Bisphenol A Incorporated into Eggs from Parent Fish Persists for Several Days

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Chronological changes of bisphenol A (BPA) concentration were investigated for a week in mature medaka (*Oryzias latipes*) and spawned eggs (embryo) after exposing the fish to BPA at a concentration of 100 μg/l in water. The BPA concentrations in mature fish and spawned eggs increased beginning at the day after initial exposure, and reached an approximately constant level on the second day. On the other hand, the decrease in BPA concentration in the parent body was rapid after being placed back into pure water (average decrease was 87% on the first day, and 98% on the second day), while approximately 24% of the BPA in the spawned eggs from the parents remained after the fourth day in pure water. Thus, it was assumed that there is no system for BPA excretion from eggs. Another experiment was conducted, in which eggs spawned from a parent on the fourth day of exposure were raised in pure water. The BPA concentration in the eggs on the sixth day was approximately 29% lower when compared to that at spawning. Therefore, it was assumed that the embryo cannot conjugate BPA incorporated into the egg due to an underdeveloped metabolic or excretion mechanism.

Key words —— bisphenol A, medaka, embryo

INTRODUCTION

Humans have long been releasing large amounts of various chemical compounds into the environment, and various wild creatures have been exposed to these chemical compounds. Among these chemicals, some have been reported not to be lethal at low doses, but it has now been suggested by many researchers that some chemicals affect reproduction by disturbing sexual differentiation. Such chemical compounds are called endocrine-disrupting chemicals (EDCs).[1–3)]

EDC activity evaluation has been conducted on various chemical compounds using fish. It has been confirmed that some chemical compounds, such as bisphenol A (BPA),[4–8)] nonylphenol (NP)[9–12)] and octylphenol (OP),[10)] exhibit activity, even at relatively low concentrations. As short-term experimental methods, sex reversal and reproduction tests have been conducted by exposing parent fish to chemical compounds in water. As experimental methods to observe long-term effects, partial life cycle and full life cycle tests have been conducted in order to clarify the effects of chemical compounds over several generations.[11, 13, 14)] It is known that the influence of chemical exposure appears more significantly in juveniles and embryos during developmental stages than in mature parents; thus, several methods, such as exposing fry just after hatching[7, 8, 15–17)] and exposing fertilized eggs,[6, 10, 11, 16–20)] as well as microinjection into fertilized eggs,[21, 22)] are also utilized. In a special case, artificially inseminated eggs were exposed.[23, 24)] Most reports have adopted methods to observe biological influences, and thus have endpoints in biological outcomes, such as malformation, vitellogenin production levels as an indicator of male-to-female sex reversal, number of spawned eggs and hatching rate.

On the other hand, while a large number of biological experimental methods have been adopted, as described above, the number of studies that have examined the chemical compounds in fish[5, 8, 15–17, 25, 26)] and eggs[16, 18)] is relatively small, and those that have examined the concentration in eggs are particularly rare. Therefore, the present study conducted detailed investigations on a chemical compound by exposing parent medaka to BPA, and investigated the transfer from parent fish to the eggs, as well as excretion from the eggs after discontinuation of exposure.

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MATERIALS AND METHODS

Reagents and Water —— BPA was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). BPA-d_{16}, which was used as an internal standard for analysis, was also purchased from Kanto Chemical. *N*,*O*-bis(trimethylsilyl)trifloorooacetamide (BSTFA) which was used as a trimethylsilyl agent, was purchased from SUPELCO (Sigma-Aldrich, (St. Louis, MO, U.S.A.), MO, U.S.A.). Organic solvents were of residual pesticide test grade. Other reagents were of special grade, unless otherwise noted. Water for treatment prior to analysis was obtained through the Milli-Q purification system (Millipore Co., Billerica, MA, U.S.A.).

Medaka and Rearing Conditions —— The d-Rr strain of medaka (*Oryzias latipes*) was used, in which sex is easily distinguished, and is maintained by multi-generation breeding in our laboratory. The water for medaka rearing was obtained using a reverse osmosis (RO) water purification system (Kankyo Technos Co., Wakayama, Japan). One male fish and 2 female fish were placed into a 2-l beaker filled with RO water. For 1 week before initiation of the experiment, individuals that spawned eggs everyday were selected and used in the experiment. Twenty-two sets were prepared. Water temperature was kept at room temperature for 24 hr prior to use in experiments. Water was replaced every morning and no air was supplied. Water was kept at 25 ±1°C with an air-conditioner and a water jacket. During the rearing period, fish were fed approximately 10% of their body weight in freeze-dried baby artemia in the morning and evening. The photoperiod was light 16 hr-dark 8 hr.

Exposure Conditions —— After changing the water in the morning, 0.1 ml of 20 ppm-BPA dimethyl sulfoxide (DMSO) solution was added to 2 l of water, followed by stirring, before the fish were transferred. The BPA concentration was 100 μg/l. In the control group, 0.1 ml of DMSO solution was added to the water.

Concentration levels of BPA in river water were reported by Crain *et al.*[27] to vary from 0.016 to 21 ppb. In this study, the level of exposure for the fish was set at 100 ppb, which is higher than environmental concentrations. If the exposure level was set at actual environmental concentrations, for example, 1 or 10 ppb, 10 or 100 times more fish would be required. If the aim of our study was an evaluation of the biological effects of BPA, the exposure level would be set at environmental concentrations; however, as we were examining the persistence of BPA in adult fish and eggs, the exposure level was set at 100 ppb.

Measurement of BPA Concentrations in Water —— Concentrations of BPA in water, fish and eggs were analyzed according to previous methods.[26] The pH of rearing water was adjusted to approximately 3 by adding 0.7 ml of 1 M HCl to a 100-ml sample. After 1.5 g of NaCl, 1 μg of BPA-d_{16}, which is a surrogate compound, and approximately 5 ml of dichloromethane (DCM) were added, BAP in water was extracted by stirring for 30 min. This process was repeated twice. The collected DCM layers were dehydrated with anhydrous sodium sulfate. This was further concentrated to approximately 1 ml by evaporation under nitrogen gas. After approximately 200 μl of BSTFA was added, the sample was heated at 60°C for 30 min for derivatization, and was subjected to GC/MS analysis.

Measurement of BPA in Medaka and Eggs —— Wet weights of selected fish and spawned eggs were measured after the moisture on the surface was removed. Samples were kept at −30°C until analysis. Analyses were conducted on a whole fish or the eggs from 2–3 fish.

BPA levels in medaka were analyzed as follows. A whole medaka (0.369 ± 0.0828 g) was homogenized after addition of 1 μg of BPA-d_{16} and 5 ml of methanol. Homogenized samples were subjected to centrifugation at 3000 rpm for 10 min, and supernatants were collected. This process was repeated twice. After 10 ml of hexane was added to the collected methanol fraction, the mixture was shaken for 5 min, and the hexane layer was discarded. This process was repeated twice. After 60 ml of water, 500 μl of 1 M HCl, 1.5 g of NaCl and 5 ml of DCM were added to the methanol layer, the mixture was shaken for 10 min, and the DCM layer was collected. This process was repeated twice. The collected DCM layers were combined, and dehydrated with anhydrous sodium sulfate. After the sample was further concentrated to approximately 1 ml under a stream of nitrogen gas, it was subjected to a cleanup process using a silica-gel column. The silica gel was heated at 130°C for 17 hr, and added water at 5% (v/w) after the silica gel cooled. The BPA fraction from the silica gel was dried under a stream of nitrogen gas, and was re-dissolved into acetonitrile. This sample was purified by isolating interfering substances in a cleanup process using a Gel Permeation Chromatography (GPC) column, which is described below. For analysis of

eggs, eggs from 2–3 fish (0.0274 ± 0.00748 g) were analyzed together using the above-mentioned procedures. For BPA concentration analysis, the number measured for both fish and eggs was 5 (n = 5). Upon completion of the experiment, surviving fish that were not used for BPA analysis were kept separately in an aquarium until they died.

GPC Conditions —— Because numerous compounds that contain the same fragment ions as BPA (they are assumed to be fatty acids) are present in fish, microanalysis of BPA is difficult. Such compounds cannot be completely removed in the above cleanup process using the silica-gel column. Therefore, size exclusion chromatography was conducted using a GPC column.

In the GPC cleanup process, a GF-310HQ column (300 mm × 7.6 mm i.d.; Shodex, Tokyo, Japan), and a guard column GF-1G7B (Shodex) were used with a mobile phase of 100% acetonitrile. The injection volume was 50 µl. Because the BPA fraction appears in approximately 15 min, fractions eluted before and after 2 min were collected. After water was added to the fractions, the BPA components were extracted by DCM. Subsequent steps from condensation to measurement were the same as those described above for water samples.

GC/MS —— The gas chromatography system was a Varian GC-3800. The capillary column was a J&W DB-5MS (30 m × 0.25 mm i.d.; thickness of liquid phase, 25 µm). Measurements were performed with increasing column temperature from 35°C to 300°C. The mass detector was an ion-trapping mass spectrometer, Saturn 2000 (Varian Inc., (Palo Alto, CA, U.S.A.)). The sums of m/z = 357 and 358, and of m/z = 368 and 369 were used for quantification of BPA and BPA-d_{16}, respectively.

RESULTS AND DISCUSSION

Confirmation of Concentrations in Water

The BPA concentrations in the beaker while rearing medaka were analyzed 3 times daily; at 0, 12 and 24 hr. The results are shown in Fig. 1. The concentrations in the water were a few % higher than the set concentration of 0.1 ppm. However, because the concentration was almost constant for 24 hr between water changes, this variation was not considered to affect the experiment. In the present study, RO water was used. However, when tap water, which was kept at room temperature prior to use, was used in the preliminary experiment, it was found that the BPA concentration sometimes decreased by approximately 20–30% after 24 hr.

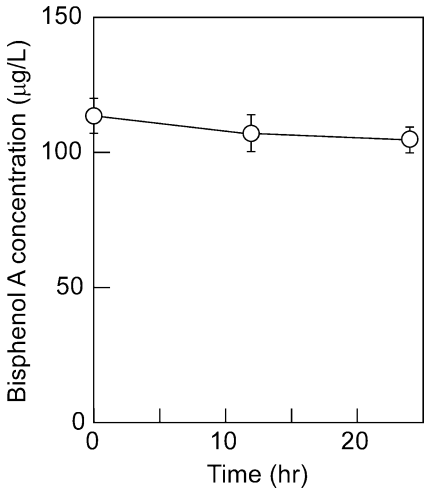

Fig. 1. Bisphenol A Concentration in the Beaker While Rearing Medaka

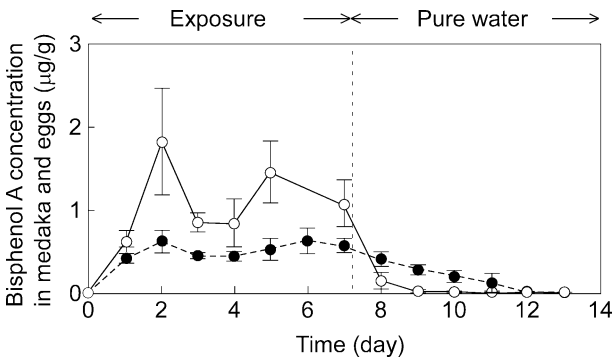

Fig. 2. Chronological Changes in Bisphenol A Concentration in Medaka Fish (Open Circles) and Eggs (Closed Circles) Spawned from These Medaka

Chronological Changes in BPA Concentrations in Medaka and Spawned Eggs

In Fig. 2, the chronological changes in BPA concentration in medaka for 7 days after the initiation of exposure and those after the fish was put back into pure water are indicated by open-circle plots (n = 5). Concentrations increased markedly from the day after exposure initiation, and maximum levels were observed on the 2nd day. On the 3rd and 4th days, the concentration decreased to approximately that of the 1st day, and then increased slightly again on the 5th and 7th days. Regarding the temporary decrease around the 3rd and 4th days, similar data has been reported in another study of fish exposed to BPA in water.[15, 26] The bioconcentration factor on the 7th day of the exposure was

approximately 11. On the 4th day of the experiment, the BPA concentration in medaka reared in an aquarium without BPA (blank test) was measured. The BPA concentration in all individuals was below the detection limit (0.01 μg/g).

BPA concentration in medaka that were placed back into pure water after 1 week of exposure decreased by 87% on the 1st day, and 98% on the 2nd day. The rate of BPA decrease in the fish body was thus rapid.

The chronological changes in BPA concentration in the eggs spawned from these medaka are plotted with closed circles. Although the concentration in the eggs during the exposure period fluctuated similarly as observed in parent fish, the degree of fluctuation was smaller; thus, the concentration was stable. A constant level of BPA was present in the eggs from the day after exposure initiation.

On the other hand, the BPA concentration in the eggs spawned from the fish put back into pure water after 1 week of exposure showed a different pattern of chronological changes from the parent fish. Although BPA was almost completely eliminated from the parent fish on the 2nd day, that in the eggs decreased only by 28% on the 1st day after the fish were put back into pure water, by 50% on the 2nd day, by 65% on the 3rd day, and by 76% on the 4th day.

These results suggested that BPA incorporation into and excretion from adult fish are extremely rapid. The rate of BPA incorporation into spawned eggs was also extremely rapid. However, even after the BPA concentration in parent fish decreased, it was found that considerable levels of BPA remained in the eggs. Therefore, it is assumed that when BPA is incorporated into the eggs from parent fish, there is no mechanism to excrete the incorporated BPA from the eggs.

BPA Concentration Change in Eggs in Pure Water

Figure 3 shows the results of daily BPA measurement in the eggs spawned from the parents on the 4th day of exposure and placed in pure water. Although some variation was observed, it was found that the BPA concentration in the eggs decreased slightly after 6 days in pure water. For example, when the averages are compared, the concentration in the eggs on the 6th day in pure water decreased by only 29% from the 1st day. Based on these results, it was assumed that the embryo cannot metabolize the BPA that was incorporated into the egg due to

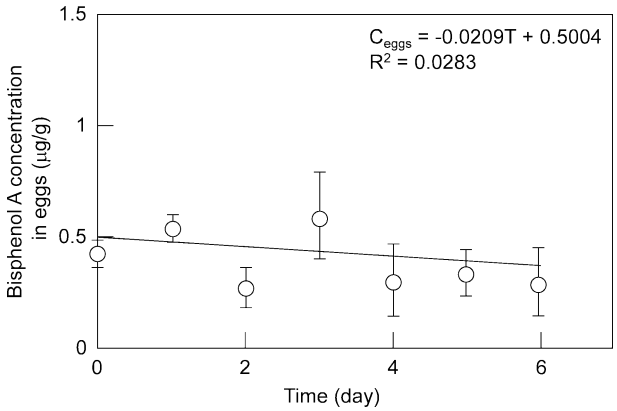

Fig. 3. Daily Bisphenol A Measurement in Eggs Spawned from the Parents on the 4th Day of Exposure and Placed in Pure Water

an underdeveloped metabolic or excretion mechanism. Thus, embryos that develop in spawned eggs are constantly exposed to such chemical compounds (BPA) passed on by the parents. Such chemical exposure in the developmental process increases the risk of malformation and developmental abnormalities.

REFERENCES

1) McLachlan, J. A. (2001) Environmental Signaling: What Embryos and Evolution Teach Us About Endocrine Disrupting Chemicals, *Endocr. Rev.*, **22**, 319–341.
2) Campbell, C. G., Borglin, S. E., Green, F. B., Grayson, A., Wozei, E. and Stringfellow, W. T. (2006) Biologically Directed Environmental Monitoring, Fate, and Transport of Estrogenic Endocrine Disrupting Compounds in Water: A Review. *Chemosphere*, **65**, 1265–1280.
3) Mills, L. J. and Chichester, C. (2005) Review of Evidence: Are Endocrine-Disrupting Chemicals in the Aquatic Environment Impacting Fish Populations? *Sci. Total Environ.*, **343**, 1–34.
4) Kang, I. J., Yokota, H., Oshima, Y., Tsuruda, Y., Oe, T., Imada, N., Tadokoro, H. and Honjo, T. (2002) Effects of Bisphenol A on the Reproduction of Japanese Medaka (*Oryzias latipes*). *Environ. Toxicol. Chem.*, **21**, 2394–2400.
5) Lindholst, C., Pedersen, K. L. and Pedersen, S. N. (2000) Estrogenic response of bisphenol A in rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.*, **48**, 87–94.
6) Yokota, H., Tsuruda, Y., Maeda, M., Oshima, Y., Tadokoro, H., Nakazono, A., Honjo, T. and

Kobayashi, K. (2000) Effect of Bisphenol A on the Early Life Stage in Japanese Medaka (*Oryzias latipes*), *Environ. Toxicol. Chem.*, **19**, 1925–1930.
7) Pastva, S. D., Villalobos, S. A., Kannan, K. and Giesy, J. P. (2001) Morphological effects of Bisphenol-A on the Early Life Stages Medaka (*Oryzias latipes*). *Chemosphere*, **45**, 535–541.
8) Honkanen, J. O., Holopainen, I. J. and Kukkonen, V. K. (2004) Bisphenol A Induces Yolk-sac Oedema and Other Adverse Effects in Landlocked Salmon (*Salmo salarm. sebago*). *Chemosphere*, **55**, 187–196.
9) Kang, I. J., Yokota, H., Oshima, Y., Tsuruda, Y., Hano, T., Maeda, M., Imada, N., Tadokoro, H. and Honjo, T. (2003) Effects of 4-Nonylphenol on Reproduction of Japanese Medaka, *Oryzias latipes. Environ. Toxicol. Chem.*, **22**, 2438–2445.
10) Seki, M., Yokota, H., Maeda, M., Tadokoro, H. and Kobayashi, K. (2003) Effects of 4-Nonylphenol and 4-*tert*-Octylphenol on Sex Differentiation and Vitellogenin Induction in Medaka (*Oryzias latipes*). *Environ. Toxicol. Chem.*, **22**, 1507–1516.
11) Yokota, H., Seki, M., Maeda, M., Oshima, Y., Tadokoro, H., Honjo, T. and Kobayashi, K. (2001) Life-Cycle Toxicity of 4-Nonylphenol to Medaka (*Oryzias latipes*). *Environ. Toxicol. Chem.*, **21**, 2552–2560.
12) Lin, B.-L., Hagino, S., Kagoshima, M., Ashida, S., Hara, T., Iwamatsu, T., Tokai, A., Yoshida, K., Yonezawa, Y., Tominaga, M. and Nakanishi, J. (2004) Three-Generation Full-Life-Cycle Study on 4-Nonylphenol Using S-rR Strain Medaka (*Oryzias latipes*). *J. Jpn. Soc. Water Environ.*, **27**, 727–734.
13) Seki, M., Yokota, H., Matsubara, H., Maeda, M., Tadokoro, H. and Kobayashi, K. (2003) Fish Full Life-Cycle Testing for the Weak Estrogen 4-tert-Pentylphenol on Medaka (*Oryzias latipes*). *Environ. Toxicol. Chem.*, **22**, 1487–1496.
14) Hutchinson, T. H., Yokota, H., Hagino, S. and Ozato, K. (2003) Development of fish tests for endocrine disruptors. *Pure Appl. Chem.*, **75**, 2343–2353.
15) Lindholst, C., Pedersen, S. N. and Bjerregaard, P. (2001) Uptake, Metabolism and Excretion of Bisphenol A in the Rainbow Trout (*Oncorhynchus mykiss*). *Aquat. Toxcol.*, **55**, 75–84.
16) Mäenpää, K. A., Penttinen, O.-P. and Kukkonen, V. K. (2004) Pentachlorophenol (PCP) Bioaccumulation and Effect on Heat Production on Salmon Eggs at Different Stages of Development. *Aquat. Toxcol.*, **68**, 75–85.
17) Petersen, G. I. and Kristensen, P. (1998) Bioaccumulation of Lipophilic Substances in Fish Early Life Stages. *Environ. Toxicol. Chem.*, **17**, 1385–1395.
18) Honkanen, J. O., Heinonen, J. and Kukkonen, J. V. K. (2001) Toxicokinetics of Waterborne Bisphenol A in Landlocked Salmon (*Salmo salar m. sebago*) Eggs at Various Temperatures. *Environ. Toxicol. Chem.*, **20**, 2296–2302.
19) Iwamatsu, T., Kobayashi, H., Hamaguchi, S., Sagemami, R. and Shuo, T. (2005) Estradiol-17b Content in Developing Eggs and Induced Sex Reversal of the Medaka (*Oryzias latipes*). *J. Exp. Zool.*, **303A**, 161–167.
20) Helmstetter, M. F. and Alden III, R. W. (1995) Toxic Responses of Japanese Medaka (*Oryzias latipes*) Eggs Following Topical and Immersion Exposures to Pentachlorophenol. *Aquat. Toxcol.*, **32**, 15–29.
21) Colman, J. R., Twinre, M. J., Hess, P., McMahon, T., Satake, M., Yasumoto, T., Doucette, G. J. and Ramsdell, J. S. (2005) Teratogenic Effects of Azaspiracid-1 Identified by Microinjection of Japanese Medaka (*Oryzias latipes*) embryos. *Toxicon*, **45**, 881–890.
22) Villalobos, S. A., Papoulias, D. M., Pastva, S. D., Blankenship, A. L., Meadows, J., Tillitt, D. E. and Giesy, J. P. (2003) Toxicity of *o*, *p*′-DDE to Medaka d-rR Strain after a One-time Embryonic Exposure by in Ovo nanoinjection: an Early through Juvenile Life Cycle Assessment. *Chemosphere*, **53**, 818–826.
23) González-Doncel, M., de la Peña, E., Barrueco, C. and Hinton, D. E. (2003) Stage Sensitivity of Medaka (*Oryzias latipes*) Eggs and Embryos to Permethrin. *Aquat. Toxcol.*, **62**, 255–268.
24) González-Doncel, M., Fernández-Trija, C., Hinton, D. E. and Tarazona, J. V. (2004) Stage-Specific Toxicity of Cypermethrin to Medaka (*Oryzias latipes*) Eggs and Embryos Using a Refined Methodology for an *In Vitro* Fertilizaion Bioassay. *Arch. Environ. Contam. Toxcol.*, **48**, 87–98.
25) Lindholst, C., Wynne, P. M., Marriott, P., Pedersen, S. N. and Bjerregaard, P. (2003) Metabolism of Bisphenol A in Zebrafish (*Danio Rerio*) and Rainbow Trout (*Oncorhynchus mykiss*) in Relation to Estrogenic Response. *Comp. Biochem. Physiol. C*, **135**, 169–177.
26) Lee, H. C., Soyano, K., Ishimatsu, A., Nagae, M., Kohra, S., Ishibashi, Y., Arizono, K. and Takao, Y. (2004) Bisphenol A and Nonylphenol Bioconcentration in Spotted Halibut *Verasper variegates. Fisheries Sci.*, **70**, 192–194.
27) Crain, D. A., Eriksen, M., Iguchi, T., Jobling, S., Laufer, H., LeBlanc, G. A. and Guillette, L. J. (2007) An ecological assessment of bisphenol-A: Evidence from comparative biology. *Reprod. Toxicol.*, **24**, 225–239.