の時期を迎え、春に高く、秋（10月）に低い傾向が認められた。この季節変化の特徴は釜房ダム湖における藻類個体数の変化に一致し、AOC の動態に藻類が関与している可能性が考えられた。

② *Phormidium tenue* 及び *Melosira granulata* が増殖過程で代謝する EOM には AOC 成分が含まれることが明らかになった。AOC 成分の代謝は藻類の増殖の時期によって異なり、AOC 濃度は安定期に最も高くなることが分かった。M-11培地による *Phormidium tenue* の培養において EOM として放出される AOC は分子量1,000以下の P17成分が主であることが明らかになった。AOC の代謝は環境因子としての培地の有機物質濃度に影響を受け、より低濃度の条件において AOC の生産が大きい可能性が示された。

謝辞：本研究を行うにあたり多大なるご協力をいただきました仙台市水道局の皆様に心より感謝いたします。

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Dose-related changes of oxidative stress and cell proliferation in kidneys of male and female F344 rats exposed to potassium bromate

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It is still of importance to investigate renal carcinogenesis by potassium bromate ($KBrO_3$), a by-product of water disinfection by ozonation, for assessment of the risk to man. Five female F344 rats in each group were given $KBrO_3$ at a dose of 300 mg/kg by single i.g. intubation or at a dose of 80 mg/kg by single i.p. injection, and were killed 48 h after the administration for measurements of thiobarbituric acid-reactive substances (TBARS) and 8-oxodeoxyguanosine (8-oxodG) levels in the kidney. Both levels in the treated animals were significantly elevated as compared with the control values. In a second experiment, 5 male and female F344 rats in each group were administered $KBrO_3$ at concentrations of 0, 15, 30, 60, 125, 250 and 500 ppm in the drinking water for 4 weeks. $KBrO_3$ in the drinking water did not elevate TBARS in either sex at any of the doses examined, but 8-oxodG formation in both sexes at 250 ppm and above was significantly higher than in the controls. Additionally, the bromodeoxyuridine-labeling index for proximal convoluted tubules was significantly increased at 30 ppm and above in the males, and at 250 ppm and above in the females. α2u-Globulin accumulation in the kidneys of male rats was increased with statistical significance at 125 ppm and above. These findings suggest that DNA oxidation induced by $KBrO_3$ may occur independently of lipid peroxidation and more than 250 ppm $KBrO_3$ in the drinking water can exert a carcinogenic effect by way of oxidative stress. (Cancer Sci 2004; 95: 393–398)

Potassium bromate ($KBrO_3$) was at one time widely used as a maturing agent for flour and as a dough conditioner.[1] It was, however, demonstrated to induce renal cell tumors in male and female F344 rats after oral administration for 2 years in the drinking water[2] and the use of $KBrO_3$ as a food additive is now limited or prohibited, so that exposure of humans via food is very low.[3] Nevertheless, there is still concern regarding this chemical in the environment. In order to avoid the formation of trihalomethanes, major by-products in the process of drinking water chlorination[4] that are carcinogenic in rodents,[5] ozone disinfection has been proposed as an alternative method.[6] However, it has been shown that ozonation of surface water can generate bromate as one of various by-products in treated drinking water,[7] implying a potential hazard.

$KBrO_3$ has been classified as a genotoxic carcinogen based on positive mutagenicity in the Ames,[8] chromosome aberration[9] and micronucleus tests.[10] It has the potential to induce 8-oxodeoxyguanosine (8-oxodG) formation both *in vitro* and *in vivo*,[11–14] and since ribo- and deoxyribonucleosides of 8-oxodG induce sister chromatid exchange in human lymphocytes[15] and 8-oxodG pairs with adenine as well as cytosine, generating GC-to-TA transversion upon replication by DNA polymerases,[16] it has been postulated that this oxidized base is responsible for the mutagenicity and carcinogenicity.[17,18] The formation of oxidized base also indicates that the intra-nuclear redox status is altered in an oxidative direction, and this may lead to the induction of aberrant transcriptional events. However, except for our previous paper,[19] we know of no data showing a direct correlation between actual carcinogenic doses and 8-oxodG formation in kidney DNA. Likewise, although it has been proposed that reactive free radicals resulting from the oxidizing property of $KBrO_3$ also attack membrane lipids to induce cellular lipid peroxidation (LPO) in male rats,[20,21] it remains uncertain whether LPO indeed occurs concomitantly with DNA oxidation during carcinogenesis. In view of the possible role of various reactive aldehydes as end products of LPO in tumorigenesis,[22,23] it is necessary to assess their participation in $KBrO_3$ carcinogenesis.

A two-stage model using *N*-ethyl-*N*-hydroxyethyl-nitrosamine (EHEN) as an initiator has supplied clear evidence that $KBrO_3$ has promoting activity for renal carcinogenesis in male and female rats.[24,25] We have also shown that numbers of bromodeoxyuridine (BrdU)-incorporating cells in kidney tubules are elevated in male and female rats exposed to $KBrO_3$ at a dose of 500 ppm in the drinking water.[19,26] While we have hypothesized the involvement of oxidative stress induced by $KBrO_3$ in the promoting activity, our previous data also suggest that the promoting action observed in male rats may be dependent on cell proliferation due to accumulation of α2u-globulin, a male rat specific urinary protein.[26] Elimination of this possibility as a factor contributing to $KBrO_3$ promoting activity is a prerequisite for accurate assessment of the carcinogenic risk in humans.

In the present study, in order to confirm a positive correlation between oxidized DNA base formation and occurrence of LPO, we measured the levels of 8-oxodG and thiobarbituric acid-reactive substances (TBARS) in kidneys of F344 female rats given $KBrO_3$ by single administration at high doses. Secondly, we examined the dose-response effects with reference to 8-oxodG levels, TBARS, BrdU-labeling and α2u-globulin accumulation in kidney, as well as serum creatinine (CRN) level, of male and female rats, employing the same doses and route as used in the previous carcinogenicity tests and promoting activity assays. The aim was to clarify the possibility that LPO and oxidative DNA damage participate in $KBrO_3$ initiation and to cast light on the effects of oxidative stress in the promotion phase.

Materials and Methods

Chemicals. $KBrO_3$ was purchased from Wako Pure Chemical Industries, Ltd. (Osaka). Alkaline phosphatase and BrdU were obtained from Sigma Chemical Co. (St. Louis, MO) and nuclease P1 was from Yamasa Shoyu Co. (Chiba).

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Animals, diet and housing conditions. The protocols for this study were approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences. Five-week-old male and female F344 rats (specific pathogen-free) were purchased from Charles River Japan (Kanagawa) and housed in polycarbonate cages (5 rats per cage) with hardwood chips for bedding in a conventional animal facility maintained under conditions of controlled temperature (23±2°C), humidity (55±5%), air change (12 times per h) and lighting (12 h light/dark cycle). The animals were given free access to CRF-1 basal diet (Charles River Japan) and tap water, and were used after a 1-week acclimation period.

Animal treatments.

Experiment I: Five female rats in each group were given $KBrO_3$ at a single dose of 300 mg/kg by i.g. administration or 80 mg/kg by i.p. injection. Control animals received saline at the same volume as the i.p. administration group. All animals were killed 48 h after the administration under ethyl ether anesthesia, and the right and half of the left kidneys were immediately removed and frozen with liquid nitrogen and stored at −80°C until measurement of 8-oxodG in nuclear DNA and TBARS levels. The remaining kidney tissue was fixed in buffered formalin and then routinely processed for embedding in paraffin, sectioning and H&E staining. The doses and experimental period followed reported conditions under which the 8-oxodG and TBARS levels in kidney were significantly increased.[27]

Experiment II: Five male and female rats in each group were administered $KBrO_3$ solution at concentrations of 0, 15, 30, 60, 125, 250 and 500 ppm in the drinking water for 4 weeks. All animals were injected with BrdU (100 mg/kg) i.p. twice a day for the final 2 days of the exposure and once on the day of termination, 2 h before killing. For analysis of CRN, the animals were anesthetized with ethyl ether and blood was collected from the aorta. Determination of CRN was carried out at SRL, Inc. (Tokyo). At necropsy, the right kidneys were fixed in ice-cold acetone for 3 days and processed for embedding in paraffin, sectioning (4 μm), and immunostaining for BrdU after histochemical demonstration of γ-glutamyltranspeptidase (γ-GT) activity. The left kidneys were frozen and stored as in Experiment I until measurement of 8-oxodG in nuclear DNA, and TBARS levels and α2u-globulin contents in the homogenates.

Measurement of nuclear 8-oxodG. The 8-oxodG levels in kidney DNA were determined according to the method of Nakae *et al.*[28] Nuclear DNA was extracted with a DNA Extracter WB Kit (Wako Pure Chemical Industries, Ltd., Osaka). The DNA was digested to deoxynucleotides with nuclease P1 and alkaline phosphatase and levels of 8-oxodG (8-oxodG/10^5 deoxyguanosine) were assessed by high-performance liquid chromatography (HPLC) with an electrochemical detection system (Coulochem II, ESA, Bedford, MA).

Measurement of TBARS. Malondialdehyde (MDA, nmol/g) was assessed as an index of LPO by the method of Uchiyama and Mihara.[29] A 0.1 g portion of kidney was homogenized with 0.9 ml of 1.15% KCl solution and the TBARS content was measured.

α2u-Globulin content. α2u-Globulin accumulation in kidneys was measured using a commercially available ELISA kit (Quatikinine M, R&D Systems, Inc., MN). Absorbance at 450 nm was determined using a microplate reader (Thermo Labsystems, Vantaa, Finland), with the reference wavelength set at 590 nm.

Immunohistochemical procedures. For immunohistochemical staining of BrdU, sections were treated sequentially with normal horse serum, monoclonal mouse anti-BrdU (Becton Dickinson) (1:100), biotin-labeled horse anti-mouse IgG (1:400) and avidin-biotin-peroxidase complex (ABC) after denaturation of DNA with 4 *N* HCl. Before the denaturation step, sections were processed histochemically for demonstration of γ-GT activity by the method of Rutenburg *et al.*[30] using L-glutamyl-4-methoxy-β-naphthylamide (Polysciences, Ltd., Warrington, PA) as a substrate in order to assist in distinguishing the three kinds of tubules, as previously described.[26] The sites of peroxidase binding were demonstrated by incubation with 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.). The immunostained sections were lightly counterstained with hematoxylin for microscopic examination.

Cell proliferation quantification. Cells of the three kinds of tubules in the kidney were identified on the basis of γ-GT activity and morphology as previously described.[31] At least 3000 tubular cells in each kidney were counted. The labeling index (LI) was calculated as the percentage of cells positive for BrdU incorporation.

Statistics. The significance of differences in the results from Experiment I was evaluated with Student's *t* test. For Experiment II ANOVA was used, followed by Dunnett's multiple comparison test.

Results

Experiment I. The data for 8-oxodG and TBARS levels in kidneys of female rats given $KBrO_3$ by single administration at doses of 300 mg/kg (i.g.) or 80 mg/kg (i.p.) are summarized in Fig. 1. Values for both parameters were significantly ($P<0.01$) elevated as compared with the controls, in line with previous data. Histopathological examination revealed severe nephrotoxicity characterized by hemorrhage and protein diapedesis in Bowman's capsule, accumulation of hyaline droplet-like material and basophilic alteration in proximal tubules, and extensive necrosis of collecting ducts (Fig. 6, A and B).

Experiment II. As shown in Fig. 2, $KBrO_3$ in the drinking water did not cause elevation of TBARS in kidneys of either sex at any of the doses examined. However, 8-oxodG levels in male

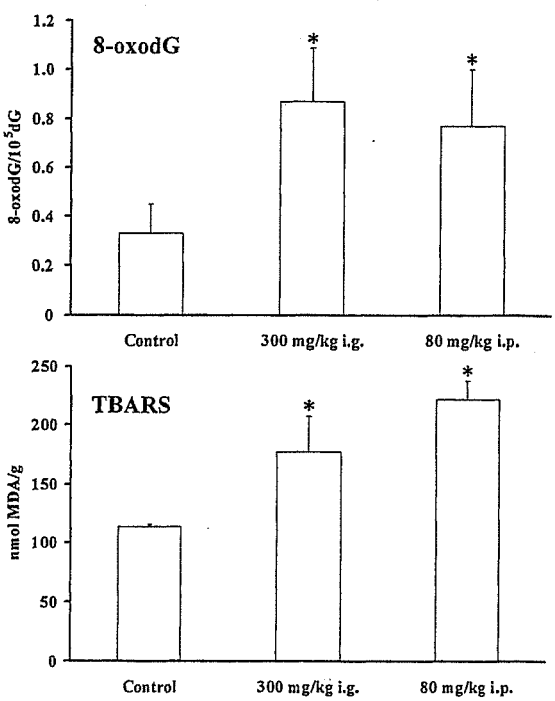

Fig. 1. 8-OxodG and TBARS levels in kidneys of female rats 48 h after single i.g. or i.p. administration of $KBrO_3$ at the dose of 300 or 80 mg/kg, respectively. Values are means±SD of data for 5 rats. Significantly different (* $P<0.01$) from the control group treated with saline alone.

rats exposed to $KBrO_3$ in the drinking water were elevated at concentrations of 250 ppm and above in a clearly dose-dependent manner (250 ppm, $0.57\pm0.19/10^5$ dG, $P<0.01$; 500 ppm, $0.71\pm0.21/10^5$ dG, $P<0.01$) as compared to the control value ($0.31\pm0.06/10^5$ dG). Likewise, 8-oxodG levels in female rats were $0.51\pm0.10/10^5$ dG at 250 ppm and $0.70\pm0.16/10^5$ dG at 500 ppm, which were statistically significantly higher ($P<0.01$) than the control value ($0.25\pm0.05/10^5$ dG). Histopathologically, although degeneration of proximal tubules was dose-dependently observed in the males at 60 ppm and above, there were no overt nephrotoxicity in the females at any of the doses examined. Fig. 3 illustrates changes in BrdU-LI for each tubule type in male and female rats treated with $KBrO_3$ in the drinking water at concentrations of 0, 15, 30, 60, 125, 250 and 500 ppm for 4 weeks. BrdU-LIs of proximal convoluted tubular cells (PCT) in the males were elevated in a dose-dependent manner, with significant increases at 30 ppm ($1.69\pm0.32\%$, $P<0.01$), 60 ppm ($2.67\pm0.45\%$, $P<0.01$), 125 ppm ($4.23\pm0.80\%$, $P<0.01$), 250 ppm ($6.11\pm2.23\%$, $P<0.01$) and 500 ppm ($9.10\pm1.40\%$, $P<0.01$), as compared to the control value ($0.87\pm0.32\%$). In the females, although there was no change up to 125 ppm, dose-dependent increase was subsequently observed to $1.29\pm0.39\%$ at 250 ppm and $2.22\pm0.37\%$ at 500 ppm, both of which were statistically siginificant ($P<0.01$) as compared to the control value ($0.59\pm0.14\%$) (Fig. 6C). On the other hand, no change in BrdU-LIs for other tubules was found at any dose in either sex. Fig. 4 summarizes data for α2u-globulin accumulation in kidneys of male and female rats given $KBrO_3$ in the drinking water. In the males, increase was evident at 30 ppm and above in a dose-dependent fashion, the elevation being statistically significant at 125 ppm (1.37 ± 0.18 mg/ml, $P<0.01$), 250 ppm (1.96 ± 0.24 mg/ml, $P<0.01$) and 500 ppm (3.50 ± 0.26 mg/ml, $P<0.01$) as compared to the control value (0.80 ± 0.16 mg/ml). In contrast, α2u-globulin contents in the females were much lower than those in the males and were not changed by $KBrO_3$ exposure. Fig. 5 shows the changes of serum CRN levels in rats of both sexes given $KBrO_3$ in the drinking water. In contrast to the male data, revealing a slight, but statistically significant elevation at 250 ppm and above, there was no change among the female groups.

Discussion

It is generally accepted that oxygen radicals can attack DNA to produce damaged bases, including 8-oxodG, and/or initiate the oxidative decomposition of cellular membranes by LPO,[23]

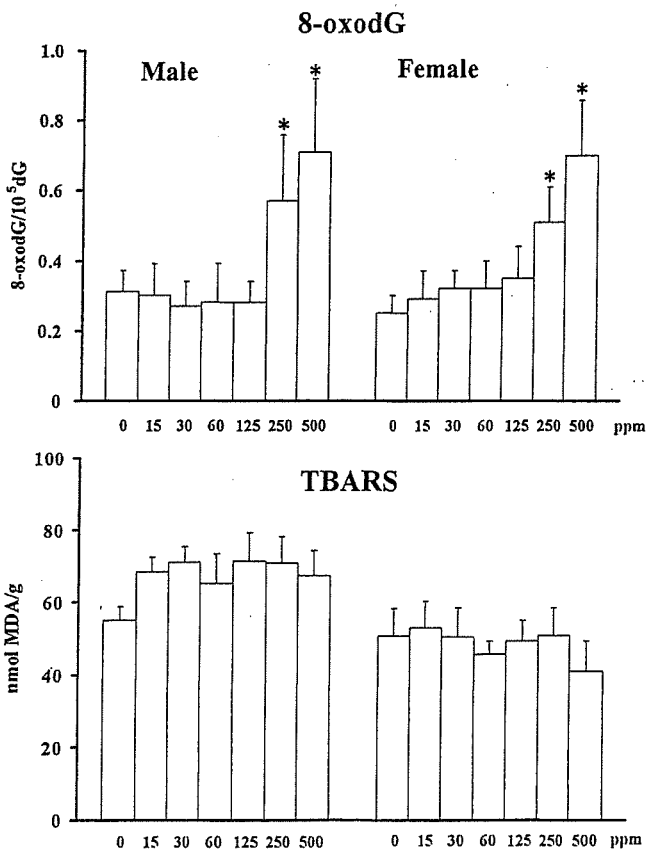

Fig. 2. 8-OxodG and TBARS levels in kidneys of male and female rats given $KBrO_3$ in the drinking water for 4 weeks at doses of 0–500 ppm. Values are means±SD of data for 5 rats. Significantly different (* $P<0.01$) from the control group (0 ppm).

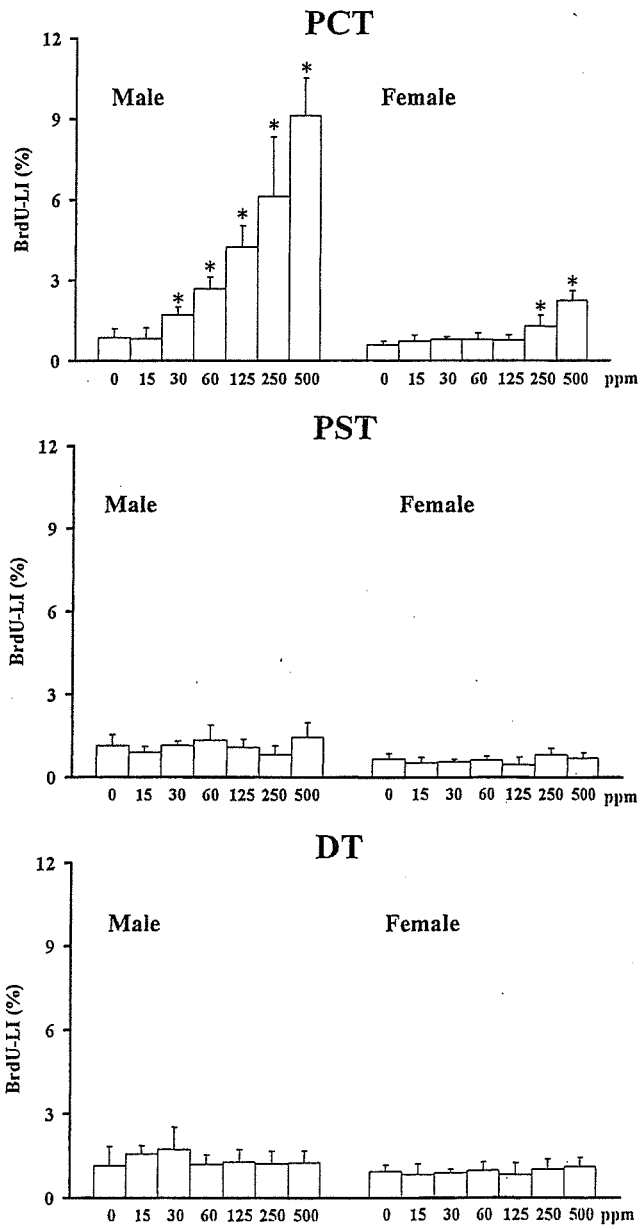

Fig. 3. BrdU-LIs for the proximal convoluted, straight and distal tubules (PCT, PST, DT) of male and female rats given $KBrO_3$ in the drinking water for 4 weeks at doses of 0–500 ppm. Values are means±SD of data for 5 rats. Significantly different (* $P<0.01$) from the control group (0 ppm).

which not only act as intermediates for free radical chain reactions, but also generate various reactive aldehydes, such as malondialdehyde and *trans*-4-hydroxy-2-nonenal, which directly form exocyclic DNA adducts.[22,32] A single exposure of male rats to $KBrO_3$ at high doses causes an increase of TBARS along with 8-oxodG formation,[27] which was also confirmed in the present study using female rats. However, exposure to carcinogenic doses in the drinking water failed to increase TBARS, in spite of the elevation of 8-oxodG levels. Another group has also reported that a single dose of $KBrO_3$ at a low dose did not elevate etheno-DNA adducts formation or TBARS levels in the kidneys of male rats.[21] In the light of the finding of no initiating activity of $KBrO_3$ with a single i.g. administration at 300 mg/kg,[33] our present data indicate that LPO might not be involved in the renal carcinogenesis due to this compound. Instead, histological findings in the present study suggest an involvement of LPO in the nephrotoxicity induced by $KBrO_3$. It has recently been reported that reduction of $KBrO_3$ by sulfhydryl compounds such as glutathione and cysteine yields bromine oxides and bromine radicals, which can effectively oxidize guanine.[34] A large amount of cysteine is supplied as a result of metabolism of glutathione by γ-glutamyltransferase on the proximal tubule brush borders,[35] where $KBrO_3$ reduction might give rise to bromine oxides. Since they are stable in comparison with radicals, they might move into the nuclei, where further reduction could generate bromine radicals in close proximity to nuclear DNA, leading to formation of 8-oxodG without any necessity for intervention of cellular LPO.

Kurokawa *et al.* earlier reported significantly elevated incidences of renal cell tumors in male and female rats given $KBrO_3$ at 250 and 500 ppm in the drinking water for 110 weeks.[2] A further dose-response study using only male rats showed 125 ppm to also be a carcinogenic dose.[36] However, a recent study by another group demonstrated that while $KBrO_3$

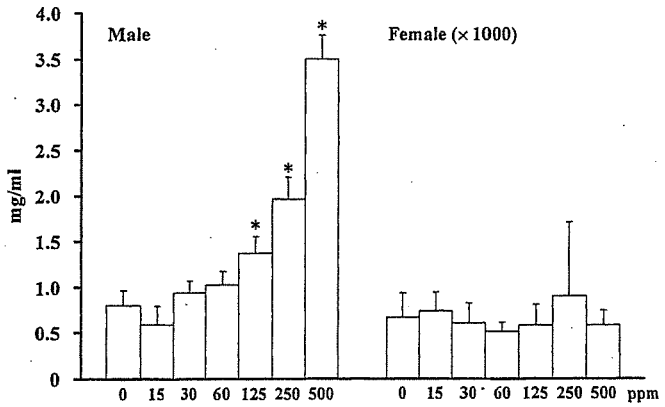

Fig. 4. α2u-Globulin levels in kidneys of male and female rats given $KBrO_3$ in the drinking water for 4 weeks at doses of 0–500 ppm. Values are means±SD of data for 5 rats. Note the values for females are μg/ml. Significantly different (* $P<0.01$) from the control group (0 ppm).

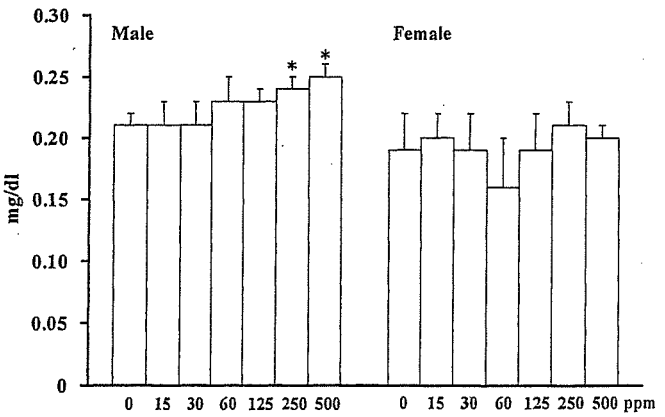

Fig. 5. Serum CRN levels in male and female rats given $KBrO_3$ in the drinking water for 4 weeks at doses of 0–500 ppm. Values are means±SD of data for 5 rats. Significantly different (*, ** $P<0.05$, 0.01) from the control group (0 ppm).

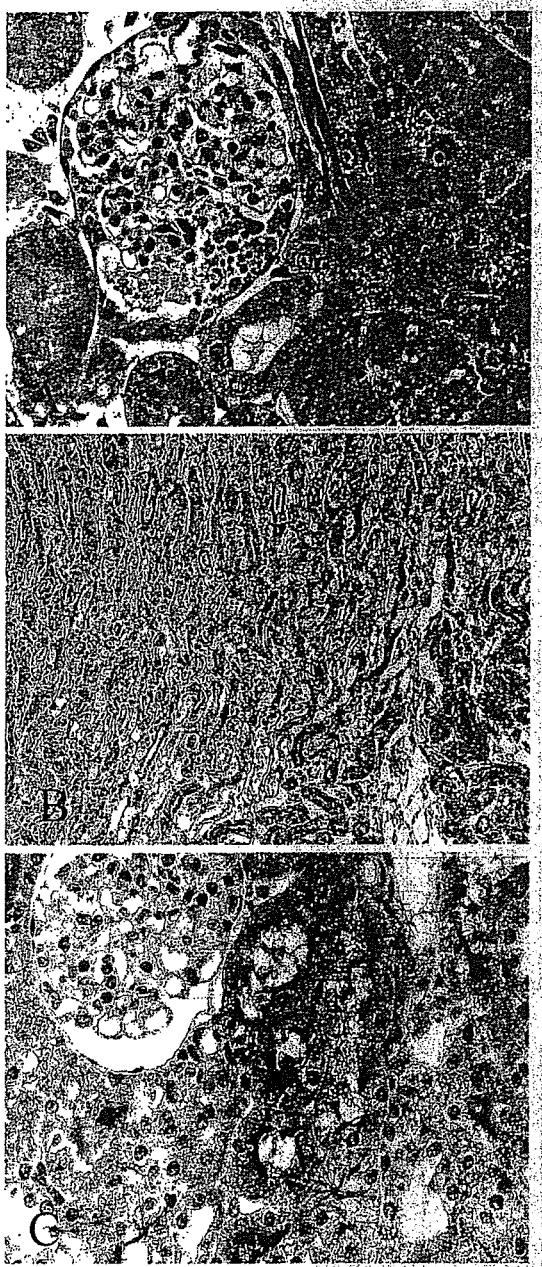

Fig. 6. (A) Renal cortex of a female rat treated with $KBrO_3$ at 80 mg/kg by single i.p. injection. Note hemorrhage and protein diapedesis in Bowman's capsule, accumulation of hyaline droplet-like material and basophilic alteration in proximal tubules. H&E staining at ×720 original magnification. (B) Renal medulla of a female rat treated with $KBrO_3$ at 80 mg/kg by single i.p. injection. Note extensive necrosis of collecting ducts. H&E staining at ×180 original magnification. (C) Renal cortex of a female rat treated with $KBrO_3$ at 500 ppm in drinking water for 4 weeks. BrdU-positive cells were seen in PCT (positive enzymatic reaction for γ-GT), but not in DT (negative enzymatic reaction for γ-GT). γ-GT-BrdU immunohistochemical staining at ×720 original magnification.

at 400 ppm in the drinking water was able to induce tumors in male rats with significant incidences, this was not the case with 200 ppm.[37] For the present, it seems equivocal whether 125 ppm has a carcinogenic potential. Accordingly, the present demonstration of increased 8-oxodG formation in kidney DNA of male and female rats given $KBrO_3$ at 250 and 500 ppm, but not at 125 ppm and below, seem to be in accordance with the carcinogenic data. In addition, the fact that $KBrO_3$-induced renal cell tumors originate from the proximal tubules[37] allows us to hypothesize that oxidative stress participates in the carcinogenesis. In a previous carcinogenicity study, the mean induction time for tumors in males was much shorter than in females,[2] but there was no sex difference with regard to doses inducing 8-oxodG formation in the present study. Therefore, variation in the tumor latency period might be explained by differential susceptibility to $KBrO_3$-induced cell proliferation, rather than oxidative stress.

In the two-stage rat renal carcinogenesis model using EHEN as an initiator, promoting activity of $KBrO_3$ was apparent in both sexes of rats.[24,25] In particular, in males, a dose of 30 ppm in the drinking water was sufficient for development of dysplastic foci from initiated cells. We also showed, in the present study, that $KBrO_3$ at the same dose was able to cause degeneration and increase of BrdU-LI in the PCT in the males. α2u-Globulin accumulation in the kidney of male rats exposed to $KBrO_3$ was also observed in a dose-dependent manner at 30 ppm and above, even though the increases at 30 and 60 ppm were not statistically significant. It has been established that this is associated with eventual cell death and subsequent cell proliferation.[38] Despite negative mutagenicity,[39,40] exposure to this kind of chemical can lead to renal cell tumors in male rats, which implies that α2u-globulin-mediated cell proliferation might be sufficient for tumor development.[41] Thus, it is highly probable that $KBrO_3$-induced cell proliferation in PCT and subsequent tumor-promoting activity observed in males might involve α2u-globulin accumulation. However, the finding that $KBrO_3$ exposure of female rats also increases BrdU-LI in PCT at doses of 250 and 500 ppm in spite of the absence of α2u-globulin indicates an involvement of some other mechanism. Considering that $KBrO_3$ might be reduced to form more reactive species at PCT,[34] the good correlation between the doses inducing 8-oxodG formation and elevation of BrdU-LI enables us to hypothesize that the cell proliferation observed in female rats might result from oxidative stress.[19,25] Since the histopathological findings and serum biochemical parameters indicate no obvious nephrotoxicity in female rats treated with $KBrO_3$ in the drinking water at any dose tested, oxidative stress might act via mitogenic stimulation.[42–44]

Judging from the female data, it appears that the cell proliferation observed in male rats at 125 ppm and below might be attributed to α2u-globulin accumulation and not to oxidative stress. In other words, the increase at 250 ppm and above in the males might reflect the combined effects of the two. For risk assessment of $KBrO_3$ in the human situation, it is essential to focus on oxidative stress and to ignore α2u-globulin-mediated effects.[45] The overall data allow us to hypothesize that more than 250 ppm of $KBrO_3$ in the drinking water is able to exert both initiating and promoting activities in the kidney of rats of both sexes by means of the generated oxidative stress. Long-term studies now appear warranted for confirmation.

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Study of 1,4-Dioxane Intake in the Total Diet Using the Market-Basket Method

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1,4-Dioxane has been classified by the US Environmental Protection Agency and the International Agency for Research on Cancer as a compound that may be carcinogenic in humans. Although there are several reports of 1,4-dioxane being detected in the environment, such as in tap water, there have been few reports on the content of 1,4-dioxane in food. We therefore studied the intake of 1,4-dioxane in food based on the average intake of food in the Kanto area of Japan as reported by the Ministry of Health, Labor and Welfare. The food was cooked in the normal manner and then homogenized in a mixer. A 20 g of sample of the homogenate was added to a solution of the purified water with 0.2 μg of 1,4-dioxane-d_8 as a surrogate and the 200 ml azeotropic solution was recovered using the steam distillation method. This solution was applied to a pair of active carbon solid-phase cartridges and the analyte was eluted from each cartridge with dichloromethane. The eluted solution was prepared for gas chromatographic/mass spectrometric analysis by reduction to a volume of 1 ml under a gentle stream of nitrogen. The detection limit of the analysis was 2 μg/kg. We found that the 1,4-dioxane content of 12 food groups ranged between 2 μg/kg and 15 μg/kg. From these results, the total daily intake of 1,4-dioxane was calculated to be 0.440 μg. An intake of this magnitude corresponds to 0.055% of the calculated total daily intake (TDI) (16 μg/kg body weight/day). This study indicates that the amount of 1,4-dioxane intake contributed by food is very low and that this value does not represent a potential problem as it does not raise the risk of carcinogenesis.

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Key words —— 1,4-dioxane, total diet, risk

INTRODUCTION

1,4-Dioxane is used extensively as an industrial solvent in dyes, paints, lacquers, varnishes, oils, waxes, and resins and is also added as a stabilizer to chlorinated solvents.[1)] 1,4-Dioxane is highly soluble in water, forming an azeotropic mixture, and when discharged into the atmosphere it returns to the surface as rainwater. As a property of its low adsorption to soil, 1,4-dioxane then permeates into the groundwater and causes water pollution over the long term. Therefore 1,4-dioxane has the potential to cause widespread contamination of the environment. There are reports of 1,4-dioxane being detected in river water at levels between 0.1 and 16.0 mg/l, and in groundwater at a maximal concentration of 94.8 mg/l in Japan.[2)] Several other studies have also shown high levels of 1,4-dioxane pollution in groundwater.[2–4)] As the removal of 1,4-dioxane in water purification systems is difficult, these findings raise concerns regarding chronic exposure to 1,4-dioxane in drinking water.

1,4-Dioxane has been classified as a carcinogenic compound by both the USA Environmental Protection Agency[5)] and the International Agency for Research on Cancer (IARC).[6)] Long-term oral administration of 1,4-dioxane has been shown to cause tumors in the liver and gallbladder in guinea pigs,[7)] and in the nasal cavity and liver of rats.[8–11)] 1,4-Dioxane has also demonstrated promoter activity in studies in mice using a two-stage carcinogenic test.[12)]

Levels of 1,4-dioxane between 0.2 mg/l and 1.5 mg/l were also detected in tap water samples collected during 1995 and 1996 from six cities in Kanagawa prefecture, Japan.[13)] This finding raises the possibility that food may also have become con-

taminated. Although 1,4-dioxane is now included in the quality standards for drinking water in Japan, there have been few reports on the contents and intake of 1,4-dioxane in food. To safeguard human health, it is important to determine the Japanese intake levels of 1,4-dioxane through food. This paper describes a study on the intake of 1,4-dioxane through food in Japan using the market-basket method.

MATERIALS AND METHODS

Chemicals —— 1,4-Dioxane was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan), 1,4-dioxane-d_8 from Sigma-Aldrich Co. Ltd. (St. Louis, MO, U.S.A.), dichloromethane from Kanto Chemical Industry Co. Ltd. (Tokyo, Japan), ethanol from Katayama Chemical Industry Co. Ltd. (Osaka, Japan), acetonitrile and acetone from Wako Chemical Industry Co. Ltd. (Osaka, Japan), and the antifoaming agent silicon TAS730 from Toshiba Silicon Co. Ltd. (Tokyo, Japan). All solvents were of the highest reagent grade. Purified water was prepared using a Milli-Q water purification PSS20 system (Millipore Corp., Bedford, MA, U.S.A.).

Preparation of Standard Solutions —— A stock solution of 1,4-dioxane (1 mg/ml) was prepared in dichloromethane, and a stock solution of 1,4-dioxane-d_8 (10 mg/ml) was prepared in ethanol. The stock solution of 1,4-dioxane-d_8 was then diluted with dichloromethane to a final concentration of 40 μg/ml. The 1,4-dioxane stock solution was diluted with dichloromethane and used to prepare the working standard solutions containing 1,4-dioxane-d_8 0.2 μg/ml in the concentration range of 0.04–1 μg/ml. A solution of 1,4-dioxane-d_8 2 μg/ml was prepared in acetonitrile and was added to samples to determine the recovery rate of the analytical procedure.

Preparation of Food Samples —— The food ingredients were purchased at a general market in Setagaya-ku, Tokyo, Japan in April and May 2000. The quantity of each food item was determined on the basis of the results of the average intake of food in the Kanto area reported by the Ministry of Health, Labor and Welfare (Table 1), using six times the weight of each food group for preparation. Food was cooked in a manner similar to that used in normal homes and was then homogenized in a mixer (Hamilton Beach/Proctor-Silex, Inc., Washington, NC, U.S.A., Model 911). Food that was difficult to homogenize was made uniform by the addition of purified water. In the preparation and cooking of the food, utensils made from wood, aluminum, fluororesin iron, and plastic were used and included items such as a chopping block, pots, and pans. These utensils were washed in a manner similar to that used in normal homes, and tap water was used for cooking. The food homogenates of each group were stored in glass bottles with silicon seals and were kept frozen at –20°C until analyzed.

Extraction of 1,4-Dioxane —— A 20 g of sample of each homogenate was placed in a 500 ml eggplant-type flask, followed by the addition of 150 ml of purified water and 100 μl of 2 μg/ml 1,4-dioxane-d_8 solution. Two hundred milliliters of aqueous solution was recovered from the sample mixture using the steam distillation method. This solution was then passed through a pair of active carbon solid-phase cartridges equilibrated with 20 ml of dichloromethane, 30 ml of acetone, and then 40 ml of purified water. Extraction of the water samples was carried out at a flow rate of 10 ml/min. After the water sample had passed through the cartridges, the cartridges were washed with 5 ml of purified water at the same flow rate. Dried nitrogen gas was then passed through the cartridges for 30 min, and the analyte was eluted from the each cartridge, with the cartridge for the water adsorption attached at the bottom with dichloromethane 3 ml. The cartridge for water adsorption was washed with dichloromethane 20 ml prior to use. The eluted solution was then reduced to a volume of 1 ml under a gentle stream of nitrogen for gas chromatographic/mass spectrometric (GC/MS) analysis.

GC/MS Analysis —— An Agilent 6890/5973N (Agilent Technologies Inc., Palo Alto, CA, U.S.A.) instrument was used for the GC/MS analysis, with separation carried out on a SPB-624 capillary column (60 m × 0.25 mm i.d. × 1.4-μm film thickness) (Sigma-Aldrich Co. Ltd.). Helium was used as the carrier gas with a column flow rate of 1 ml/min in the constant flow mode. The column temperature was kept at 60°C for 1 min, then programmed to increase by 5°C per minute to 130°C and then 20°C per minute to 230°C. Pulsed splitless injection was used with a pulse pressure of 400 kPa (1 min). The ion source temperature was kept at 230°C with the mass spectrometer operated in the EI mode. In the selected ion monitoring (SIM) mode, the monitoring ions were 58 and 88 for 1,4-dioxane and 64 and 96 for 1,4-dioxane-d_8. The injection volume was 2.0 μl. A calibration curve was prepared from the ratio of the peak height of 1,4-dioxane and 1,4-di-

Table 1. List of Food in the Total Diet Study

Group	Food Group	Food	Daily Intake (g)
I	Rice	Rice	149.0
	Rice products	Rice vermicelli	4.1
II	Barley	Oatmeal	0.2
	Flour	Wheak flour	8.3
	Bread	Bread	36.1
	Sweet bun	Bean-jam bun	8.6
	Noodles	Japanese wheat noodles	39.2
	Noodles, macaroni	Buckwheat	6.5
	Instant noodles	Instant noodles	3.7
	Grain	Cornflakes	2.5
	Seed	Crushed almonds	2.0
	Sweet potato	Sweet potato	8.6
	Potato	Potato	35.9
	Tubers and roots	Taro	10.4
	Product of tubers and roots	Konjak	13.5
III	Sugar	Granulated sugar	7.6
	Jam	Strawberry jam	1.6
	Candy	Caramel	0.3
	Rice cracker	Rice cracker	2.0
	Cake	Pound cake	4.0
	Biscuit	Biscuit	3.3
	Other snacks	Japanese fried-dough cookies	15.0
		Azuki bean jelly	
		Chocolate	
IV	Butter	Butter	1.3
	Margarine	Margarine	1.7
	Vegetable oil	Soybean oil	10.4
	Animal oil	Lard	0.2
	Mayonnaise	Dressing	5.7
V	Soybean paste	Soybean paste	12.8
	Beancurd (tofu)	Beancurd (tofu)	35.4
	Product of beans	Deep-fried beancurd	6.0
	Soybean products	Freeze-dried beancurd	9.9
	Beans		2.6
VI	Citrus	Navel orange	30.6
	Apple	Apple	26.6
	Banana	Banana	7.7
	Strawberry	Strawberry	0.2
	Fruit	Watermelon	49.0
		Loquat	
		Japanese apricot	
	Juice	Tomato juice	17.0
VII	Carrot	Carrot	24.0
	Spinach	Spinach	19.7
	Green pepper	Green pepper	4.7
	Tomato	Tomato	21.7
	Green and yellow vegetables	Broccoli	30.8
		Celery	
		Okra	

Table 1. Continued

Group	Food Group	Food	Daily Intake (g)
VIII	Japanese radish (daikon)	Japanese radish	36.0
	Onion	Onion	27.7
	Cabbage	Cabbage	24.8
	Cucumber	Cucumber	14.7
	Napa cabbage	Napa cabbage	18.2
	Vegetables	Burdock (gobo root)	44.5
		Beansprouts	
		Eggplant	
	Pickles	Pickles (nozawa-na)	6.5
	Pickled Japanese radish	Fukujinn-zuke	15.5
	Mushrooms	Mushrooms	12.8
	Seaweeds	Green laver	5.6
IX	Soy sauce	Soy sauce	19.9
	Sauces	Ketchup	5.4
	Salt	Salt	1.3
	Seasoning	Sauce	11.6
	Sake	Sake	15.5
	Beer	Beer	64.2
	Liquor	Wine	12.4
	Soft drinks	Soft drinks	69.4
		Tea	
X	Salmon and trout	Salmon	3.3
	Tuna	Tuna	8.8
	Bream and flatfish	Flatfish	8.0
	Horse mackerel and sardine	Horse mackerel	12.5
	Raw fish	Ayu	6.4
		Kisu	
		Halfbeak	
	Cuttlefish, octopus, and crab	Octopus	13.6
	Shellfish	Scallop	4.7
	Salted fish	Salted cod	8.7
	Dried fish	Dried sardine	9.5
	Canned fish	Bonito	2.9
	Cooked fish	Smelt	0.7
	Cooked fish paste	Hannpen	12.2
	Fish product	Fish sausage	0.3
XI	Beef	Beef	21.7
	Pork	Pork	31.9
	Chicken	Chicken	19.8
	Whale		0.0
	Other animal meat	Lamb	0.9
	Ham and sausage	Pork loin ham	11.0
	Eggs	Chicken eggs	38.3
XII	Milk	Milk	116.4
	Cheese	Cheese	2.8
	Daiury products	Yoghurt	21.1
	Others	Sake sediment	5.8

oxane-d_8. Quantitative analysis of the food samples was carried out using methodology identical to that used in the preparation of the calibration curve.[13]

RESULTS AND DISCUSSION

Detection Limit in Food Samples

The minimum detection level of 1,4-dioxane-d_8 added as an internal standard was 0.04 μg/l (S/N = 10). The minimum detection limit of 1,4-dioxane in the prepared food was calculated to be 2 μg/kg using the following formula: (0.04 μg/l × 1 ml)/20 g = 0.002 μg/g = 2 μg/kg , in which 1 ml indicates the final volume for GC/MS analysis and 20 g indicates the weight of the food homogenate.

Recovery Test of 1,4-Dioxane

The concentration of 1,4-dioxane in the purified water and tap water used in the analysis and in the preparation of the food samples was less than 0.04 μg/ml. This level represented the minimum detection limit when the analysis was carried out in the manner used for the food samples.

After the addition of 1,4-dioxane 0.2 μg and 1,4-dioxane-d_8 1 μg to 4 g of the prepared food samples, the recovery rate of 1,4-dioxane was obtained using the method described in the MATERIALS AND METHODS section. Table 2 shows that the recovery rate was between 99% and 111% in the 12 groups. These results indicate there was no problem with the efficiency of extraction when 1,4-dioxane was added to the food samples at a concentration < 0.2 μg/4 g (50 μg/kg).

Table 2. Recovery Rate of 1,4-Dioxane Added to Food Samples

Group	Recovery Rate (%)
I	104
II	101
III	105
IV	101
V	106
VI	100
VII	100
VIII	100
IX	99
X	102
XI	104
XII	111

Table 3. Content of 1,4-Dioxane in Food Samples

Group	Content (mg/kg)
I	ND
II	6
III	6
IV	8
V	3
VI	4
VII	3
VIII	8
IX	7
X	5
XI	6
XII	13

ND: not detectable.

Content of 1,4-Dioxane in the Food Samples

The extraction of 1,4-dioxane from each 20 g prepared food sample was carried out according to the method described in the MATERIALS AND METHODS section. Table 3 shows that the content of 1,4-dioxane in the 12 food groups was between 2 μg/kg, the detection limit of the analysis, and 15 μg/kg. If the food sample was difficult to homogenize after cooking, an appropriate quantity of purified water was added to achieve homogeneity (*i.e.*, groups I, II, III, and X in Table 4). The weight of the food used for the extraction of 1,4-dioxane before and after cooking was then calculated.

The intake of 1,4-dioxane was calculated based on the average intake of food in the Kanto area as reported by the Ministry of Health, Labor and Welfare. For example, the calculation in group II included a food sample of 1061.6 g that was added to 600.0 g of purified water for cooking and then homogenized. The actual weight of food for extraction was 18.34 g calculated as (20 g/1157.5 g) × (1061.6 g + 600.0 g) × {1061.6 g/(1061.6 g + 600.0 g)}. Since the intake of group II food was 175.5 g, the intake of 1,4-dioxane from food in this group was 0.057 μg calculated as (175.5 g/18.34 g) × 6 μg/kg × (1/1000). From the results of the content of each group in Table 4, the daily total intake of 1,4-dioxane from food was calculated to be 0.440 μg.

Risk from 1,4-Dioxane in Food

There is evidence that long-term oral administration of 1,4-dioxane causes hepatic and nasal cavity tumors in rodents,[8–12] and accordingly the IARC has classified 1,4-dioxane as a group 2B carcino-

Table 4. Intake of 1,4-Dioxane from Food

Group	Weight before Cooking (g)	Weight of Added Water (g)	Weight after Cooking (g)	Actual Weight of Food for Extraction (g)[a)]	Intake of Food (g)	Content in Food (μg/kg)	Intake of 1,4-Dioxane (μg)
I	894.0	1143.3	1715.0	10.43	153.1	ND	0.000
II	1061.6	600.0	1157.5	18.34	175.5	6	0.057
III	202.8	100.0	202.8	20.00	33.8	6	0.010
IV	1114.0		1114.0	20.00	19.3	8	0.008
V	727.9		400.2	36.38	66.7	3	0.006
VI	981.5		981.5	20.00	131.1	4	0.026
VII	605.4		609.0	19.88	100.9	3	0.015
VIII	1237.8		1143.7	21.65	206.3	8	0.076
IX	416.4		416.4	20.00	199.7	7	0.070
X	550.2	300.0	524.0	21.00	91.6	5	0.022
XI	608.9		908.9	13.40	123.6	6	0.055
XII	1019.4		1019.4	20.00	146.1	13	0.095

a) 1 : 20 g was used for extraction.

gen.[6)] With regard to a cancer endpoint, a total daily intake (TDI) of 16 μg of 1,4-dioxane/kg body weight/day has been calculated by applying an uncertainty factor of 1000 that incorporates 100 for inter- and intraspecies variation and 10 for nongenotoxic carcinogenicity to the no observed adverse effect level of 16 μg/kg body weight/day, as found in a long-term study involving drinking water in rats.[14,15)] The 0.440 μg intake of 1,4-dioxane we measured in our study corresponds to 0.055% of the calculated TDI (0.440 μg/{16 μg/kg body weight/day × 50 kg}). We therefore conclude that the intake of 1,4-dioxane from food appears to be very low and that this value does not increase the risk of carcinogenicities.

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Detection System of *Cryptosporidium parvum* Oocysts by Brackish Water Benthic Shellfish (*Corbicula japonica*) as a Biological Indicator in River Water

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Abstract. The brackish water benthic shellfish, *Corbicula japonica*, was experimentally exposed to *Cryptosporidium parvum* oocysts at 1.51×10^4 oocysts/clam/day for 7 or 14 days. Oocysts were predominantly eliminated through the feces of *Corbicula japonica* in both cases by microscopic and PCR methods. The fecal excretion rates of oocysts within 4 days after the last exposure to *Corbicula japonica* were 87.6% for the 7-day exposure group and 86.0% for the 14-day exposure group. The tissue residue level of oocysts in the gastrointestinal tract 3 days after the last exposure was 2.7% of total exposed oocysts and that of 7 days was 1.1% for the 7-day exposure case and 1.6 and 0.5% for the 14-day exposure case, respectively, maintaining infectivity to cultured cells (HCT-8) *in vitro*. At the same time, field tests of *Corbicula japonica* for collecting oocysts showed that this clam could certainly collect *Cryptosporidium parvum* oocysts in the natural river and, furthermore, the gene type of *C. parvum* could be also identified proving its effectiveness as a biological indicator. The present study showed that the brackish water benthic shellfish *Corbicula japonica* may be capable of gathering and preserving *Cryptosporidium parvum* oocysts to a considerable extent under the natural ecological conditions, and further suggests the effectiveness of *Corbicula japonica* as a practical and general bioindicator for estimates of river water contamination by oocysts of *Cryptosporidium parvum*.

There are a number of reports of the detections of oocysts of the coccidian protozoan genus *Cryptosporidium parvum* (*C. parvum*) in aquatic life, especially in sea water shellfish (Fayer *et al.* 1998, 1999; Graczyk *et al.* 1998, 1999, 2001; Gomez-Bautista *et al.* 2000; Freire-Santos *et al.* 2001, 2002; Traversa *et al.* 2004; Giangaspero *et al.* 2005; Gomez-Couso *et al.* 2005). Previous studies of these shellfish have suggested that some of the shellfish offer the potential to be biological indicators of contamination of their habitat by *C. parvum* oocysts. However, quantitative time-course balance study is limited. The amount of oocysts detected in sea water shellfish is assumed to depend on the surrounding ocean currents (Gomez-Bautista *et al.* 2000), and while these sea water clams appear to be a possible hygienic biological indicator for perishables, they could not be a practical biological indicator for estimating vicinal river water contamination by *C. parvum* in its habitat. To enable a more practical and effective estimate of river water contamination by *C. parvum*, the aquatic life in fresh or brackish water must be taken into account, because it presents a more limited and stable habitat than the sea environment.

In Japan, there are a number of fresh or brackish water benthic shellfish, which feed on suspended plankton by filtration with gills. Of these, three *Corbiculidae* species are commonly consumed and considered edible: *Corbicula leana* and *Corbicula sandai*—known to be hermaphroditic, ovoviviparous, and gonochorismal, oviparous clams—live in fresh water throughout their lives, inhabiting the middle reaches of rivers and lakes, respectively. *Corbicula japonica* (*C. japonica*) is a gonochorismal, oviparous clam, which lives in brackish water and is widely bred in river-mouth brackish water areas at the estuaries of rivers. Therefore, it may offer the potential to be a biological indicator for estimates of contamination in river water by *C. parvum* oocysts, and it is available for sampling throughout the year as well as being a convenient size for experiments in the laboratory.

In a previous study on the role and the usefulness of *C. japonica* as a biological parasite indicator by single exposure of *C. parvum* oocysts to the clam, the effectiveness of *C. japonica* as a biological indicator for oocysts of *C. parvum* was suggested (Izumi *et al.* 2004). The present study investigates the role of *C. japonica* as a practical oocyst collection system in further detail by consecutive exposures of *C. parvum* oocysts to the clam.

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Materials and Methods

Keeping of the Shellfish

C. japonica with a body size of 31.2–37.4 × 27.3–32.0 × 18.2–25.0 mm and a body weight of 11.5–18.1 g were collected from the Ishikari River, Hokkaido, Japan. Ninety *C. japonica* clams were placed in a stainless steel cage so as not to overlap in a 12-L inner cistern filled with dechlorinated 10% artificial sea water (Tetra Marinsalt, Tetrawerke, Germany) of pH 7.4 and d = 1.002 equipped with an air pump (Inno β6000, Nisso, Japan) with two stick type air stones (15 × 15 × 150 mm) set under the stainless steel cage for aeration and circulation of the water, floated in an insulated outer cistern filled with fresh water maintained at 15°C by a cooler (RZ-90, REI-SEA, Japan). To remove ammonia and nitrites, an adequate amount of oxidizing bacteria (Aquarium Pharmaceuticals) was added to the water of the inner cistern. The clams were acclimatized for 6 weeks in the aquarium prior to the oocyst introduction, and they were satiated daily with proper amounts of both liquid-type chaw for invertebrates (Tetrawerke) and powdered chlorella (*Chlorella pyrenoidosa*, Sun Chlorella, Japan) suspended in water, which had a negative IFA reaction against *C. parvum* oocysts. The water of the inner cistern was periodically changed, at 9:30 a.m. every morning, with pre-adjusted dechlorinated 10% artificial sea water (15°C) to protect the shellfish from temperature shock, and the feeding was carried out about 30 min after the water change.

Administration and Recovery of C. parvum *(Balance Study)*

Oocysts of *C. parvum* (bovine type, Lot No. 8-27) were purchased from Waterborne Inc. and stored at 4°C. After 6 weeks of acclimation, 20 randomly selected control clams were removed from the aquarium, and the water had a daily dose of 1.36×10^6 oocysts of *C. parvum* (1.13×10^5 oocysts/L, 1.51×10^4 oocysts/clam) added with chaw at 10:00 a.m. For both the 7- and 14-times exposure tests, clam fecal samples and cistern water were collected daily and examined after the start of the *C. parvum* exposure, and ten randomly selected clams were removed and examined at 1, 2, 3, 4, 7, 10, and 14 days after the final addition of *C. parvum*. The treatment of the clam feces and water in the aquarium during the experiment was carried out in the same manner as the previous report with the above-mentioned time intervals.

Detection of C. parvum *Oocysts*

The details of the detection of oocysts from the clam bodies, fecal samples, and cistern water are as described previously (Izumi *et al.* 2004). Prior to the staining of the surface of the fecal oocysts by fluorescent dye, the anti-*Cryptosporidium* kit (Dynabeads GC-Combo Kit, Dynal Biotech Inc.) was used to purify the samples, followed by boiling treatment for 5 min (Inomata *et al.* 1999). The microscopic observations were carried out for the identification and determination of the *C. parvum* oocysts under a fluorescence microscope (DM LB2, Leica) with 200 times magnification.

Field Survey

A field experiment was carried out to estimate the ability of the clams to collect protozoa in the actual river. First, the shellfish was acclimatized for 6 weeks in the aquarium in the laboratory and checked to be *C. parvum* free, then groups of 60 clams were put in closed stainless-steel mesh cages and set at the three different locations (total 180 clams) in the river-mouth area of the Yanbetsu River in Hokkaido, Japan. The cages were positioned as follows: one in the center of the river-flow, and the others about 5 m from the center, to form a line across the river. The transport of the clams from the laboratory to the river was rapid in an insulated box kept at about 4°C with frozen gel packs and without water. On the 7th day after the placement in the river, the three cages were removed from the river and carried in an insulated box to the aquarium in the laboratory under the above conditions. Thereafter, fecal samples of the bivalves were collected daily for 3 days and analyzed for oocysts of *C. parvum* in the manner described previously. To estimate the relationship between the density of oocysts of *C. parvum* in the water and the actual amount trapped by the clams, the protozoa in the river water were checked twice during the survey, at the start and at the end (the 7th day) of the experiments with the clams in the river. The treatment of the water sample (10 L) was processed by a mixed cellulose ester membrane filter (A100A090C, ADVANTEC) filtration method (membrane filtration method), followed by acetone dissolution, oocyst purification by the above-mentioned anti-*Cryptosporidium* kit, and staining with fluorescent dyes. The trapping ability of *C. japonica* for oocysts of *C. parvum* in the river was made by an approximate estimate calculating the volume of water filtered by a clam per day and the density of the protozoa in the river water. Fecal samples collected in the same way at the other time from the same field were used in the following infectivity assay, DNA sequencing, and PCR-RFLP analysis.

Oocyst Infectivity Assay by Cultured Cells (HCT-8)

The *in vitro* infectivity of the oocysts recovered from the clam feces was determined by the infectivity to cultured cells (HCT-8). The basic procedure was detailed in previous reports (Upton *et al.* 1995; Slifko *et al.* 1997; Hirata *et al.* 2001; Izumi *et al.* 2004). The infection of the cultured cells was evaluated by observation of the proliferation of sporozoits stained with immunofluorescent agent in the HCT-8 cells under the fluorescence microscope with 200–1,000 times magnification.

DNA Extraction and PCR

In both tests, an aliquot of sample of excreta and the GI tract after the final exposure were checked for protozoan DNA by PCR, as reported previously, while the internal control (IC: 542 bp) was applied to only the negative control (Peng *et al.* 1997; Sulaiman *et al.* 1998).

DNA Sequencing and Analysis

The PCR product was extracted by the QIAquick Gel Extraction Kit (Qiagen) followed by purification with HPLC (TOSO 8020 DNA NPR column: 4.6 mm × 75 mm) equilibrated with 20 mM Tris-HCl/1 mM EDTA/pH 8.0. The product was eluted by a 0.200–0.275 M NaCl gradient and then ligated to a pGEM T-Easy Vector (Promega) by a Rapid DNA Ligation Kit (Roche Diagnostics), followed by transformation to the JM109 Competent Cell (Promega). The DNA sequencing of recombinant clones that held the corresponding insert of *C. parvum* was performed on an ABI 377 Automated Sequencer (Perkin Elmer) using a BigDye Primer Cycle Sequencing FS Ready Reaction Kit with a primer set of -21M13 and M13Rev (Applied Biosystems). The representative human and bovine genotypes were

deposited in GenBank with access numbers AF082521 to AF082524 showing typical human type sequence #51:G, #78:C, #100:T, #147:C and #280:T/C including the *Hae*III site (#76–79:GGCC), and typical bovine type sequence #51:A, #78:T, #100:G, #147:T and #280:C including *Bst*EII site (#76–82:GGTNACC), respectively (see Table 2).

PCR RFLP

To supplement the DNA sequencing study for differentiating the genotypes of *C. parvum*, PCR amplification of the TRAP-C2 gene was followed by RFLP (Sulaiman *et al.* 1998). Restriction enzymes, *Bst*EII and *Hae*III (Roche Diagnostics), were applied for classification between bovine and human genotypes, respectively. For the restriction fragment analysis, about 1.5 μg of amplified PCR product in a total of 20 μl of the respective restriction buffer was digested by 10 U of *Bst*EII or *Hae*III under the conditions recommended by the supplier. The digested product was fractionated on 2.0% agarose gel and visualized by ethidium bromide staining.

Measurement of the Water Filtration Rate of C. japonica

To obtain basic ecological data of *C. japonica* for the following field survey, an attempt was made to determine the water filtration rate of the clams. Ten randomly selected clams were placed in a stainless steel cage in a glass beaker with 2 L of 10% artificial seawater. The beaker was kept cooled at 15°C, and a ball-type air stone and a stirrer stone were set under the stainless steel cage to prevent chlorella powder sedimentation during the test. Two hundred milligrams of chlorella powder were suspended in water and added to the beaker. Subsequently, 10 ml of water were collected every 10 min after the addition of the chlorella; after centrifugation (3,000 rpm, 10 min) of the collected water, the chlorophyll in the precipitate was extracted with 5 ml of acetone. The extracted chlorophyll was determined as chlorophyll A and B by measurement of the wavelengths at 664, 647, and 630 nm, as *Chlorella pyrenoidosa*, a *Chlorophyceae* that contains both chlorophylls A and B (Greenberg *et al.* 1992). The calculation of the two chlorophyll concentrations was made with the following numerical formula: chlorophyll A (μg/ml) = $11.85Abs_{664} - 1.54Abs_{647} - 0.08Abs_{630}$, and chlorophyll B (μg/ml) = $-5.43Abs_{664} + 21.03Abs_{647} - 2.66Abs_{630}$. The total chlorophyll content was calculated as the sum of chlorophyll A and B. We measured the time to the plateau level where the total chlorophyll had been removed by the clam feeding, and then estimated the filtration rate of the clams.

Analysis of Metals and Nitrogen

The basic procedures of the analysis of the metals (Al, Ba, Ca, Cd, Cr, Cu, Fe, Mg, Mn, Pb, and Zn) in the water of the cistern were as described in the previous report (Izumi *et al.* 2004). The analysis of nitrogen for ammonia and nitrites in the water of the cistern was carried out with indophenol and colorimetric methods, respectively (Greenberg *et al.* 1992).

Results

Overall the data for maintaining the shellfish show that the conditions for this study were adequate. The basic data for maintaining the shellfish containing the metal concentration, and the nitrogen values of ammonia and nitrites in the water, which are both harmful to clams, showed almost the same values as the previous report (Izumi *et al.* 2004). None of the control clams contained *C. parvum* oocysts, confirmed both microscopically and by PCR. The aquarium water was oocyst-negative before the start of the experiment. During the experimental period of both consecutive exposure tests, the clam mortalities were below 2.0% for both the 7- and 14-day studies.

The two exposure studies reported here show that *C. parvum* oocysts were rapidly ingested in *C. japonica* and nearly all excreted in the feces irrespective of the length of the oocyst exposure period. Figure 1 shows the balance of *C. parvum* oocysts detected in the clam excreta and the gastrointestinal tracts (GI tract). In the exposure periods, the fecal excretion of oocysts was almost linear with steep slopes, showing relatively constant daily excretion rates in both tests. After the final of the 7 or 14 consecutive daily doses of *C. parvum* oocysts to *C. japonica*, the excretion patterns of oocysts were similar to that of the single administration (Izumi *et al.* 2004). Thus, the oocysts were predominantly excreted in the feces in at least three phases: a more rapid first phase for several days after cessation of the exposure, and 6 or 7 days after the last dose (the last phase), in which a small quantity of oocysts was microscopically detected, while the protozoan DNA was identified by PCR on the 7th day after the last dose in both tests. It was observed that the excretion rate in feces seemed to be slightly faster in the 7-times exposure group than in the 14-times exposure group. However, there were statistically no significant differences between the two groups (Fig. 1A–D).

Overall, the excretion of oocysts by the fecal route was predominant, and the total recovery of oocysts within the 4 days after the final dose was 87.6% in the feces and 1.5% in the water for the 7-times exposure test, and 86.0% in the feces and 1.9% in the water for the 14-times exposure test.

The oocyst residue in the tissue of the two groups after the final exposure showed that most of the oocysts in the shellfish were in the GI tract, and the amount of oocysts in the mantle and gills was negligible compared to that in the GI tract in both tests (data not shown).

The decrease rate of oocysts in both the 7- and 14-times exposure tests in the GI tract was slightly different at different time points, with the decrease in the first 3 days more rapid than later (Fig. 1E, F). The slope of the curve in the later phase was flatter and reached a plateau level 10–14 days after the final exposure. The decrease in oocysts in the GI tract appears mostly to correspond to the fecal excretion, in both magnitude and reduction pattern, like that observed in the single administration test.

The oocyst residue levels in the GI tract 1 day after the last exposure were 6.6% of the total dose for the 7-times exposure test and 4.2% for the 14-times exposure test, and 14 days later the residual levels were 0.7 and 0.2%, respectively. At 14 days after the last dose, the residue of *C. parvum* oocysts in the GI tract was microscopically and genetically detected in both dose tests, while fecal DNA was not identified at that time. This may account for some nonspecific absorbance of oocysts to the GI tract (Fig. 2A). In the exposure studies, the total amount of oocysts detected microscopically in both the excreta and water amounted to about 89.9% of the oocysts introduced during the 7-times exposure test and 88.3% during the 14-times exposure

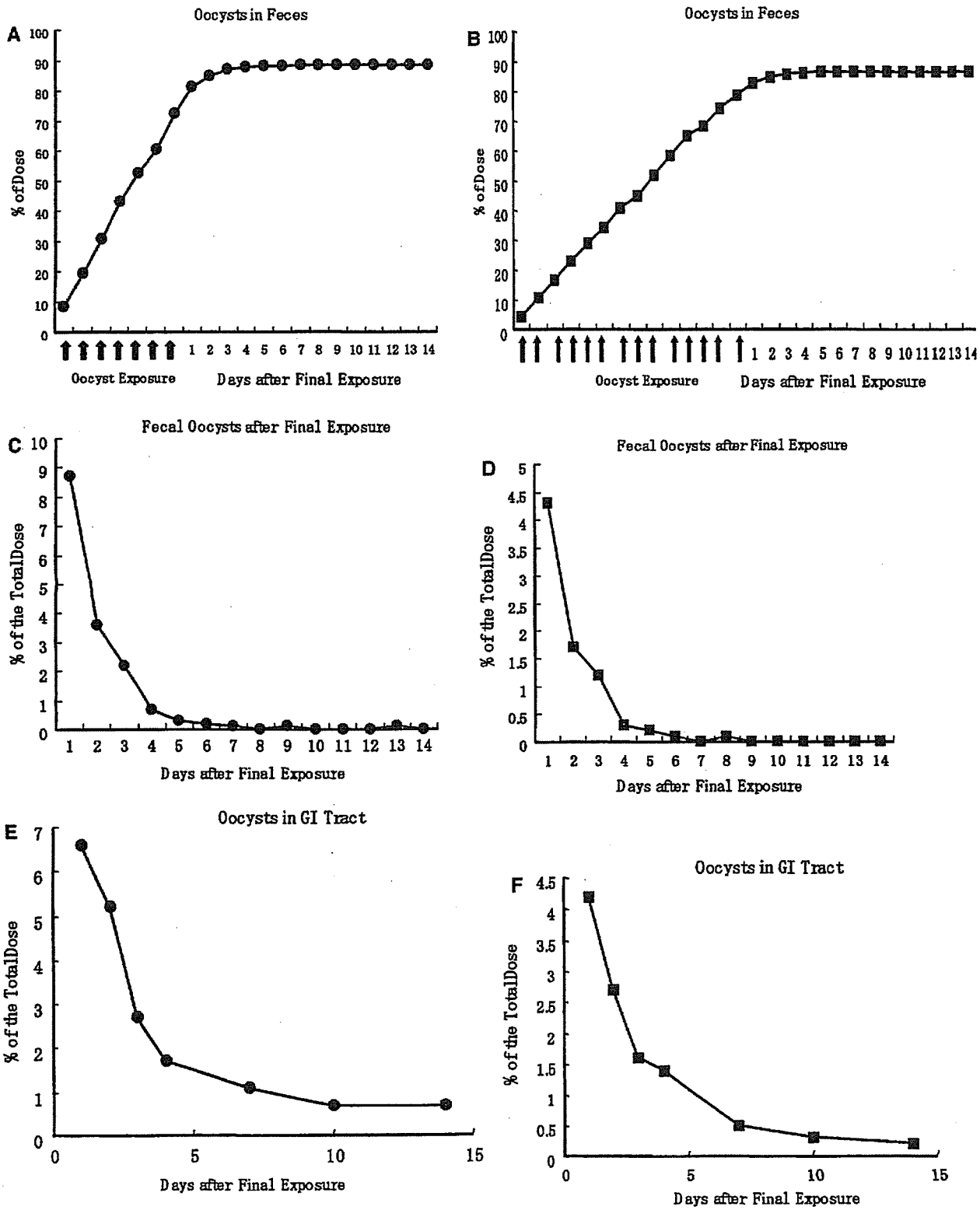

Fig. 1. The relative amounts of *Cryptosporidium parvum* oocysts detected in the excreta and GI tract of *Corbicula japonica* in the studies by consecutive oocysts exposure tests (A, C, and E: 7 doses; B, D, and F: 14 doses). (A,B) The cumulative percent amount of oocysts detected in the clam excreta with the exposure rate of 1.51×10^4 oocysts/clam/day of oocysts for 7 or 14 days. (C–F) The percent amounts of oocysts determined in clam feces and GI tract following 7 or 14 consecutive daily exposures of oocysts