Genotoxicity and Estrogenic Activity of 3,3'-Dinitrobisphenol A in Goldfish

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3,3'-Dinitrobisphenol A (dinitro-BPA) is formed in a mixture of bisphenol A (BPA) and nitrite under acidic conditions. It shows genotoxicity in male ICR mice on a micronucleus test, but its estrogenic activity has not been examined in vivo. We examined its estrogenic activity using goldfish (Carassius auratus) by measuring plasma levels of vitellogenin (VTG) by the ELISA method. Expression of VTG didn't increase in the plasma of goldfish intraperitoneal injected with dinitro-BPA at a dose of 10 mg/kg of body weight.

We also examined the genotoxicity of dinitro-BPA by single-cell gel electrophoresis (comet assay) and a micronucleus test using goldfish. The DNA tail moment of blood cells increased after intraperitoneal injection of dinitro-BPA. Dinitro-BPA at the same dose significantly increased micronucleus frequency in gills of goldfish. On the other hand, BPA did not significantly increase the frequency of micronucleated cells.

In conclusion, we found that dinitro-BPA did not show estrogenic activity, but had genotoxic potency stronger than that of BPA.

Key words: goldfish; 3,3'-dinitrobisphenol A; vitellogenin; comet assay; micronucleus test

It is known that various chemicals in our environment show substantial influences on aquatic animals and mammals, including humans. Among these compounds, bisphenol A (2,2-bis(4-hydroxyphenyl)propane, BPA), which is used as an ingredient in the manufacture of epoxy carbonate, polycarbonate and polyester styrene, exhibits estrogenic activity. The estrogenic activity of BPA has been demonstrated using various assays. Krishnan et al. found that BPA showed estrogenic activity in a culture assay using MCF-7 human breast cancer cells.3,5) Hashimoto et al. revealed estrogenic activity of BPA using the yeast two-hybrid system.21) It has been reported that BPA bound with human estrogen receptor alpha, beta, and gamma.3-5) Hence it is said to be an endocrine-disrupting chemical (EDC). It is also used in food packaging and can-coating agents and in dental sealants, and is readily orally-ingested by humans. Consequently, it is important to examine the toxicity of BPA in human body after oral intake.

BPA has not been recognized as a mutagen by several in vitro and in vivo mutagenicity assays. Haworth et al. reported that it showed negative results in bacterial reverse mutation tests using Salmonella typhimurium TA98, TA100, TA1535, and TA1537.6) Yuett et al. reported that it did not exhibit mutagenic activity in an in vitro genotoxicity test with Chinese hamster ovary cells.7) Gudi et al. also reported that it did not increase the frequency of micronucleated reticulocytes (MNREts) in bone marrow of mice.8) Recently, however, there have been positive results to the effects that it shows mutagenic activity. Hilliard et al. reported that BPA exhibited positive effects in an in vitro chromosome aberration test using CHO cells without S9 mix.9) Tayama et al. demonstrated that it induced genotoxicity in CHO-K1 cells using sister-chromatid exchange and comet assay.10) Tsutsui et al. found that quinone compounds formed from BPA formed DNA adducts in an in vitro 32P-postlabeling assay.11) Thus BPA shows opposite effects on mutagenic activity in different test systems.

Humans regularly consume nitrite and nitrate through vegetables and tap water, and in their daily diet as food additives.12) Nitrate is readily reduced to nitrite by oral bacteria. Some scientists have reported that mutagenic/carcinogenic nitrosamines are formed by the reaction of nitrite and secondary amines in foodstuffs under acidic conditions.13) Several phenolic compounds also show mutagenic activity after nitrite treatment. Wakabayashi et al. found that some phenol and indole derivatives present in the environment are changed to mutagenic compounds by nitrosation.14) Kikugawa and Kato found

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that diazoquinone compounds, which showed strong mutagenicity, were formed by interaction between phenol and nitrite.\textsuperscript{15,16} We have found that BPA showed mutagenic activity on treatment with nitrite under acidic conditions, and dinitro-BPA, which induced micronuclei in peripheral erythrocytes of ICR male mice, was formed in a reaction mixture of BPA and nitrite.\textsuperscript{17} Additionally, we found that dinitro-BPA did not show estrogenic activity on \textit{in vitro} assay. However, we have not yet determined whether dinitro-BPA shows estrogenic activity on an \textit{in vivo} assay.

Some scientists have examined the estrogenic activity of endocrine disruptors by \textit{in vivo} tests using aquatic organisms. Warner \textit{et al.} demonstrated the estrogenic activity of BPA using fathead minnow.\textsuperscript{18} Tabata \textit{et al.} used Japanese medaka (\textit{Oryzias latipes}) to confirm the endocrine disrupting action of BPA.\textsuperscript{19} Ishibashi \textit{et al.} also confirmed the estrogenic activity of nonylphenol by detecting the vitellogenin concentration in plasma of goldfish.\textsuperscript{20} Goldfish can be bred easily and cheaply and are available all over the world. We have also examined the genotoxicity of various chemicals in river water using goldfish. Judging by these reports, goldfish is a useful organism in investigating the mutagenic and estrogenic activities of various chemicals.

In the present study, we examined the mutagenic and estrogenic activities of BPA and dinitro-BPA with goldfish.

**Materials and Methods**

\textbf{Chemicals.} BPA, 17β-estradiol (E2), acridine orange, methyl metahnesulfonate (MMS), and dimethylsulfoxide (DMSO) were purchased from Wako Pure Chemicals (Osaka, Japan). 3,3’-Dinitro-bisphenol A (dinitro-BPA) was synthesized as described by Masuda \textit{et al.}\textsuperscript{17} Figure 1 shows the chemical structure of dinitro-BPA.

Low melting point (LMP) agarose and normal melting point (NMP) agarose were from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was from Gibco (Grand Island, NY). Mitomycin C (MMC) and ethidium bromide were from Kyowa Hakko Kogyo (Tokyo) and Merck (Darmstadt, Germany) respectively.

\textbf{Fish.} We obtained goldfish weighing 11 ± 2 g from a local dealer in Hamamatsu, Japan. Before the experiment, goldfish were acclimatized for 2 weeks in a well-aerated aquarium at 18 ± 2°C.

![Chemical Structure of Dinitro-BPA](image)

\textbf{Fig. 1.} Chemical Structure of Dinitro-BPA.

\textbf{Treatment of fish.} In a previous study, we injected chemicals from a river intraperitoneally to examine their mutagenic activities.\textsuperscript{21} In a similar way, BPA and dinitro-BPA were dissolved in DMSO and injected intraperitoneally once at 1 and 10 mg/kg of body weight. Three to 5 fish were used in each group. In the negative control group, DMSO was injected intraperitoneally in place of the test chemical. In the positive control group, we injected intraperitoneally E2 (1.0 mg/kg of body weight), MMS (50 mg/kg of body weight) and MMC (4.0 mg/kg of body weight) for VTG determination and in comet assay and micronucleus test. For determination of VTG in plasma, at 96h after injection blood was collected and centrifuged at 3,000 rpm for 20 min, and the plasma was divided into aliquots and stored at −20°C until use. In the comet assay, 3h after injection of chemicals, peripheral blood was collected. Peripheral blood and gills were obtained at 96h after injection of chemicals in the micronucleus test.

\textbf{Vitellogenin analysis.} Several researchers have found that the VTG molecule structure of carp (\textit{Cyprinus carpio}) is similar to that of other cyprinid fish. Zhong \textit{et al.} determined VTG production of hepatocytes in a rare minnow (\textit{Gobiocypris rarus}) by ELISA using an antibody against carp (\textit{Cyprinus carpio}) VTG.\textsuperscript{22} This antibody has been found to bind to VTG produced by other cyprinid species, such as goldfish and fathead minnow.\textsuperscript{23} Hence the carp VTG antibody is used to measure VTG concentration in plasma of a wide variety of cyprinid fish, such as goldfish. In this study, a Carp VTG ELISA Kit (Trans Genic, Japan) was used to determine VTG contents in plasma of goldfish according to the manufacturer’s instructions. The concentrations of VTG were calculated from the linear part of the log-transformed VTG standard curve. The detection limit of VTG was 0.04 μg/ml plasma.

\textbf{Alkaline comet assay.} The alkaline comet assay was performed according to the method of Tice \textit{et al.}, with some modifications.\textsuperscript{24} Five μl of blood was diluted with Ca\textsuperscript{2+} and Mg\textsuperscript{2+}-free phosphate-buffered saline (PBS(-)); 7.5 g of NaCl, 0.2 g of KCl, and 0.2 g of sodium bicarbonate dissolved in 1 liter of water), and samples were mixed with 75 μl of 1% low melting point (LMP) agarose. The mixture (75 μl) was layered on a 1% LMP agarose layer and covered with 75 μl of 1% LMP agarose. After preparation, the slide was placed in alkaline electrophoresis buffer for 10 min to allow salt equilibration and further DNA unwinding. Electrophoresis was performed at 30 V, 300 mA for 15 min at 4°C. The slide was then neutralized with 0.4 M Tris buffer (pH 7.5) for 10 min. The cells were stained with 50 μl of ethidium bromide (20 μg/ml). Comet images were analyzed using a fluorescence microscope (magnification 200×) equipped with CCD camera. One hundred cells were examined...
per fish. The tail moment of DNA was measured using Comet Analyzer Youworks Bio Imaging Software.

**Micronucleus test.** The micronucleus test was performed according to the methods of Ueda et al.,25) and Hayashi et al.,26) with some modifications. Peripheral blood of goldfish was collected from a caudal vein using a heparinized syringe. Ten μl of peripheral blood was diluted with 40 μl of fetal bovine serum. Nine μl of diluted blood was spread on an acridine orange-coated glass slide. Three gills on each side of the were excised and washed with PBS(-). Gills were transferred to 5 ml of PBS(-) and broken up with forceps. Tissue clumps and gill arches were removed and discarded. Free cells were collected by centrifugation (1,000 rpm, 5 min) and treated with 2 ml of 75 mM KCl hypotonic solution for 5 min. Then 0.1 ml of Carnoy fixative A (acetic acid and methanol, 1:3 by vol.) was added and centrifuged at 1,000 rpm for 5 min. A half volume of the supernatant with cells was suspended in 5 ml of Carnoy fixative B (acetic acid and methanol, 1:99 by vol.) and centrifuged. Then three fourth of the supernatant was removed and the cell suspension was dropped on a slide glass smeared with 0.0025% acridine orange solution. At least 1,000 erythrocytes and gill cells were observed with a fluorescence microscope (magnification, ×400/C2)28) and the numbers of micronucleated cells were recorded.

**Statistical analysis.** Dunnett’s test after one-way ANOVA was used to evaluate the significance of differences in VTG contents, tail moment in the comet assay, and micronucleus frequency in the micronucleus test between fish treated with BPA and with dinitro-BPA and the untreated group. A p-value lower than 0.05 was considered to be statistically significant.

**Results and Discussion**

VTG a phospholipoglycoprotein precursor of egg yolk protein, is synthesized in the liver of sexually mature oviparous females.27) It is normally undetectable in the plasma of males and immature females. However, when male fish were treated with EDCs, VTG was detectable in their plasma. Hence VTG in plasma of male and juvenile fish treated with chemicals is used as a biomarker in evaluation of estrogenic activity of chemicals.28)

In this study, we examined the estrogenic activities of BPA and dinitro-BPA by quantifying of VTG by the ELISA method with carp VTG antibody. The VTG concentration (113.5 μg/ml) in plasma of goldfish was significantly increased by treatment with BPA (10 mg/kg of body weight) (p < 0.01) (Fig. 2), but dinitro-BPA did not show any significant expression of VTG protein in plasma (n.d.). We have also found that the estrogen (α) binding potency of dinitro-BPA was weaker than that of non-treated BPA by in vitro assay.17) In addition, we have reported that the estrogenic activity of 2-nitro-17β-estradiol, which is formed in a reaction mixture of 17β-estradiol and nitrite, was weaker than that of E2 using an ELISA kit.29) Hence it is possible that binding potency to the estrogen receptor changed because the physico-chemical properties of BPA were transformed by nitrination.

Comet assay is a rapid and sensitive method of detecting DNA single-strand breaks and alkali-labile sites in individual cells. Since these types of DNA damage can be the initial damage induced by genotoxic chemicals, comet assay is generally used as a method of detecting DNA damage due to exposure with mutagens and carcinogens.30) Figure 3 shows the mean values of DNA tail moment in peripheral erythrocytes induced by dinitro-BPA and BPA. The mean tail moment values were increased by intraperitoneal injection of BPA (5.06 ± 0.37) and dinitro-BPA (8.20 ± 0.67) at 10 mg/kg of body weight. Variance analysis (p < 0.01) of these values showed a significant difference in DNA damage as between the dinito-BPA treated group and BPA-treated group.

Figures 4 and 5 show the frequencies of micronuclei in peripheral erythrocytes and gills of goldfish respectively.
The frequency of micronuclei in gills increased significantly after intraperitoneal injection of dinitro-BPA at a dose of 10 mg/kg of body weight (8.00 ± 1.00) as compared with the control group (p < 0.01), but dinitro-BPA did not induce micronuclei in peripheral erythrocytes. Hayashi et al. have reported that mutagen-treated fish showed higher frequencies of micronucleated cells in gills than in peripheral erythrocytes, and recommended the use of gill cells in fish micronucleus assays. MMC did not induce micronuclei in either gills or erythrocytes. Michael et al. have reported that BPA caused DNA damage due to apoptosis induced by BPA.\textsuperscript{31} In this study, BPA showed DNA-damaging potency on the comet assay, but did not induce micronuclei on the micronucleus test at significant frequency. On the other hand, dinitro-BPA showed DNA damaging potency and induced micronuclei. These results suggest that the toxicity of BPA is different from that of dinitro-BPA. Therefore, dinitro-BPA is assumed to induce abnormal chromosomes and to act as a genotoxic chemical.

The genotoxic effects of nitro compounds are generally linked to nitrate reductase. There are several kinds of NADPH-cytochrome c reductase,\textsuperscript{32} xanthine oxidase,
DT-diaphorase, and other enzymes. Nitro compounds show genotoxic activity through two pathways. The first reduction step is the formation of the nitrogen radical anion. Under aerobic conditions, this radical is reoxidized by O$_2$ and produces superoxide and hydroxyl radical anions. Under anaerobic conditions, reductive reaction leads to the formation of nitroso and hydroxylamine derivatives. Hydroxyl radicals cause DNA strand breaks. Hydroxyl amino groups induce the transformation of DNA adducts. These metabolic responses can cause DNA damage and abnormal chromosomes in vivo. Hence it is necessary to measure nitrogen reductase activity in goldfish.

Recently, many problems of nitrogen pollution have appeared in the water environment and in the acidification of freshwater. When these environmental conditions occur simultaneously, a nitro compound might be generated in the environment. Telcher et al. reported the formation of a nitrogen metabolite of nonylphenol isomer in soil/sewage sludge mixtures. In the water phase, nitrated polycyclic aromatic hydrocarbons (nitro-PAHs) can be formed by a photochemical reaction of PAH, with nitrite donating the nitro group. There are various nitro compounds in the urban air and soil.

The present study indicates that the estrogenic activity of dinitro-BPA was lower than that of BPA in goldfish. However, the genotoxic potency of dinitro-BPA was stronger than that of BPA. We also found that E2 and nitro-E2 showed similar behaviors. Other nitro compounds might be formed and flow into the water environment. As a result, it is possible that aquatic organisms and human beings are exposed to nitro compounds. Hence we must investigate the formation of nitro compounds and evaluate their estrogenic and genotoxic activities in vivo. In addition, we must monitor dinitro-BPA and other nitro compounds in the environment.

References


