 0.07 | 31 (28.2) | 0.29 ± 0.05** | 8 (20.5) | 0.07 ± 0.05 | 47 (30.9) | 0.43 ± 0.15†† |
| G:C to C:G | 4 (9.8) | 0.04 ± 0.05 | 28 (25.5) | 0.26 ± 0.06** | 3 (7.7) | 0.02 ± 0.03 | 38 (25.0) | 0.37 ± 0.17†† |
| A:T to T:A | 0 | 0 | 5 (4.5) | 0.05 ± 0.07 | 1 (2.6) | 0.01 ± 0.02 | 7 (4.6) | 0.07 ± 0.06† |
| A:T to C:G | 1 (2.4) | 0.01 ± 0.02 | 2 (1.8) | 0.02 ± 0.04 | 0 | 0 | 3 (2.0) | 0.02 ± 0.03 |
| Deletion |
| Single bp | 4 (9.8) | 0.04 ± 0.04 | 7 (6.4) | 0.06 ± 0.05 | 9 (23.1) | 0.09 ± 0.09 | 10 (6.6) | 0.09 ± 0.05 |
| Over 2 bp | 2 (4.9) | 0.03 ± 0.05 | 2 (1.8) | 0.02 ± 0.03 | 1 (2.6) | 0.02 ± 0.03 | 3 (2.0) | 0.03 ± 0.04 |
| Insertion | 0 | 0 | 1 (0.9) | 0.01 ± 0.02 | 1 (2.2) | 0.01 ± 0.02 | 5 (3.3) | 0.04 ± 0.06 |
| Complex | 1 (2.4) | 0.01 ± 0.03 | 4 (3.6) | 0.04 ± 0.07 | 0 | 0 | 9 (5.9) | 0.08 ± 0.10 |
| Total | 41 | 0.38 | 110 | 1.04 | 39 | 0.39 | 152 | 1.40 |

**P<0.01 vs. respective control group; †P<0.05 vs. respective control group; ††P<0.01 vs. respective control group. aSingle base pair deletion mutation; bDeletion mutation of more than two base pairs.
in KBrO₃-treated mice, specific MFs of deletion mutations were increased (Table 7 and 8), consistent with the spectrum analysis of gpt mutants. In both genotypes and at both time points, NFT treatment did not change Spi– MFs (Table 6).

**Discussion**

It is well known that transcriptional upregulation of various antioxidant enzymes, including NQO1 and HO1, is regulated by NRF2, which protect cells from oxidative stress25, 26. In several studies, Nrf2⁻/⁻ mice showed higher sensitivity to various toxicants that induced oxidative stress27-31. In fact, the mRNA expression level of Nqo1 in the kidneys of vehicle-treated Nrf2⁻/⁻ mice was significantly lower than that of vehicle-treated Nrf2⁻/⁻ mice, and there was no elevation of the level in KBrO₃- or NFT-treated Nrf2⁻/⁻ mice despite the elevation in Nrf2⁻/⁻ mice. Thus, in the present study, Nrf2⁻/⁻ mice were confirmed to be susceptible to oxidative stress. As previously reported, using this highly oxidative stress-sensitive animal gives us important knowledge about the involvement of oxidative stress in chemical-induced genotoxicity and carcinogenesis27-31.

Four or thirteen weeks of administration of 1,500 ppm KBrO₃ in drinking water significantly increased the level of 8-OHdG in the kidneys of both genotypes. At both time points, the degree of 8-OHdG increase was higher in Nrf2⁻/⁻ mice than in Nrf2⁺/⁺ mice. Meanwhile, increases in gpt and Spi– MFs were detected, and the tendencies of the degrees of increase in gpt and Spi– MFs at 13 weeks of exposure were the same as those of 8-OHdG. In the spectrum analysis of gpt and Spi– mutants in KBrO₃-treated Nrf2⁻/⁻ mice, specific MFs of deletion mutations were increased, consistent with a previous study of rats24, and they were accompanied by an increase in the frequencies of deletion mutations of more than two base pairs. An *in vitro* report demonstrated that error in the repair process of 8-OHdG induced by KBrO₃ treatment caused double-strand breaks (DSBs) in human cells and that DSBs resulted in a large deletion38. Considering these mechanisms, the increase in size of deletion mutations might reflect the accumulation of high amounts

**Table 6.** Spi– MFs in the Kidneys of Nrf2⁺/⁺ or Nrf2⁻/⁻ gpt Delta Mice Treated with KBrO₃ or NFT for 4 or 13 Weeks

| Treatment period | Genotype | Treatment | No. of animals | MF (%) |
|------------------|----------|-----------|----------------|--------|
| 4 weeks          | Nrf2⁺/⁺  | Control   | 4              | 0.24 ± 0.13 |
|                  |          | 1,500 ppm KBrO₃ | 5              | 0.43 ± 0.16 |
|                  |          | 2,500 ppm NFT  | 5              | 0.30 ± 0.13 |
|                  | Nrf2⁻/⁻  | Control   | 4              | 0.18 ± 0.05 |
|                  |          | 1,500 ppm KBrO₃ | 4              | 0.36 ± 0.10 |
|                  |          | 2,500 ppm NFT  | 5              | 0.26 ± 0.07 |
| 13 weeks         | Nrf2⁺/⁺  | Control   | 8              | 0.31 ± 0.06 |
|                  |          | 1,500 ppm KBrO₃ | 8              | 0.85 ± 0.16 ** |
|                  |          | 2,500 ppm NFT  | 7              | 0.33 ± 0.07 |
|                  | Nrf2⁻/⁻  | Control   | 8              | 0.28 ± 0.06 |
|                  |          | 1,500 ppm KBrO₃ | 8              | 0.92 ± 0.28 †† |
|                  |          | 2,500 ppm NFT  | 8              | 0.36 ± 0.14 |

*P<0.05 vs. respective control group; ** P<0.01 vs. respective control group. ††P<0.01 vs. respective control group; MF, Mutant frequency; *Means ± SD. Spi– assays were performed in two divided groups: the 4-week treatment group and 13-week treatment group.

**Table 7.** Mutation Spectra of Spi– Plaques in the Kidneys of Nrf2⁺/⁺ or Nrf2⁻/⁻ gpt Delta Mice Treated with KBrO₃ or NFT for 4 Weeks

| Treatment | Control | 1,500 ppm KBrO₃ | 2,500 ppm NFT |
|-----------|---------|-----------------|--------------|
| Number (%) | Number frequencies (10⁻⁵) | Number (%) | Number frequencies (10⁻⁵) | Number (%) | Number frequencies (10⁻⁵) |
| Nrf2⁺/⁺   | **Simple**
| G or C    | 6 (25.0) | 0.06 ± 0.04     | 6 (9.2) | 0.04 ± 0.05     | 6 (12.0) | 0.03 ± 0.03     |
| A or T    | 0              | 0              | 5 (7.7) | 0.03 ± 0.04     | 1 (2.0)  | 0.01 ± 0.01     |
| In run    | G or C  | 4 (16.7) | 0.04 ± 0.03     | 17 (26.2) | 0.12 ± 0.08     | 15 (30.0) | 0.09 ± 0.09     |
| A or T    | 5 (20.8) | 0.05 ± 0.03 | 18 (27.7) | 0.11 ± 0.09     | 13 (26.0) | 0.08 ± 0.04     |
| 2 to 1 kb deletiona | 0              | 0              | 5 (7.7) | 0.03 ± 0.04     | 0              | 0              |
| Over 1 kb deletiona | 8 (33.3) | 0.08 ± 0.10 | 12 (18.5) | 0.08 ± 0.11     | 12 (24.0) | 0.07 ± 0.07     |
| Complex   | 1 (4.2)  | 0.01 ± 0.02   | 2 (3.1)  | 0.01 ± 0.03     | 1 (2.0)  | 0.01 ± 0.01     |
| Insertion | 0                | 0              | 0                | 1 (2.0)  | 0.01 ± 0.02     |
| Base substitution | 0                | 0              | 0                | 1 (2.0)  | 0.00 ± 0.01     |
| Total     | 24      | 0.24           | 65              | 0.43     | 50              | 0.30     |
| Nrf2⁻/⁻   | **Simple**
| G or C    | 0              | 0              | 7 (20.6) | 0.08 ± 0.05     | 3 (7.9)  | 0.02 ± 0.02     |
| A or T    | 2 (8.0)  | 0.01 ± 0.02   | 2 (5.9)  | 0.02 ± 0.02     | 0              | 0              |
| In run    | G or C  | 7 (28.0) | 0.04 ± 0.03     | 9 (26.5) | 0.10 ± 0.09     | 12 (31.6) | 0.08 ± 0.05     |
| A or T    | 8 (32.0) | 0.06 ± 0.08 | 8 (23.5) | 0.09 ± 0.06     | 11 (28.9) | 0.08 ± 0.05     |
| 2 to 1 kb deletiona | 0              | 0              | 3 (8.8)  | 0.03 ± 0.04     | 0              | 0              |
| Over 1 kb deletiona | 8 (32.0) | 0.07 ± 0.06 | 4 (11.8) | 0.04 ± 0.04     | 12 (31.6) | 0.08 ± 0.05     |
| Complex   | 0                | 0              | 1 (2.9)  | 0.01 ± 0.01     | 0              | 0              |
| Insertion | 0                | 0              | 0                | 0              | 0              | 0              |
| Base substitution | 0                | 0              | 0                | 0              | 0              | 0              |
| Total     | 25      | 0.18           | 34              | 0.36     | 38              | 0.26     |

*aDeletion mutation of two or more but less than one thousand base pairs. bDeletion mutation of more than one thousand base pairs.*
of 8-OHdG in the nuclei due to KBrO₃. These results suggested that the formation of 8-OHdG induced by oxidative stress was directly involved in the increase of deletion mutations in KBrO₃-treated animals. It was suspected that the formation of high amounts of 8-OHdG owing to the strong oxidizing potential of KBrO₃, as an oxidizing agent might exceed the repairing capacity of base excision repair enzymes.

Four or thirteen weeks of administration of 2,500 ppm NFT in diet did not increase the level of 8-OHdG in the kidneys of either genotype. In our previous study, the level of 8-OHdG was increased in the kidneys of Nrf2−/− mice by oral administration of NFT at 70 mg/kg. The lower exposure levels of NFT in the present study compared with those in our previous study might be a cause of the static level of 8-OHdG. On the other hand, 13 weeks administration of 2,500 ppm NFT in diet significantly increased gpt MFs with guanine base substitution mutations in the kidneys of both genotypes. The degree of increase in gpt MFs was higher in Nrf2−/− mice than in Nrf2+/− mice. These results implied that the vulnerability to oxidative stress caused by the deficiency of Nrf2 leads to more mutations in NFT-treated mice. Thus, in the genotoxic mechanism of NFT, the formation of 8-OHdG induced by oxidative stress might not be involved in the increase of guanine base substitution mutations. Considering our previous studies, which suggested the involvement of oxidative stress in the chemical structure-related genotoxicity of NFT in rodents, factors other than 8-OHdG might be crucial to the genotoxicity of NFT, though the present study did not identify them.

In recent years, the level of 8-OHdG has been frequently used as a marker of oxidative stress in human diseases. In addition, some reports demonstrated the involvement of oxidative stress in chemical-induced genotoxicity and carcinogenesis using the increase in 8-OHdG level as a parameter of oxidative stress in experimental animals. However, the relationship between the formation of 8-OHdG and subsequent mutations, including deletion mutations and G:C to T:A transversions, had not been clarified. The revelation of the relationship between 8-OHdG and several types of mutations induced by KBrO₃ or NFT provides new insight into oxidative stress-related in vivo mutagenicity.

The present study demonstrated that the formation of 8-OHdG, which resulted from the oxidizing potential of KBrO₃, was directly involved in the increase of deletion mutations; however, oxidative stress-related factors other than 8-OHdG might play a critical role in NFT-induced guanine base substitution mutations. This was the first study to investigate the relationship between 8-OHdG and several types of mutations caused by oxidative stress-inducing chemicals. Accumulation of detailed examinations like this, such as further research on 8-OHdG with respect to individual chemical substances, will lead to accurate risk assessment of oxidative stress in carcinogenicity.

**Disclosure of Potential Conflicts of Interest:** The authors declare that they have no competing interests.

### Table 8. Mutation Spectra of Spi− Plaques in the Kidneys of Nrf2+/+ or Nrf2−/− gpt Delta Mice Treated with KBrO₃ or NFT for 13 Weeks

|                | Control                  | 1,500 ppm KBrO₃ | 2,500 ppm NFT | 2,500 ppm NFT |
|----------------|--------------------------|-----------------|---------------|---------------|
| **Nrf2−/−**    |                          |                 |               |               |
| Single bp deletion | G or C                   | 10 (13.7)       | 24 (12.8)     | 6 (12.8)      | 0.05 ± 0.05   | 0.11 ± 0.07*  |
|                | A or T                   | 2 (2.7)         | 13 (7.0)      | 2 (4.3)       | 0.01 ± 0.02   | 0.05 ± 0.04*  |
| In run         | G or C                   | 20 (27.4)       | 60 (32.1)     | 22 (46.8)     | 0.16 ± 0.10   | 0.27 ± 0.09** |
|                | A or T                   | 28 (38.4)       | 63 (33.7)     | 10 (21.3)     | 0.06 ± 0.06   | 0.29 ± 0.10** |
| 2 to 1 kb deletion | G or C                   | 1 (1.4)         | 12 (6.4)      | 0            | 0             | 0.05 ± 0.05*  |
|                | A or T                   | 0.00 ± 0.01     | 4 (8.5)       | 0.02 ± 0.03   | 0.08 ± 0.06   | 0            |
| Insertion      | 0                        | 1               | (2.1)         | 0.00 ± 0.01   | 0             | 0            |
| Base substitution | 0                        | 0.00 ± 0.01     | 2 (4.3)       | 0.01 ± 0.03   | 0             | 0            |
| Total          | 73                       | 187             | 47            | 0.33          | 0.85          |               |
| **Nrf2+/+**    |                          |                 |               |               |
| Single bp deletion | G or C                   | 13 (28.3)       | 17 (10.8)     | 5 (7.0)       | 0.03 ± 0.05   | 0.10 ± 0.09   |
|                | A or T                   | 1 (2.2)         | 15 (9.6)      | 3 (4.2)       | 0.02 ± 0.02   | 0.10 ± 0.11†  |
| In run         | G or C                   | 6 (13.0)        | 44 (28.0)     | 24 (33.8)     | 0.12 ± 0.07†  | 0.23 ± 0.09†† |
|                | A or T                   | 22 (47.8)       | 50 (31.8)     | 20 (28.2)     | 0.10 ± 0.06   | 0.31 ± 0.13†† |
| 2 to 1 kb deletion | G or C                   | 0              | 19 (12.1)     | 3 (4.2)       | 0.02 ± 0.03   | 0.13 ± 0.09†† |
|                | A or T                   | 0.02 ± 0.04     | 11 (7.0)      | 14 (19.7)     | 0.08 ± 0.07   | 0.05 ± 0.07   |
| Insertion      | 0                        | 0              | 1 (1.4)       | 0.00 ± 0.01   | 0             | 0.01 ± 0.02   |
| Base substitution | 0                        | 0              | 1 (1.4)       | 0.00 ± 0.01   | 0             | 0            |
| Total          | 46                       | 157             | 71            | 0.36          | 0.92          | 0.00 ± 0.01   |

*P<0.05 vs. respective control group; **P<0.01 vs. respective control group. †P<0.05 vs. respective control group; ††P<0.01 vs. respective control group. aDeletion mutation of two or more but less than one thousand base pairs. bDeletion mutation of more than one thousand base pairs.
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