INDUCTION OF VITELLOGENIN mRNA IN JUVENILE CHINESE STURGEON (ACIPENSER SINENSIS GRAY) TREATED WITH 17β-ESTRADIOL AND 4-NONYLPHENOL

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Abstract—It has been demonstrated that 4-nonylphenol (4-NP) exerts estrogenic effects in diverse fishes. The present study investigated the effects of 4-NP on Chinese sturgeon (Acipenser sinensis Gray) vitellogenin (VTG) gene expression. By reverse transcription–polymerase chain reaction (RT-PCR) using degenerate primers, a 462–base pair fragment of Chinese sturgeon VTG, corresponding to a 154–amino acid sequence, was amplified and sequenced. This sequence exhibited 152/154 identity to the amino acid sequence of white sturgeon (A. transmontano) VTG. Conventional RT-PCR and quantitative real-time RT-PCR were established and used to study the VTG and β-actin gene expression in the liver of juvenile Chinese sturgeon injected three times with 17β-estradiol (5 mg/kg body wt/week) or 4-NP (10 or 100 mg/kg/week) in the course of three weeks. Significant induction of VTG gene expression was detected in all treated groups, and no VTG mRNA was detected in the control group. The ratio of VTG to β-actin analyzed from the results of quantitative real-time RT-PCR reached 0.041 ± 0.024 (mean ± SD) in the group receiving 10 mg/kg/week of 4-NP and 4.51 ± 1.68 in the group receiving 100 mg/kg/week of 4-NP. Chemical analysis of 4-NP showed that the concentrations of 4-NP in the 10 mg/kg/week group and the 100 mg/kg/week group were 2.78 ± 2.41 and 31.38 ± 0.26 μg/g wet weight, respectively. Compared with the 4-NP concentrations (0.8–1.92 μg/g wet wt) in fish from the Yangtze River, China, a potential hazard exists regarding 4-NP in Chinese sturgeon. These results represent the first indication of the risk of endocrine-disrupting chemicals for Chinese sturgeon.

Keywords—Endocrine-disrupting chemicals Real-time polymerase chain reaction Vitellogenin 4-Nonylphenol

Acipenser sinensis

INTRODUCTION

The past decade has seen great concern regarding endocrine-disrupting chemicals (EDCs) [1]. These chemicals have the ability to mimic the action of endogenous estrogen and to disrupt the endocrine system, development, and reproduction of fish and wildlife [2–5]. Endocrine-disrupting chemicals include man-made and naturally occurring compounds, such as 4-nonylphenol (4-NP) and 17β-estradiol (E2) [6–9]. Of concern is 4-NP, a degradation product of nonylphenol polyethoxylates (NPEOs), that is commonly used as a nonionic surfactant and is ubiquitous in aquatic environments. Several reports have shown that 4-NP can inhibit testicular growth [6], induce intersexuality–ova development, reduce viability of spermatozoa [7,8], alter the sex ratio, and cause dysplasia of the gonadal lumen and testicular agenesis in teleosts [9]. A number of studies have reported the estrogenic effects of NP on several species of fish, but to our knowledge, no report concerning the effects of 4-NP on sturgeon has been published.

Sturgeon belong to one of the most ancient groups of the Osteichthyes and are of extremely great commercial value. Steep declines have occurred in sturgeon populations: all 25 extant sturgeon species are listed as protected under the Convention on the International Trade of Endangered Species. Chinese sturgeon, an anadromous fish that has survived at the edge of extinction, is listed among the first class of protected animals in China. Chinese sturgeon spawn and hatch mainly in the Yangtze River, China, and their early development also takes place in the Yangtze River [10]. The extinction risk often is attributed to overfishing and habitat destruction by dam construction. Recently, intersexual testis–ova phenomenon was found both in shovelnose sturgeon (Scaphirhynchus platyrhynchus) from the Mississippi River, USA [11], and in Chinese sturgeon in the Yangtze River [12]. Declining activity of sperm and a significant decline in the ratio of males in the anadromous Chinese sturgeon (Acipenser sinensis) population also have been reported [12,13]. Exposure to EDCs possibly is related to the above phenomena. In fact, high concentrations of 4-NP (0.8–1.92 μg/g wet wt) were detected in fish and water taken from the Yangtze River [14].

Vitellogenin is a widely used marker gene in assessing endocrine disruption, particularly estrogenic stimulation, in all aquatic oviparous vertebrates [15–18]. In the present study, 24 one-year-old juvenile Chinese sturgeon were used to assess the effect of 4-NP on Chinese sturgeon with VTG mRNA as a marker.

MATERIALS AND METHODS

Chemicals

The E2 (purity, 98%) and peanut oil were purchased from Sigma (St. Louis, MO, USA). The 4-NP (technical grade) was purchased from Tokyo Chemical Industry (Tokyo, Japan).

Fish and experimental design

Chinese sturgeon is a fish with a late sexual maturity; the average age of initial reproduction is approximately 14.3 years [12]. One-year-old juvenile fish (body wt, 1.7–2.8 kg) were used. The fish were purchased in October 2002 from a Fisheries Research Institute in Wuhan (Hubei, China). They were cul-
tured indoors in a large fishery room with deep groundwater until the experiment was completed. Fish were cultured for one whole winter at 4 to 8°C before the experiment began. In the spring, the temperature was adjusted to between 16 and 18°C. Then, the fish were randomly divided into four groups of six, and each group was maintained separately in a concrete pond (width, 1.6 m; length, 2.5 m; depth, 1.0 m) with continuously running freshwater (16–18°C; hardness, 8.1 mg/L; pH 7.8–8.1; dissolved oxygen, 8.3 to 9.0 mg/L) and a photoperiod of 8:16-h dark:light. After one week of acclimatization, all groups were treated as follows: Group 1 was the negative control; this group was injected only with a vehicle (peanut oil) at 2 ml/kg body weight/week. Group 2 was the positive control; this group was injected with 5 mg/kg/week of E2 dissolved in the same volume of vehicle as used in group 1. Group 3 was injected with 10 mg/kg/week of 4-NP, and group 4 was injected with 100 mg/kg/week of 4-NP. For groups 3 and 4, the 4-NP was dissolved in the same volume of peanut oil as used in group 1. Injections occurred a total of three times in the course of three weeks. After 3 d from the last injection, the fish were sampled. The liver tissues were quickly taken out, immediately frozen in liquid nitrogen, and kept until isolation of RNA and NP analyses. Fish were not fed during the experimental period because of the possibility of interference with phytoestrogens in the food.

Gas chromatography–mass spectrometry of NP

The frozen liver sample for each negative control and 4-NP–treated fish (1 g wet wt) was homogenized, extracted, and cleaned up mainly according to the method described by Rice et al. [19]. Gas chromatography–mass spectrometry (GC-MS) was performed with a Hewlett-Packard 5890 gas chromatograph connected to a Hewlett-Packard 5971 mass spectrometer (Avondale, PA, USA). The operation conditions of GC-MS have been reported previously [14].

RNA isolation and DNase I digestion

Total RNA from 50-μg frozen liver samples of each fish was isolated by 1 ml of Trizol reagent (Gibco BRL, Life Technologies, Gaithersburg, MA, USA) according to the manufacturer’s instructions. To remove the possibility of contamination with genomic DNA, total RNA (50 μg) was digested with 50 U of RNase-free DNase I according to the manufacturer’s instructions (TaKaRa Biotechnology, Dalian, China). Total RNA was purified after DNase I digestion by two phenol/chloroform/isoamylalcohol extraction steps, precipitated with isopropanol, and dissolved in diethylpyrocarbonate-treated distilled water. The purified total RNA was measured at 260 and 280 nm with a spectrophotometer. The 260-nm reading was used to estimate the concentration of total RNA recovered from the isolation and DNase I digestion. The 260:280-nm ratios, as well as a 1% agarose-formaldehyde gel stained with ethidium bromide, were used to verify the quality of the RNA in each sample.

Reverse transcription

Reverse-transcription (RT) reagents were from Applied Biosystems (Foster City, CA, USA). The purified total RNA (0.4 μg) was incubated at 70°C for 10 min, then spun briefly and immediately placed in ice. Syntheses of cDNA were carried out in a 20-μl reaction mixture containing RNase-free water; 1× TaqMan RT buffer; 5.5 mM magnesium chloride; 0.5 mM of each dATP, dGTP, dCTP, and dTTP; 2.5 μM oli-
go(dT)16; 0.4 μl of RNase inhibitor; and 1.25 U/μl of MultiScribe Reverse Transcriptase. The RT reaction was carried out for 10 min at 25°C and 30 min at 48°C and then inactivated for 5 min at 95°C. A control without reverse transcriptase was performed in each group tested, and the results confirmed that no DNA contamination occurred in the RNA.

Partial amplification and sequencing of Chinese sturgeon VTG and β-actin cDNA

To amplify a partial VTG gene of Chinese sturgeon, degenerate primers were designed from a 582-base pair (bp) conserved region by alignment of available VTG mRNA sequences from white sturgeon (Acipenser transmontano, GenBank accession no. U00455), Acanthogobius flavimanus (AB088473), Pimephales promelas (AF130354), common carp (Cyprinus carpio, AF414432), zebrafish (Danio rerio, AF406784), Sillago japonica (AB081299), Oreochromis aureus (AF514281), killifish (Fundulus heteroclitus, U07055), medaka (Oryzias latipes, AB064320), and haddock (Melanogrammus aeglefinus, AF284034). Amplification of the Chinese sturgeon β-actin gene was performed according to a similar strategy. Two degenerate primers were designed from a conserved region obtained from mRNA sequence alignment of β-actin from zebrafish (AF025305), common carp (Carassius auratus, AB039726), rainbow trout (Oncorhynchus mykiss, AF157514), Atlantic salmon (Salmo salar, AF012125), Mozambique tilapia (Oreochromis mossambicus, AB037865), chicken (Gallus gallus, L08165), horse (Equus caballus, AF035774), and human (Homo sapiens, BC001301). These alignments were done using ClustalW alignment in MacVector 6.5 (Oxford Molecular, San Diego, CA, USA). The degenerate primers are listed in Table 1.

The cDNA of the E2-treated group was used to amplify the VTG gene. The PCR reactions were performed under the following conditions: 95°C for 1 min; followed by 35 cycles of 95°C for 30 s, 55°C for 10 s, and 72°C for 30 s; and then 72°C for 5 min. After 1.2% agarose gel electrophoresis, the anticipated bands were cut and purified from the agarose gel using the Wizard® PCR Prep DNA Purification System (Promega, Madison, WI, USA). The purified DNA fragments were sequenced with corresponding PCR primers using an ABI PRISM™ 377XL DNA Sequencer (Applied Biosystems).

Conventional PCR

For conventional PCR, pairs of specific primers of Chinese sturgeon VTG and β-actin (Table 1) were designed from the obtained sequences using Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA). The PCR reaction solution, with a final volume of 50 μl, was obtained by mixing with 1 μl of cDNA; 1× PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM MgCl2); 0.2 mM of each dATP, dGTP, dCTP, and dTTP; and 0.4 μM each VTG and β-actin primer. After a denaturation step at 95°C for 1 min, amplification occurred for 30 cycles of 95°C for 30 s and 58°C for 60 s, followed by a final extension step at 72°C for 7 min. No template was added in the negative-control tube. The PCR products were electrophoresed in 1.8% agarose gel. The gel was stained in ethidium bromide and then exposed to ultraviolet radiation and scanned.

Quantitative real-time RT-PCR

The specific forward and reverse primers (listed in Table 1) for VTG and β-actin were designed from the known se-
sequences using the Primer Express program (Applied Biosystems). The concentration of the purified PCR product DNA was estimated by optical density at 260 nm (OD260), and the number of copies per milliliter of standard was calculated according to the following formula:

\[
copies/ml = \frac{6.023 \times 10^{23} \cdot C \cdot OD_{260}}{MWt}
\]

where \(C = 5 \times 10^{-4} \text{g/ml for DNA and MWt} = \text{the molecular weight of the PCR product (number of base pairs} \times 6.58 \times 10^{2} \text{g})[20].\) To prepare a quantification curve, the purified PCR products were serially diluted as follows: VTG with seven dilutions from \(1 \times 10^{5}\) to \(1 \times 10^{1}\) copies/\(\mu\)l and \(\beta\)-actin with five dilutions from \(1 \times 10^{5}\) to \(1 \times 10^{0}\) copies/\(\mu\)l. All samples were analyzed in duplicate from RT to the end.

The SYBR® Green real-time PCR was performed in MicroAmp® optical 96-well reaction plates with optical adhesive covers using an ABI Prism™ 7000 sequence detection system (Applied Biosystems). The PCR assays were carried out under the following conditions: 10 \(\mu\)l of SYBR® Green PCR master mix (Applied Biosystems), 250 nM each of forward and reverse primers, and 0.5 \(\mu\)l of cDNA template were added to a 20-\(\mu\)l total reaction volume. The reactions were incubated at 50°C for 2 min to activate the uracil N9-glycosylase (in SYBR® Green PCR master mix) and then for 10 min at 95°C to inactivate the uracil N9-glycosylase and activate the AmpliTaq Gold DNA polymerase (in SYBR® Green PCR master mix), followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Following the final cycle of the PCR, the reactions were denatured over a 35°C temperature gradient at 0.03°C/s from 60 to 95°C to determine the quality of PCR products.

Statistical analyses

The statistical program SPSS® (Ver 11.0; Chicago, IL, USA) was used to collate and analyze all the collected data. Independent \(t\) test analysis was used to determine whether the results of the analysis were statistically significant \((p < 0.05)\). All data are expressed as the mean ± standard deviation.

RESULTS

Treatment of Chinese sturgeon with triple injections of \(E_2\) or 4-NP did not elicit overt toxicity. No mortality occurred, and no obvious loss of body weight was observed after injection with the appropriate doses of \(E_2\) and 4-NP throughout the duration of the experiment.

Partial amplification and sequencing of Chinese sturgeon VTG and \(\beta\)-actin cDNA

Degenerate primers were designed to a 582-bp conserved region near the N-terminus of the amino acid sequences by alignment of VTG from 10 different fish species. A fragment from Chinese sturgeon liver RNA was amplified by RT-PCR using the degenerate primers. The expected fragment was purified and sequenced. A 462-bp sequence corresponding to a 154-amino acid sequence was obtained (Fig. 1). In the comparison of the resulting Chinese sturgeon VTG sequence (AJ745099) with those of white sturgeon (U00455) and zebrafish (AF406784), the amino acid sequences exhibited 152/154 and 115/154 sequence identities, respectively (Fig. 2). Using the same strategy, a 226-bp \(\beta\)-actin fragment corresponding to a 75-amino acid sequence (AJ745100) (Fig. 3) was found to exhibit 100% amino acid sequence identity to those of the \(\beta\)-actin mRNA from yellow perch (AY332493), zebrafish (AF025305), chicken (L08165), and human (X00351).

Quantiﬁcation of VTG mRNA in Chinese sturgeon by conventional RT-PCR and quantitative real-time RT-PCR

Before real-time RT-PCR, conventional RT-PCR was performed to qualify the induction of VTG mRNA. With both pairs of VTG- and \(\beta\)-actin–specific primers, conventional RT-PCR was carried out, and two bands were generated by agarose gel electrophoresis as shown in Figure 4A. The larger band was vitellogenin cDNA (462 bp), and the smaller band was the \(\beta\)-actin internal control cDNA (226 bp). Results show that both \(E_2\) and 4-NP induced expression of VTG mRNAs, and the bands of VTG cDNA in the groups receiving 5 mg/kg/week of \(E_2\) or 100 mg/kg/week of 4-NP were more intense than those in the group receiving 10 mg/kg/week of 4-NP. No VTG cDNA was detected in the control group.

For absolute quantification of VTG mRNA and \(\beta\)-actin mRNA, all samples were reverse transcribed in duplicate, and real-time PCR with SYBR Green detection was used to determine the absolute quantities of VTG and \(\beta\)-actin cDNA. The purified conventional RT-PCR products were quantified by 260-nm reading using a spectrophotometer and serially diluted. Both standard curves for quantification of VTG and \(\beta\)-

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Table 1. Polymerase chain reaction (PCR) primers used for amplifying Chinese sturgeon vitellogenin (VTG) and \(\beta\)-actin partial sequences for conventional PCR and for SYBR® Green real-time PCR assay

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primerb</th>
<th>Amplicon (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degenerate primes</td>
<td>VTG</td>
<td>5'-GG[T/A/G]CATCTGAAACA[G/C]CT[G/T]CA-3'</td>
</tr>
<tr>
<td>Conventional PCR primes</td>
<td>VTG</td>
<td>5'-GAAACTGCTGCAACTAACACATC-3'</td>
</tr>
<tr>
<td></td>
<td>-Actin</td>
<td>5'-AGGGGAACCTGCTGTAAGA-3'</td>
</tr>
<tr>
<td>Real-time PCR primes</td>
<td>VTG</td>
<td>5'-GGCGGCTATGTTGGTAT-3'</td>
</tr>
<tr>
<td></td>
<td>-Actin</td>
<td>5'-TGTTGCGCTGGGTGTTTGACA-3'</td>
</tr>
</tbody>
</table>

a Applied Biosystems, Foster City, California, USA.

b Forward (upper) and reverse (lower) primers.
**Fig. 1.** Partial nucleotide and protein sequence of the Chinese sturgeon vitellogenin gene (GenBank accession no. AJ745099). The numbers above the sequence indicate the nucleotide position, and the values on the right correspond to the amino acid residues.

**Fig. 2.** Comparison of the partial amino acid sequence of Chinese sturgeon vitellogenin with those of white sturgeon and zebrafish. Different residues are shaded in dark gray. The alignment was generated using the ClustalW alignment.
actin mRNA were linear over five orders of magnitude with the linear correlation ($r > 0.99$) between threshold cycle ($C_T$) and the number of copies of target. The qualities of PCR amplifications were validated by the heat dissociation curves. The specificity of real-time RT-PCR with SYBR Green detection was ascertained by comparing the heat dissociation curves of the amplification products from different samples to those of the standards. The melting temperatures of the 74-bp VTG amplicon and the 79-bp β-actin amplicon were 77 and 82°C, respectively. All samples exhibited a melting temperature within ±1°C of those of the standards, and no hairpin or dimer was detected.

To decrease the error that occurs with incomplete RT, the ratios of VTG to β-actin cDNAs were used to assess the VTG gene expression in the groups. Consistent with the conventional PCR results, no VTG cDNA was detected in the control group. The level of VTG expression reached $17.8 \pm 13.4$ copies/copy β-actin in the group receiving 5 mg/kg/week of E$_2$. The VTG expression in the groups treated with 10 or 100 mg/kg/week of 4-NP were both significantly induced ($p < 0.05$). The former reached $0.041 \pm 0.024$ copies/copy β-actin, although it was approximately 110-fold lower than that of the latter ($4.51 \pm 1.68$ copies/copy β-actin). Results from real-time RT-PCR are summarized in Figure 4B.

Concentrations of 4-NP in livers

Chemical analysis of 4-NP showed that the residual concentrations of 4-NP were $2.78 \pm 2.41$ μg/g wet weight in the livers of the group receiving 10 mg/kg/week of 4-NP and $31.38 \pm 0.26$ μg/g wet weight in the livers of the group receiving 100 mg/kg/week of 4-NP, and the concentration in the control group was less than 5 ng/g wet weight. Figure 5 shows a plot of the relationship between the concentrations of 4-NP and the ratios of VTG to β-actin in the liver of Chinese sturgeon, and it can be seen that the ratio of VTG to β-actin obviously increased with increasing 4-NP concentration in liver.

DISCUSSION

It has been reported that the amino acid sequence of vitellogenin is poorly conserved across oviparous species except

Fig. 3. Partial nucleotide and protein sequence of the Chinese sturgeon β-actin gene (GenBank accession no. AJ745100). The numbers above the sequence indicate the nucleotide position, and the values on the right correspond to the amino acid residues.

Fig. 4. (A) Agarose (1.8%) gel image of reverse transcription–polymerase chain reaction (RT-PCR; 30 cycles) amplification products of vitellogenin gene (VTG; 462 base pairs) and internal control (β-actin; 262 base pairs) in livers of control and juvenile Chinese sturgeon treated with 17β-estradiol (E$_2$; 5 mg/kg body wt/week) or 4-nonylphenol (4-NP; 10 or 100 mg/kg/week). Lane a: DNA ladder; lane b: negative-control PCR containing no cDNA. (B) Graphical analysis of the relative VTG gene expression in livers of control and juvenile Chinese sturgeon treated with E$_2$ (5 mg/kg/week) or 4-NP (10 or 100 mg/kg/week). The results were reported as the ratio of VTG to β-actin. Each experimental group consisted of six fish. Data were given as the mean ± standard deviation.

Fig. 5. 4-Nonylphenol (4-NP) concentrations and the ratio of vitellogenin (VTG) to β-actin mRNAs in livers of control and juvenile Chinese sturgeon treated with 4-NP (10 or 100 mg/kg body weight/week). Each experimental group consisted of six fish. Data are given as the mean ± standard deviation.
for the phosvitin region near the C-terminus, so many studies have designed VTG degenerate primers based on the phosvitin region [21]. In the present study, 10 available VTG sequences of teleosts, including the white sturgeon, in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) were aligned with multiple alignment. The results showed that the 910–amino acid sequence near the N-terminus was suitable for designing degenerate primers, because fewer gaps were present and similarity was relatively high. Then, based on the alignment and, mainly, on white sturgeon VTG sequence, the VTG degenerate primers were designed (Table 1). Using the designed primers, a single VTG mRNA sequence was amplified and sequenced from Chinese sturgeon (Fig. 1). This sequence was confirmed by alignment (Fig. 2) and BLAST analyses. The primers employed in the present study also may be useful for cloning VTG mRNA segments from other sturgeon species, and using a similar strategy, a fragment of Chinese sturgeon β-actin was obtained (Fig. 3).

Vitellogenin protein has been found to last much longer in the plasma than the mRNA does in the liver [21], and VTG mRNA assay would be better in avoiding EDC interference in the culture before the experiment. In addition, it has been confirmed that VTG mRNA assay is more sensitive than the enzyme-linked immunosorbent assay in assessing EDC activity [17,18]. The quantitative real-time RT-PCR assay is a quick and accurate method for detecting gene expression [18,22]. In the present study, real-time PCR methods were established for Chinese sturgeon VTG and β-actin, and β-actin mRNA was used as internal control to normalize the data of VTG gene expression. Although Larkin et al. [23] found that β-actin had been up-regulated by estrogen exposure in sheepshead minnows [23], no significant difference among the levels of β-actin in different groups was found in the present study (p > 0.05). The gene expressions of VTG refer to β-actin from conventional RT-PCR and real-time RT-PCR and are summarized in Figure 4. Significant induction of VTG gene expression was detected in the E₂- and 4-NP-treated groups.

Vitellogenin is an important biomarker for assessing the estrogenic activity of EDCs, and it can be used as a diagnostic and prognostic tool for hazard assessment in fish stocks [15–18,21–24]. Vitellogenin is a yolk precursor protein, which is produced naturally during maturation of oocytes in the liver after stimulation from the increased endogenous estrogen. Under physiological conditions, the vitellogenin gene does not express in male and immature fish [25,26]. However, 4-NP, along with other EDCs in the environment, can promote expression of the VTG gene in teleosts, and some phenomena, such as inhibition of testicular growth [6], reduction of the viability of spermatozoa [8], testis–ova development [7], declining male ratio [9], and so on, occurred during the course of the present study. Based on the induction of VTG and zona radiata proteins, Fossi et al. [24] has assessed that the Mediterranean top predator, swordfish, was being threatened by EDCs. Chinese sturgeon is a top predator in the Yangtze River. Results of VTG expression and analyses of 4-NP showed that the residue concentration of 2.78 ± 2.41 μg/g wet weight of 4-NP in liver was sufficient to induce VTG expression (Fig. 5). However, high concentrations of 4-NP and NPEOs have been detected in fish (0.8–1.92 μg/g for NP and 20.2–48.27 μg/g for NPEOs in liver) and in water (up to 9.52 μg/L for NP and up to 37.28 μg/L for NPEOs) from the Yangtze River [14]. Although the 4-NP concentration in treated Chinese sturgeon was only a little higher than that detected in fish from the Yangtze River, Chinese sturgeon in the Yangtze River are exposed for a much longer time than the fish in the laboratory. Meanwhile, some other EDCs in the Yangtze River might produce additive or synergistic effects. Wei et al. [12] found that recruitment of males in the population was declining at a dangerous rate. Declining activity of sperm and intersexual phenomenon in Chinese male sturgeon has been reported [12,13]. These results represented the indication of the risk of EDCs for Chinese sturgeon, and further systematic studies are needed to protect this endangered species.

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