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The Influence of Bisphenol A on Selenoprotein N Expression Genes during Zebrafish Embryogenesis

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Bisphenol A가 Zebrafish 발생과정 중 Selenoprotein N 발현 유전자에 미치는 영향

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요 약

내분비계 장애물질 중 에스트로겐성 특성을 지닌 것으로 알려진 bisphenol A에 폭로되었을 때에 나타나는 이상 증상과 selenoprotein N 결핍 증상은 유사성이 있는 것으로 관찰되었다. 이에 본 연구에서는 bisphenol A에 폭로된 생물체의 selenoprotein N 유전자 발현에 영향을 미치는지에 관해 연구하였다. 실험동물은 zebrafish (*Danio rerio*, wild type)의 수정란을 사용하였다.

Zebrafish의 수정란은 서로 다른 농도의 bisphenol A (0.1, 1, 10 ppm)에 노출하였다. 각각의 폭로 환경에서 부화된 치어를 시료로 selenoprotein N 유전자 발현을 RT-PCR 방법에 의해 알아보았다. 그 결과, bisphenol A는 척추이상과 심장이상이 나타나 selenoprotein N 이상시 나타나는 현상과 유사성을 보였다. Selenoprotein N 유전자 발현은 bisphenol A 폭로 농도가 높아짐에 따라 유전자 발현 농도가 낮아지는 것으로 관찰되었다. 특히 1 ppm 농도에서는 대조군이나 0.1 ppm 농도보다 catalase의 활성이 높게 나타나 특정 농도에서 bisphenol A에 의한 영향이 나타나는 것을 알 수 있었다.

Key words : bisphenol A, selenoprotein N, zebrafish

INTRODUCTION

Endocrine disrupting chemicals (EDCs) have disturbed not only the development of the reproductive processes and endocrine systems, but also function as a hand-me-down poison. It also affects on the offspring as the abnormality on the embryogenesis and reproduction (Guillette, 1996; Cooper and Kavlock, 1997; Mantovani *et al.*, 1999; Crews *et al.*, 2000; Guillette and McLachlan, 2001). Bisphenol A, 4, 4′-isoprophylidenediphenol, is an important chemicals as composing epoxy, polycarbonate and polystyrene

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resins even used in a cock of nursing bottle, food container, beverage can and erosion protective coating. Bisphenol A (BPA) has an estrogenic property (Safe *et al.*, 2001; Kho, 2004). Since it has been used in wide-range of industries, therefore humans and wild lives have been exposed to the industrial and artificial chemicals. The variety of compounds resembles natural hormones, so they mimic or interrupt the function of hormones as changing all directions to organize and maintain organisms (Cornel *et al.*, 2000; Kim and Hwang, 2002). According to the case study of rodents, weights of uterus increased, prolactin secretion was facilitated and huge multiple-nucleuses liver cells frequently appeared in male mice by bisphenol A, in fact (Bond *et al.*, 1980; Steinmetz *et al.*, 1997; Kim and Jo, 2000; Yeo, 2003).

Zebrafish (*Danio rerio*, wild type) which used in the experiment is the vertebrate and the appropriate animal model to observe the embryogenesis (Yeo, 2003). So, zebrafish is generally used in estrogenic endocrine disrupter exposure experiment. As a common idea, bisphenol A seriously affects on the zebrafish embryogenesis, especially, it caused the vertebral disorders. Moreover, a normal incubation was limited (Kang *et al.*, 2006).

On the other hand, the preceding studies have explained that a new disease called nutritional muscular dystrophy is caused by the deficiency of selenium in an organism (Rederstorff *et al.*, 2006; Deniziak *et al.*, 2007). Several mutations on the selenoprotein N 1 (sepn 1) gene which encodes the expression of a protein containing selenium, a selenoprotein N (SelN) causes a certain muscular disease called congenital muscular dystrophy (CMD) (Deniziak *et al.*, 2007). It has been proved that a supplementary 200 μg of selenium immensely declined the probability of cancers such as prostate cancer, skin carcinoma by the research of Clarketal *et al.* (Clark *et al.*, 1996; Parker *et al.*, 2007). From the time on, current promising researches and case control studies have demonstrated that higher selenium absorption or level is relevant with reducing the risk of prostate cancer (Clark *et al.*, 1996; Combs, 2004; Meuillet *et al.*, 2004; Klein, 2004; Etminan *et al.*, 2005; Rayman, 2005; Parker *et al.*, 2007). It have confirmed that estrogen act to enhance the progression of prostate cancer at early stage from current research about animals and human pathology (Steiner *et al.*, 2001; Risbridger *et al.*, 2003; Harkonen and Makela, 2004; Ho, 2004; Parker *et al.*, 2007). Because Zn in estrogen receptor Zn fingers was eliminated and disulfide bonds were facilitated, selenium reveals property against prostate cancer by decreasing endocrine receptors binding to DNA and gene expression of endocrine receptor regulation (Thompson *et al.*, 2000; Parker *et al.*, 2007).

In summary of these results, it is hypothesized that the exposure of bisphenol A which hinders the normal bonding of estrogen receptor and the expression of selenoprotein N on zebrafish can affects on the selenium metabolism. The hypothesis may result that condition exposing bisphenol A on the animal makes a morphological abnormalities in muscles and spines, and causes the sexual ratio unbalance whenever it survives.

In other words, it seems that simply natural density of bisphenol A is so slight, zebrafish commonly survive and become a malformation or sexually unbalanced. In the process, abnormalities of selenium metabolism and selenoprotein N expression would be closely related with endocrine disrupters, for instance, bisphenol A.

In this study, the embryos of zebrafish were exposed at bisphenol A in different concentrations (0.1, 1, 10 ppm) and investigated the biological toxicity to zebrafish during embryogenesis In addition, samples from each group were used for verifying genetic disorders at genes coding selenoprotein N by RT-PCR method. We measured the hatching rate, abnormality rate, and activity of the catalase.

MATERIALS AND METHODS

1. Experimental animal

The wild-type zebrafish (*Danio rerio*) used in this

study were raised for 6~7 months. The water purified through 60L-carbon filter and its temperature was controlled at 28±1℃. Full-grown fish were raised on a 14/10 h light/dark cycle. Embryos were collected and bred by the method of Westerfield (Westerfield, 1995; Yeo, 2003; Deniziak *et al.*, 2007).

2. Bisphenol a exposure

For the control group, fertilized eggs developed in piped water which was ripened for 48 hours to eliminate disinfectants. In addition, some other fertilized eggs were exposed at 0.1 ppm, 1 ppm and 10 ppm of bisphenol A during embryogenesis for the experimental group (Yeo, 2003). It was taken about 48 hours to be fries. Because the proper water temperature for development is 28.5℃, so incubators were controlled between ±1℃. Each group contained 300 fertilized eggs (Kang *et al.*, 2006).

3. Morphological effects

By the point of 48 hours after the exposure, some of the zebrafish embryos were sampled and observed under a stereoscope (Olympus, SZ61, Japan). Especially, their spinal and cardiac development was photographed detail. After the process, fries were returned to the incubator.

4. Catalase activity analysis

The condition of experimental animals can be estimated by the activity of catalase, which is an enzyme to deprive an organism of activated oxygen. Especially, five sample fish from each group were put into micro tubes and dried by micro pipette and centrifuge. After calculating the weight of tissue, fish were homogenized with 0.1 M, pH 7.3 phosphate buffer. The solution was centrifuged at 4℃, 12,000 rpm for 5 min and the supernatant used as a resource of the enzyme. Reaction with H_2O_2 was conducted in an incubator at 37℃ for 1 min. Before measure the absorbance by UV-Vis spectrophotometer at the maximum wavelength of catalase, 405 nm, the reaction should be suspended by adding 32.4 mmol/L ammonium molybdate.

5. RT-PCR

The expression of the sepn1 gene in wild-type zebrafish was assessed by RT-PCR performed on total RNA samples isolated from 48 hpf flies (n=30), using a modification to the method described by Yeo and Kang (2008). Total RNA was obtained using the easy-BLUE™. Total RNA Extraction Kit (iNtRON Biotechnology, Inc.) according to the manufacturer's instruction. Reverse transcription was performed using 8u of a One-Step RT-PCR premix kit (iNtRON Biotechnology, Inc.), ~200~500 pg of each total RNA matrix and 10 pmol of AL 299 reverse primer hybridizing in the 3′ region of the SelN coding sequence (5′-GAGAGCTCTTGACAGAGG-3′). The reaction was conducted at 45℃ for 60 min, in a 20 μL reaction mixture, containing 50 mM KCl, 4 mM $MgCl_2$, 50 mM Tris HCl pH 8.3, 10 mM DTT, 1 mM dNTPs mix and 20u RNasin (Promega). For each RNA sample, a "-RT control" (reaction without reverse transcriptase) was performed. Reverse transcribed cDNAs (5 μL) were then amplified in a 50 μL PCR reaction, containing 10 mM Tris HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mg/mL BSA, 200 μM each dNTP, 20 pmol of each primer and 1u Taq Polymerase (Qbiogene). PCR was conducted under the following conditions: one cycle of denaturation at 94℃ for 5 min, 30 cycles of 94℃ for 45s, 65℃ for 1 min and 72℃ for 2 min; amplification was terminated by one cycle of elongation at 72℃ for 10 min. The following oligonucleotides were used in the PCR reaction: sepn-ex1, hybridizing to the 3′ region of exon1 (5′-GGGACTCCATCCAG CAGACG-3′) and sepn-ex3, are verse primer complementary to exon3 (5′-TGCAGGGTCAGCGTCTCTCC-3′). Amplification products were analyzed by electrophoresis on a 1.2% agarose gel (Deniziak *et al.*, 2007). Data were analyzed using a paire done-tailed Student's *t*-test to determine the lowest statistically significant concentration relative to an unexposed base-

line control.

RESULTS

The following photographs were taken at 48 hours after fertilization.

The control group seemed normal, especially for muscle and spines (Fig. 1A, B, C). Compare to control group, bisphenol A experimental groups contain individuals that has much more severe disorder. The figure of 0.1 ppm group (Fig. 1D, E, F), showed some fries were curved. Moreover, the fish in 1 ppm group got rumpled (Fig. 1G, H, I). For some embryos, several disorders such as cardiac dropsy, vitelline disorder were observed.

The absorbance at the 405 nm UV wavelength was the most active point of catalase, so data were used for catalase activity which implies the health of fries.

Compare to other groups, bisphenol A 1 ppm expo-

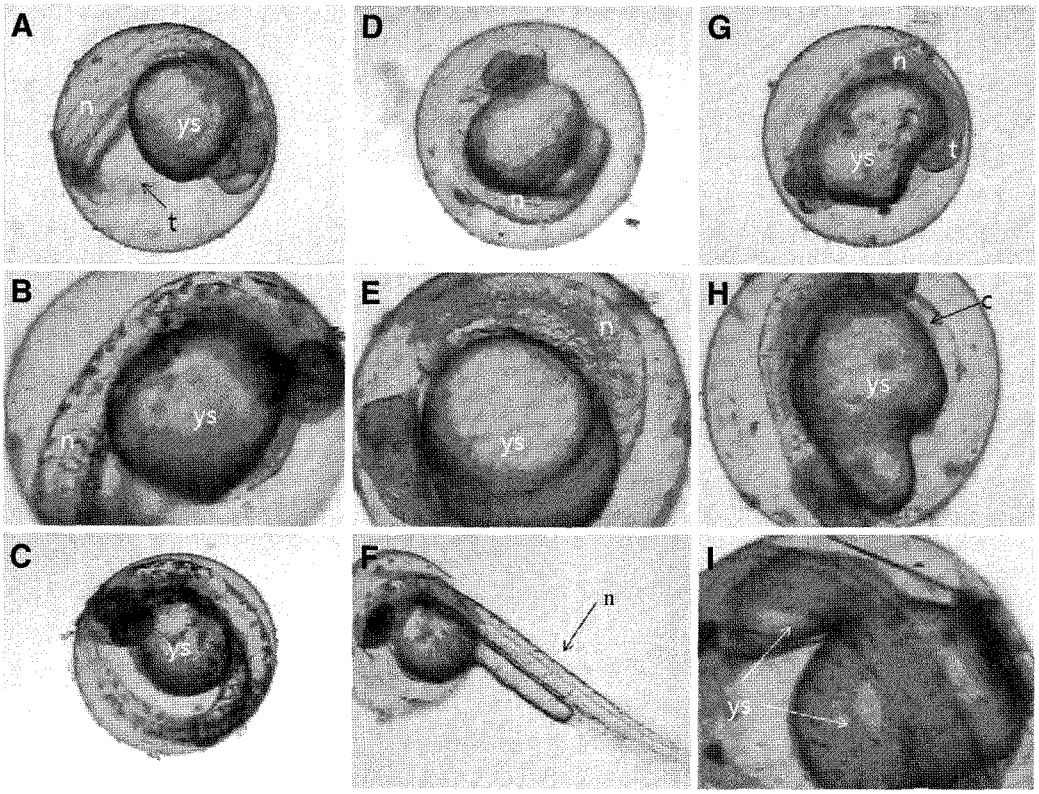

Fig. 1. The effects of bisphenol A on the development of zebrafish. Embryos were exposed to bisphenol A 0.1 ppm (D, E, F), 1 ppm (G, H, I), control (A, B, C). These images show exposed zebrafish at 48 hpf. *Abbreviations*: c, cardiac; n, notochord; t, tail; ys, yolk sac.

Table 1. The Ratio of Incubation and Handicapped

	Spinal disorder	Dropsy	Pulsation disorder	Handicapped	Survival	Final incubation ratio
Control	1	1	1	1/36	36/70	36/36
1 ppm	33	1		33/43	43/70	0/43
10 ppm	30	16	6	30/42	42/70	0/42

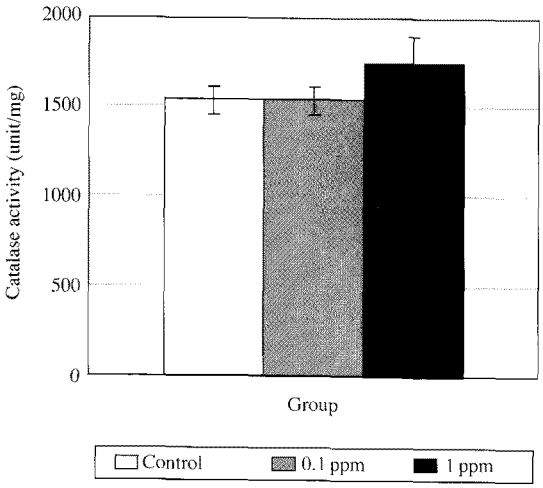

Fig. 2. The result of catalase activity. The effects of bisphenol A on the catalase activity. The catalase activities in the bisphenol A 1 ppm exposed group is shown to be increased. However there weren't significantly difference control versus experiment groups.

sure group had an extraordinarily high activity. Detail figures were here; 1,535 unit/mg for control group, 1,533 unit/mg for 0.1 ppm group and 1,750 unit/mg for 1 ppm group. This result was obviously opposite to preceding researches (Yeo, 2003), so it derived a different conclusion.

Because the data for catalase were clearly different from other researches, the experiment was conducted again on 70 embryos for each group. As a result, photographs were taken and the ratio of incubation and handicapped was added (Table 1).

Second experiment contained different experimental groups such as 10 ppm of bisphenol A group-(Fig. 3G, H, I)-instead of 0.1 ppm group. Many abnormal morphologies were seen in the 10 ppm bisphenol A group after 48 hours. As shown in Fig. 3, there were weak heart beats, abnormal vertebra, pigment deficiencies, and edema. Under the changed condition,

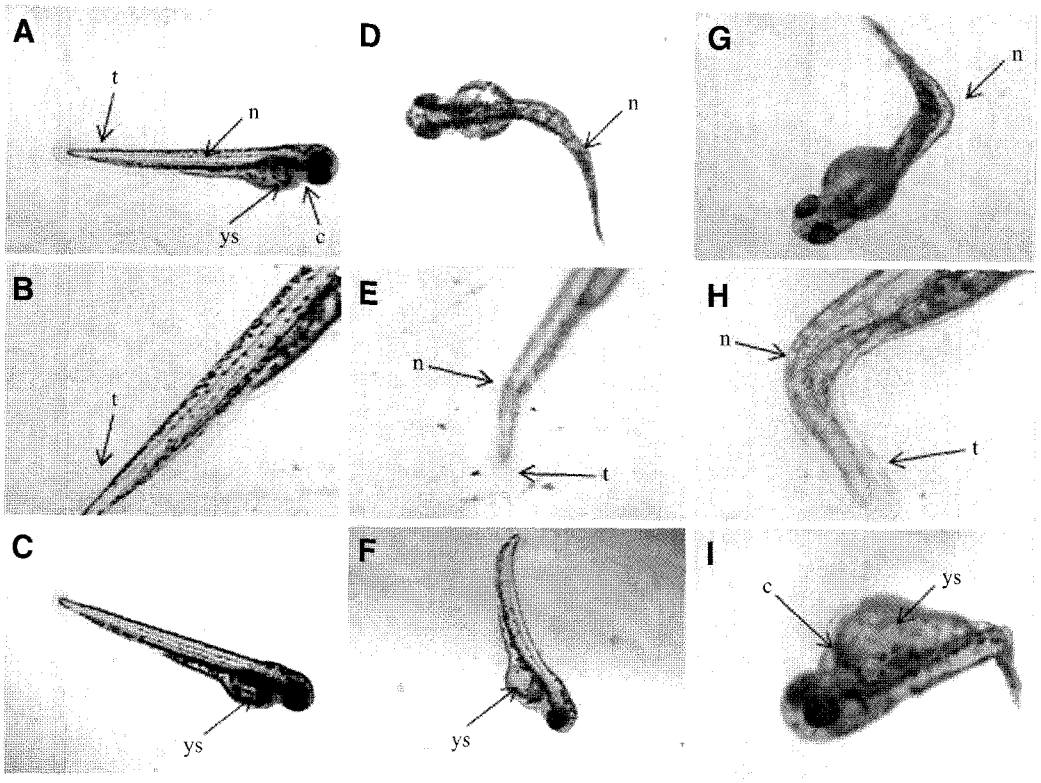

Fig. 3. Experimental groups in different condition. Control (A, B, C), 1 ppm (D, E, F), 10 ppm (G, H, I). *Abbreviations*: b, brain; e, eye; n, notochord; t, tail; ys, yolk sac.

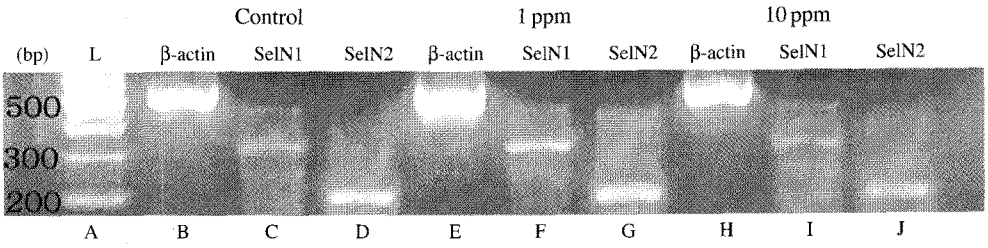

Fig. 4. RT-PCR results for the SelN-1 and SelN-2 genes in hatched zebrafish under bisphenol A exposed conditions (0.1 ppm and 1 ppm). The line L denotes the marker. β-actin is at 536 bp, SelN1 is found at 317 bp and SelN2 is at 196 bp.

the embryos morphologically twisted especially at vertebra. Since the obvious disabled were observed, they were counted for the ratio.

Compare to experimental groups, embryos from control group normally developed. Some individual having troubles were taken photographs in Fig. 3. The handicapped were sampled for RT-PCR.

The result of RT-PCR was visible by electrophoresis and it involved with selN-1 and 2. For standardization, β-actin gene primers were inserted to prove that each sample contained PCR product.

Each group had three different PCR product; β-actin, selN-1, and selN-2. The first lane showed marker (Fig. 4A). The control group products were (Fig. 4B), (Fig. 4C) and (Fig. 4D), which were ordered as β-actin, selN-1, and selN-2. All bands were looked similar as control group, however (Fig. 4E), (Fig. 4F) and (Fig. 4G) were 1 ppm group PCR products. The order was the same. Resemble to other groups (Fig. 4H), (Fig. 4I) and (Fig. 4J) were 10 ppm group.

DISCUSSION

According to the first hypothesis, estrogen-steroid hormone-receptors were involved to selenoprotein N transcription factors, so the concentration of estrogen affected on the function of selenoprotein N.

As Fig. 5 shows, estrogen helps the steroid receptor to bind on transcription factors for DNA. In the hypothesis, however, bisphenol A hindered the function of estrogen since it is a kind of steroid. In other words, bisphenol A conducted a competitive inhibitor against estrogen, so the transcription for mRNA would not be allowed. The Fig. 6 shows the processes. This hypothesis has a probability, because

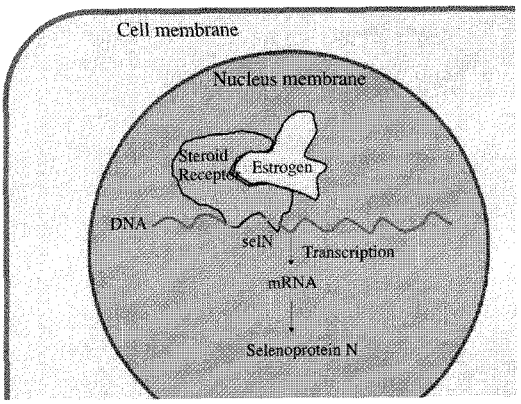

Fig. 5. The mechanism of estrogen.

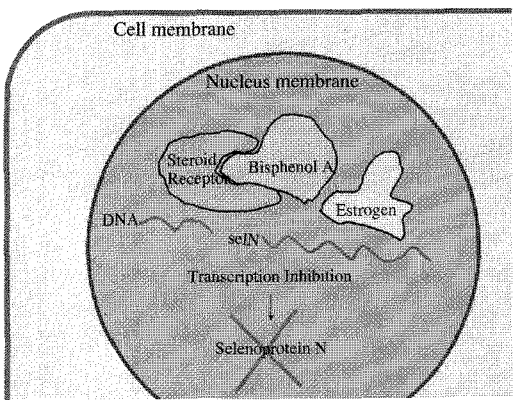

Fig. 6. The mechanism of bisphenol A to inhibit estrogen.

effects of the loss of selenoprotein N were closely similar to the consequence of bisphenol A exposure (Petit *et al.*, 2003; Wetherill *et al.*, 2007). Particularly, the expression of selN genes were associated with muscular and vertebral development that was mentioned in the part of bisphenol A researches in the case of zebrafish (Yeo, 2003; Deniziak *et al.*, 2007).

However, the result was different from the estimation. Actually, the first experiment was completely opposed to the common sense. So all the experimental conditions and instrument were analyzed. As a result, the previous experiment that used Ag (silver) nano particle affected on the surprising outcome. According to a preceding article, Ag nano particle could competitively disturb the membrane penetration of bisphenol A in the high concentration (Coleman *et al.*, 2005). In addition, it was observed that protists were eliminated in dense bisphenol A condition. Therefore, a large number of embryos in 1 ppm group could have a better condition than that of control group.

The second experiment was conducted with new appliances without pollution. Still the data of incubation and handicapped ratio were similar to expectation, electrophoresis showed unexpected result. The PCR products implied two probabilities.

The first chance means no relationship between bisphenol A and selenoprotein N. It is because bisphenol A exposure did not work on the expression of selenoprotein N gene, selN-1 and selN-2. However, the impetuous conclusion seemed an overstep jump because they had similar acts on zebrafish.

The second probability suggests that a different mechanism would work on. One of proposals is that bisphenol A does not affect on the gene, but it is involved to degradation of selenoprotein N or the metabolism of selenium. In fact, selenium is a trace element which is needed a little but not too much. So, a little amount of selenium can largely influence on selenoprotein N synthesis. Instead of the suggestion, bisphenol A can bond to a protein that decompose other products.

In conclusion, the mechanism of bisphenol A function was not clearly explained, but a certain relation between endocrine disrupters and selenoprotein N exist. This paper confirmed the probability and induce the after study. It seems that much more investigation about both bisphenol A and selenoprotein N would be needed.

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