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Short Paper

Effects of bisphenol A and DDT on mRNA expression of vitellogenin II in liver of quail embryos

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Summary

This study was conducted to reveal the estrogenic effects of bisphenol A and o, p'-DDT on quail embryos. Thirteen fertilized eggs were used as control (injected with 20 µl corn oil), 15 eggs were injected with estradiol 17β (0.04 mg dissolved in 20 µl corn oil), 20 eggs were injected with BPA (2 mg dissolved in 20 µl corn oil) and 20 eggs were injected with o, p'-DDT (2 mg dissolved in 20 µl corn oil) at day 13 of incubation. Two days later the livers of the embryos were collected. The DNA was extracted from the liver for molecular sexing, while total RNA was extracted for vitellogenin II (VTGII) mRNA expression in embryos. In female embryos, BPA and o, p'-DDT induced variable levels of VTGII mRNA expression, while in male embryos, o, p'-DDT induced a slightly VTGII mRNA expression. In contrast, there was no expression of VTGII after BPA injection. In conclusion, the estrogenicity of BPA was lower than o, p'-DDT and both of them were lower than the estradiol 17β.

Key words: DDT, Bisphenol, Quail, Embryo, Vitellogenin

Introduction

Global attention has recently become focused on environmental estrogenic compounds. The problem of endocrine disrupters is one of the five priority research areas established by the Committee on the Environment and Natural Resources of the United States (Kavlock, 1999).

Since it is difficult to predict the hormonal activity of environmental contaminants based on their chemical structures, the estrogenic potency of a substance or mixture of substances must be determined and characterized by biological methods.

The Japanese quail provides an excellent avian model for testing the endocrine-disrupting potential of chemicals *in vivo* as this species has well characterized reproductive endocrine and behavioral response (Ottinger *et al*., 2001; Mattsson *et al*., 2008; Yamashita *et al*., 2011).

Bisphenol A (4, 4′-isopropylidene-2-diphenol, BPA), suspected as an endocrine disrupter, is a major component of epoxy and polycarbonate resins which are widely used as ingredients of the protective coatings on food containers and as adhesive used in packing products, as well as in sealants and restorative materials employed for dentistry (Howe *et al*., 1998).

The parent compound of DDT and its metabolites persist in the global environment throughout the food chain and thus remain a concern (Brown, 1997). Avian species have

the possible risk of embryonic exposure to DDT by transfer of chemicals accumulated in mother birds to eggs. Transovarian exposure to o, p'-DDT (o, p′ isomer of dichlorodiphenyltrichloroethane) could bring about population declines in avian species through loss of fecundity caused by depression of hatchability and dysfunction of the reproductive tract (Kamata *et al*., 2009).

The data concerning the *in ovo* exposure of birds to endocrine disruptors is scarce, that is why the objectives of the present study were to evaluate the estrogenicity of BPA and o, p'-DDT injected into quail's embryos and to investigate their ability to induce VTGII expression in the liver of quail's embryos.

Materials and Methods

Egg incubation and injection of chemicals

Adult male and female Japanese quail were mated and the fertilized eggs were then incubated at 37.5°C and 60% relative humidity in an incubator. Birds were handled in accordance with regulations approved by the Animal Experiment Committee of Hiroshima University, Japan. At day 13 of incubation, eggs were swabbed with ethanol 70% and classified into 4 groups. Each group was injected with the chemicals according to (Halldin *et al*., 2001, 2003; Oshima *et al*., 2012) into the air sac under sterile condition. The eggs openings were sealed with paraffin and stored back in the same incubator.

DNA extraction and molecular sexing of embryos

The liver from each embryo in different treatment groups was collected and immediately put on dry ice for 1 min and then placed in a sterile autoclaved eppendorff tube and kept at -70°C until DNA and total RNA extraction. The DNA was extracted with SEPAGENE kits (Sanko-Jyunyaku Co., Tokyo, Japan). Moreover, DNA was extracted from 20 µl of peripheral blood from adult male and female quails as a control.

DNA of embryos and control adult male and female were amplified with 2550F (5'-GTT ACT GAT TCG TCT ACG AGA-3') and 2718R (5'-ATT GAA ATG ATC CAG TGC TTG-3') primers for molecular sexing according to the method of Fridolfsson and Ellegren (1999).

Reverse transcription-PCR of VGII mRNA in male and female embryos

Liver tissue was melted immediately before RNA extraction and then cut into 30 mg with sterile blades. The tissues were transferred to sterile micro-centrifuge tube. Total RNA was extracted with RNA extraction kit (RNeasy, Qiagen Inc., CA, USA).

RNA was mixed thoroughly with RT-PCR mixture and then the mixture was reverse transcribed according to the instruction of Promega Corporation (Hollow Road Madison, WI, USA). Position and sequence of synthetic oligonucleotide primers used in RT-PCR is mentioned in Table 1. Difference among control and treated groups was analysed with ANOVA (GraphPad Software, San Diego, CA, USA). It was considered significant at $P<0.05$.

Results

The male embryos as well as the control adult male showed one band of approximately 600 bp, while the female ones showed two bands of 600 bp and 450 bp. There was an evident size difference of the PCR products between the male and female Japanese quail.

There was no VTGII expression in the liver of control males and females embryos,

Table 1: Position and sequence of synthetic oligonucleotide primers used in RT-PCR assay

Gene	Location	Sequence (5' ⟶ 3')	Reference
VTGII	31-50	AGC AGT AGT GCT CAC CCT TG	Van het Schip *et al*. (1987)
	394-413	ATA GCC CAC TTG ATC TCT AT	
β-actin	168-194	ATC GTG GGT CGC CCC AGA CAT CAG GGT	GeneBank (AF199488)
	1028-1054	ATC TTG ATT TTC ATT GTG CTA GGT GCC	

whereas distinct VTGII mRNA transcripts were observed in the estradiol 17β treated groups. For the internal control, β-actin expression was detected in all samples (Figs. 1 and 2). In female embryos, a significant expression ($P<0.05$) of VTGII was observed after BPA. Moreover, o, p'-DDT injection induced remarkable expression ($P<0.01$) of VTGII (Fig. 1). In male embryos, there was no VTGII expression in BPA treated embryos, although o, p'-DDT treatment induced a slight ($P<0.05$) VTGII expression (Fig. 2). The semi-quantitative data showed that the expression of liver VTGII increased markedly ($P<0.001$) after the estradiol 17β treatment in both female and male embryos when compared to the other groups.

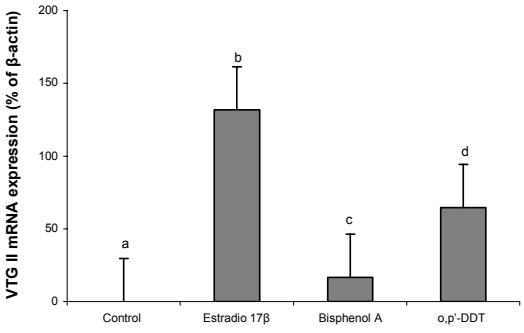

Fig. 1: VTGII mRNA expression in liver of female quail embryos. mRNA expressions were expressed as a relative density of RT-PCR products compared to β-actin

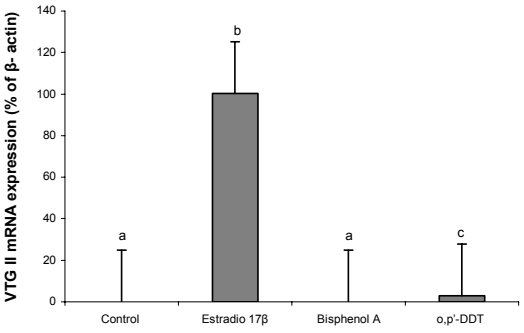

Fig. 2: VTGII mRNA expression in liver of male quail embryos. mRNA expressions were expressed as a relative density of RT-PCR products compared to β-actin

Discussion

The growing list of chemicals suspected to contribute to environmental and human health problems as obesity includes persistent organic pollutants such DDE and its precursor DDT (Casals-Casas and Desvergne, 2011). The general population is still exposed to these substances at low doses, mainly through the food chain that lead to accumulation in animal and human fat tissues over many years (Porta *et al*., 2008).

The results of embryo sexing was inconsistent with Fridolfsson and Ellegren (1999), who reported that CHD1W fragments varied between 400 and 450 bp in size, and CHD1Z fragments between 600 and 650 bp. Male birds can be recognized by a single DNA band whereas females showed two bands which distinguished the embryos before VTGII mRNA expression.

Elbrecht *et al*. (1984) demonstrated that VTGII mRNA expression in the liver of chicken embryos was induced by estrogen treatment. Ichikawa *et al*. (2003) observed that VTGII mRNA expression was inducible in the quail embryos following estrogen injection at day 13 of incubation. Furthermore, the expression of VTGII was potentiated with additional estrogen injection at day 16 of incubation in chicken embryos (Sakimura *et al*., 2001). Consistent with the previous reports, the present study also demonstrated that VTGII expression was induced in the liver of quail embryos by estradiol 17β. The expression of VTGII was observed in both female and male embryos. No expression was observed in control female and male group after injection of corn oil.

There are a few research studies on the effects of o, p'-DDT and BPA on VTGII mRNA expression in quail's embryos. In the present study, VTGII expression was induced by o, p'-DDT. In female embryos, there is a detectable expression level; however, a very slight VTGII expression was observed in male embryos. Lorenzen *et al*. (2003) reported that o, p'-DDT in a dose of 10,000 nM, induced the expression of VTGII in chicken embryo primary hepatocyte cultures. BPA has no ability to induce VTGII expression in male embryos; in contrast, a slight expression was detected in female embryos. Lorenzen *et al*. (2003) found that BPA was not estrogenic in chicken's embryo primary hepatocyte cultures.

In conclusion, BPA and o, p'-DDT

revealed estrogen-like effects in male and female embryos. Administration of BPA and o, p'-DDT affected VTGII expression in female embryos in a negative way, and this adverse effect was seen less in male embryos. However, further toxicodynamic studies are needed in order to elucidate the adverse effects of BPA and o, p'-DDT exposure in birds, with its different aspects.

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