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ScienceDirect
Ecotoxicology and Environmental Safety 66 (2007) 148–153
Ecotoxicology and Environmental Safety
www.elsevier.com/locate/ecoenv

Crucian carp (*Carassius carassius*) VTG monoclonal antibody: Development and application

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Received 19 May 2005; received in revised form 10 November 2005; accepted 22 November 2005
Available online 16 February 2006

Abstract

The vitellogenin (VTG) in fish has been used as an important biomarker for monitoring endocrine disrupting compounds (EDCs). This paper reports the development of a new monoclonal antibody (McAb) against the VTG of crucian carp *(Carassius carassius)*. The McAb has a molecular weight of 149.4 kDa (heavy chain: 53.1 kDa; light chain: 21.6 kDa), and double diffusion indicated that it belongs to the IgG1 subclass. The titer is 10^5–10^6 and the affinity constant (K_{aff}) is 7.0×10^8 L/mol, showing the high specificity and sensitivity of the antibody. The established sandwich ELISA was sensitive with a detection limit of VTG 0.98 ng/mL. The cross-reactivity of antibody was detected in cyprinids such as rare minnow, zebrafish, and carp. This ELISA was used to detect the variation of VTG in crucian carp exposed to secondary effluent and reclaimed water from the Gaobeidian sewage treatment plant (SRP). The VTG induction in secondary effluent was higher than that in reclaimed effluent and the VTG levels in juvenile crucian carp increased with increasing exposure time. The VTG concentration in male fish from downstream of the Gaobeidian STP was 88.62 ± 827.73 μg/mL, while that from a control site was undetectable.

Keywords: Vitellogenin (VTG); Monoclonal antibody (McAb); Sewage treatment plant (STP); Crucian carp (*Carassius carassius*)

1. Introduction

Vitellogenin (VTG), the precursor of egg yolk protein, is a glycophospholipoprotein produced in the liver of oviparous species in response to estrogen. Induction of VTG synthesis can occur in female fish in response to endogenous estrogen and in juvenile or male fish exposed to environmental estrogens. For example, trout exposed to sewage effluent in English rivers had a 500–100,000-fold increase in plasma VTG compared to fish upstream of the effluent (Kime et al., 1999; Sumpter and Jobling, 1995). Now, the presence of serum VTG in male or juvenile fish as well as altered plasma VTG content in female fish has been recognized as a sensitive biomarker for estrogenic exposure (Purdom et al., 1994).

VTG is easy to measure using immunoassay techniques, such as radioimmunoassay (RIA), enzyme-linked immunoassay (ELISA), immunodiffusion, and alkali-liable phosphate (Pereira et al., 1992). Among these methods, ELISA is the most favorable tool because it has no radioactivity, is easy to set up, and has high sensitivity and high throughput. Different teleost VTGs of freshwater and marine fish, such as fathead minnow (Parks et al., 1999), arctic charr (Johnsen et al., 1999), carp (Matsumoto et al., 2002) and English sole (Lomax et al., 1998), have been prepared and the corresponding ELISAs have been established.

Crucian carp, *Carassius carassius*, is a widespread, robust fish species that can inhabit various aquatic environments. The selection of a representative native species has more interest as it shows the effects on the feral fauna in the target environment and integrates the effects throughout its life history. So crucian carp can be expected to be an ideal bioindicator for monitoring

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0147-6513/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ecoenv.2005.11.005

endocrine-disrupting chemicals (EDCs) in the field, especially in heavily polluted aquatic environments, by determining its plasma VTG. The ecological impacts of EDCs are usually evaluated by determining the plasma VTG of crucian carp in waters near sewage treatment plants (STPs) (Kukkonen et al., 1999; Matsumoto et al., 2002). However, due to the unavailability of monoclonal antibody (McAb) specific to crucian carp VTG, antibody against common carp VTG has been used to measure plasma VTG of crucian carp by utilizing the cross-reactivities of this antibody.

In this study, the McAb against crucian carp VTG was prepared, and an ELISA method for measuring VTG was developed. The established sandwich ELISA was applied to evaluate the estrogenicities of effluents from the Gaobeidian STP, the largest STP in Asia. Finally, to assess the impacts of STP effluent discharge on the ecological environment of the receiving river, the estrogenic activity in water near the STP was also evaluated and was compared with that at a control site.

2. Materials and methods

2.1. Chemicals and fish

Hypoxanthine with aminopterin (HAT) and Dulbecco's Modified Eagle's Medium (DMEM) culture medium were obtained from Gibco (Grand Island, USA) and fetal bovine serum was purchased from Sijiqing (Hangzhou, China). Polyethylene glycol-1500 (PEG-1500), 17β-estradiol (E2), 3, 3′, 5, 5′-tetramethyl benzidine (TMB), second antibody conjugated horseradish peroxidase (HRP), and bovine serum albumin (BSA) were purchased from Sigma (Louis, MO, USA). All other materials were obtained from the usual commercial suppliers. Sp2/0 cells were presented by Professor Guo (Peking University). BalB/c mice were purchased from the animal center of Peking University.

2.2. Preparation of crucian carp VTG

The crucian carp ($W = 110 \pm 5.6$ g, $L = 17.6 \pm 1.1$ cm) were intraperitoneally injected with 2 mg E2/kg body weight every 5 days, a total of three times. Two days after the last injection, blood was collected from the caudal vessel with heparinized syringes and centrifuged at 12,000 rpm for 30 min (4 °C). The plasma was stored at −80 °C, mixing with 10 μL aprotinin (1%). The plasma VTG was purified and analyzed with an HPLC system (Yamanaka et al., 1998) consisting of a gradient programmer, a Waters 600 controller pump, and a Waters 2975 UV detector (USA). The purified VTG was lyophilized and stored at −80 °C. The purified VTG concentration was determined according to Bradford (1976) using commercially available reagent with BSA as standard. Working solutions of VTG for standard curve were prepared at various concentrations of VTG.

2.3. Production of monoclonal antibodies

2.3.1. Immunization, fusion, and cloning

Immunization, fusion, and cloning were performed according to the method described previously (Wang et al., 2002). Briefly, the VTG (100 μL, 500 μg/mL) of crucian carp purified above was mixed with the same volume of Freund's complete adjuvant for the first immunization and incomplete adjuvant for the following immunizations, and then booster injections were administered into the back and the back limbs of female BalB/c mice at 1-week intervals. Starting from the third immunization, blood was collected from the tail vein of mice to test the immune response. After three immunizations, the mice's spleen with the highest titer was directly immunized for 1 week. On the 8th day, the splenocytes were fused with sp2/0 cells at a ratio of 1:10 using PEG-1500 (50%, v/v). The fusion cells were slowly dispersed in HAT medium and were seeded into 96-well culture plates (100 μL/well). Cells were cultured at 37 °C in an incubator with 5% CO_2 and 95% humidity. Half of the HAT medium was changed every 2 days. After 2 weeks the HAT medium was replaced with HT medium. During the culture processes, the positive hybridoma was determined by testing the antibody in supernatant with ELISA. After 3 and/or 4 weeks the positive hybridoma was subcultured until a single colony was obtained.

2.3.2. Production and purification of antibodies

The production and purification of antibodies were according to the method described previously (Tang et al., 1999). Briefly, at first, 500 μL of paraffin was intraperitoneally injected into the mice for 1 week, and then positive hybridromas ($0.5–1.0 \times 10^6$ cell) were injected into the mice. After 2 weeks, ascites (2–4 mL) were taken from the mice's abdomens. Then the antibody was purified from the supernatants by adding caprylic acid and saturated ammonium sulfate. The purified antibodies were mixed with an equal volume of glycerol containing EDTA (0.1%, w/v) and then stored at −20 °C.

2.4. Characterization of antibody

2.4.1. Determination of antibody subclass and molecular weight

The antibody subclass was identified by double diffusion. Briefly, seven holes were drilled on the gelose gel (1%) plates. The central hole was added into antigen (VTG) and the other six holes around the central hole were added into the purified antibodies, that is, IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM, respectively. The gel was incubated at 37 °C for 24 h, and then stained with Giemsa-250.

Sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) was formed using 12% polyacrylamide gel. The antibody in the gel was stained with Coommassie blue and its molecular weight was detected.

2.4.2. Determination of the titer and K_{aff}

The titer and K_{aff} of an antibody, which reflect the affinity between the antigen and antibody, are important characteristics of an antibody. McAb of different concentrations was added into a 96-well microplate in which each well was coated with VTG (500 ng/well), and the results were detected with ELISA. The K_{aff} was drawn according to

$$K_{aff} = \frac{n-1}{2(n[Ab']_t - [Ab]_t} \quad \left(n = \frac{[Ag]_t}{[Ag']_t}\right), \tag{1}$$

where $[Ag]_t$ and $[Ag']_t$ are total concentrations of antigen coated on the bars of the plates, and $[Ab]_t$ and $[Ab']_t$ were the antibody concentrations corresponding to $[Ag]_t$ and $[Ag']_t$ when the value of OD was half the value of the OD_{max}.

2.5. Sandwich ELISA

A 96-well microplate was coated at 37 °C for 2 h with polyclonal antibody against crucian carp VTG (100 μL/well, dilution with carbonate buffer (CBS, 0.05 M, pH 9.6)). After washing three times with PBST buffer (0.05% Tween20 in PBS (0.01 M, pH 7.6), the plate was blocked with BSA (1%) for 2 h at 37 °C. After three washings with PBST buffer, VTG was added into wells and incubated at 37 °C for 1 h. Then the plate was incubated at 37 °C for 1 h with McAb and subsequently with goat anti-mouse IgG-HRP. The plate was washed, and then 100 μL/well TMB substrate was added. The color reaction was stopped after about 15 min in the dark by adding 50 μL H_2SO_4 (2 M). Absorbance (OD) was read at 450 nm with a microplate reader (Bio-rad 550). The standard curve between the antigen and antibody was defined as

$$OD = OD(samples) - OD(NSB),$$

where OD (samples) is the value of the sample and OD (NSB) is the value of the negative control.

The robustness of the ELISA was assessed through measurement of inter- and intra-assay coefficients of variation, where the intra-assay variation coefficient was measured with six replicates on each microplate. The interassay variation coefficient was measured by comparing six separate replicates of dilutions of the same standard VTG. In order to determine the interference of the plasma protein, 5 and 50 ng/mL VTG were added to the control male plasma with dilutions of 1:10, 1:50, 1:100, and 1:500. The obtained concentrations were compared to a normal standard curve performed in PBS.

The parameters such as the dilution of the polyclonal and McAb, HRP conjugated secondary antibody, range of standard curves, incubation time, and blocking buffer were optimized to develop a sensitive ELISA.

2.6. Determination of cross-reactivity

Carp (*Cyprinus carpio*) ($W = 110 \pm 10.8$ g, $L = 18.6 \pm 1.5$ cm) and sturgeon (*Acipenser sinensis*) ($W = 2.8$, $L = 86$ cm) were obtained from a farm plant in Beijing and induced with E2 for VTG. Mature female rare minnow (*Gobiocypris rarus*) ($W = 425.2 \pm 23.1$ mg, $L = 31.5 \pm 2.5$ mm) and medaka (*Oryzias latipes*) ($W = 324.3 \pm 54.3$ mg, $L = 26.8 \pm 1.5$ mm) were cultured in our lab. Purified zebrafish VTG was purchased from Biosense (Norway). Wolffish (*Odontamblyopus rubicundus*) ($W = 25.5 \pm 16.2$ g, $L = 20.4 \pm 1.8$ cm) and so-iny mullet (*Chelon haematocheilus*) ($W = 88.2 \pm 19.4$ g, $L = 21.3 \pm 2.1$ cm) were captured from Han'gu in Tianjin in September 2003. The collected plasma was stored at $-80\,^{\circ}$C as described above. The body homogenate was collected after a part of the body (less than 100 mg) was crushed in the presence of 1 mL homogenate buffer (10 mmol/L Tris-HCl (pH 7.4) containing 0.9% NaCl, 0.05% Tween20, 1% BSA, 1% aprotinin). The homogenate was centrifuged for 30 min at 4 °C (12,000 rpm). The supernatant lipids formed at the top of the tube after centrifugation, and a syringe with a pinhead was used to remove the liquid carefully. The VTG concentration was determined with HPLC to analyze cross-reactivity with crucian carp anti-VTG.

2.7. VTG analysis in crucian carp exposed to STP effluents

The Gaobeidian STP processes approximately 1 million m^3 of sewage water per day and it receives both industrial and domestic wastewater from the city of Beijing. The raw water is treated by mechanical treatment and the primary effluent is subjected to the activated sludge system. After settling in the secondary clarifier, most of the secondary effluent is discharged into Gaobaidian Lake, and the remaining part of the secondary effluent is further treated by adding 15 mg/L $AlCl_3$ coagulant, resulting in reclaimed effluent. The juvenile crucian carp ($L = 8.44 \pm 0.65$ cm, $W = 9.96 \pm 2.37$ g) were exposed to secondary effluents and reclaimed effluent on site for 8 weeks in the Gaobeidian STP. The exposure was carried out in 100-L aquaria using a flow exposure system with the air bubbling to keep dissolved oxygen and the effluents (no dilution) were exchanged 2–3 times every day. Fish were fed twice every day and the temperature ($15 \pm 0.5\,^{\circ}$C), pH (7.8 ± 0.2), and dissolved oxygen content (4–5 mg/L) were regularly monitored. The photoperiod was maintained in the natural autumn regime. After 4, 6, and 8 weeks, some fish blood was drawn and then, the fish were killed. After centrifugation, the plasma was stored for VTG analysis as described above.

2.8. Determination of plasma VTG in wild crucian carp

In March 2005, 8 male ($L = 13.72 \pm 0.89$ cm; $W = 66.75 \pm 14.86$ g) and 4 female ($L = 13.75 \pm 2.87$ cm; $W = 68.75 \pm 39.66$ g) crucian carp were collected from Gaobeidian Lake, which is located downstream of the STP (Fig. 1), and 7 males ($L = 13.87 \pm 0.96$ cm; $W = 58.40 \pm 12.90$ g) and 6 females ($L = 14.00 \pm 1.22$ cm; $W = 62.70 \pm 18.20$ g) were collected from Yuyuantan Lake, which is located upstream of the STP (Fig. 1). The plasma was provided to detect the VTG concentrations.

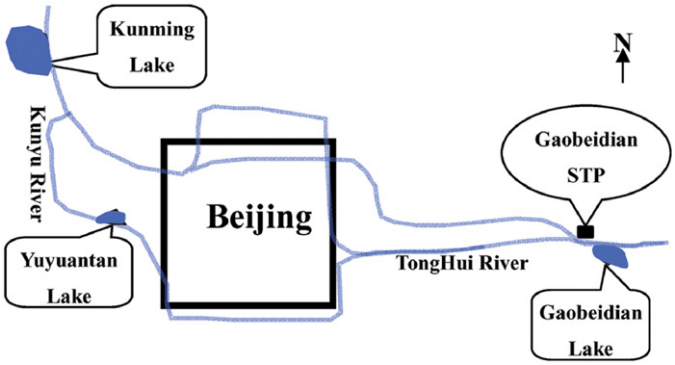

Fig. 1. Map of sampling sites. Yuyuan Lake: upstream of Gaobeidian STP, the control site; Gaobedian lake: downsteam of Gaobeidian STP, the research site.

One-way analysis of variance (ANOVA) was used to determine differences in VTG concentrations. When the value of P was below 0.05, the differences were regarded as significant.

3. Results

The titer and K_{aff} of purified antibody were about 10^{5-6} (Fig. 2) and 7.0×10^8, respectively, demonstrating the high affinity of the antibody to its antigen. The result of SDS-PAGE indicated that the antibody was composed of two different chains: a heavy chain of 53.1 kDa and a light chain of 21.6 kDa (Fig. 3). The antibody subclass was determined by double-diffusion. The line between IgG1 and the antibody indicated that the antibody belongs to the IgG1 subclass.

After the antibody of crucian carp VTG was characterized, the VTG concentration was determined using sandwich ELISA. There was a linear correlation when the VTG concentration was in the range 0.98–125 ng/mL ($r^2 = 0.99$) (the polyclonal and monoclonal antibodies were diluted at 1:500 and 1:1000, respectively) (Fig. 4). The intra-assay coefficient of variation was 5.7 ($n = 6$) and the interassay coefficient of variation was 15 ($n = 6$). The linear correlation for determination of VTG concentrations in plasma diluted by 1:100 was close to that of the standard VTG. To avoid the blood protein interference effect, a dilution of 1:100 was chosen for this ELISA method, and the detection limit for VTG in the plasma samples was determined to be 98 ng/mL plasma. The cross-reactivities of the antibody with other teleost fish VTGs were also detected. It was found that this antibody can detect VTG of carp, rare minnow, and zebrafish, which belong to the Cyprinoidae family, while no cross-reactivity was detected from others (Fig. 5), which was similar to Tyler et al. (1996).

At last, the optimized sandwich ELISA was used to determine VTG in plasma of juvenile crucian carp ($n = 5$) exposed to secondary effluent and reclaimed effluent for 4, 6 and 8 weeks (Fig. 6). The highest plasma VTG

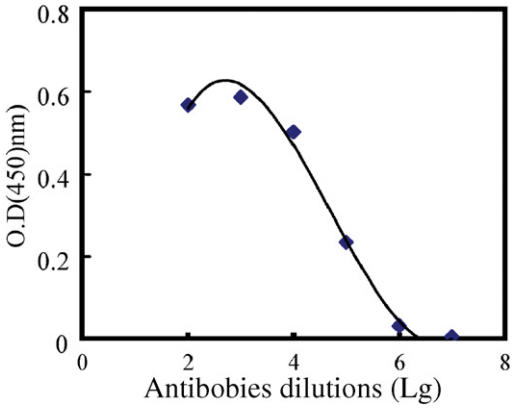

Fig. 2. Dilution curve of purified antibodies collected from hybridoma-immunized mice. Each point was measured in duplicate.

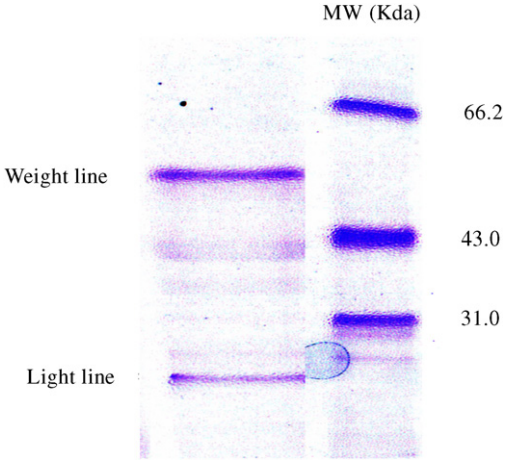

Fig. 3. Graph of anti-VTG in SDS-PAGE. Left axis: anti-VTG McAb; right axis: low-molecular-weight standard protein.

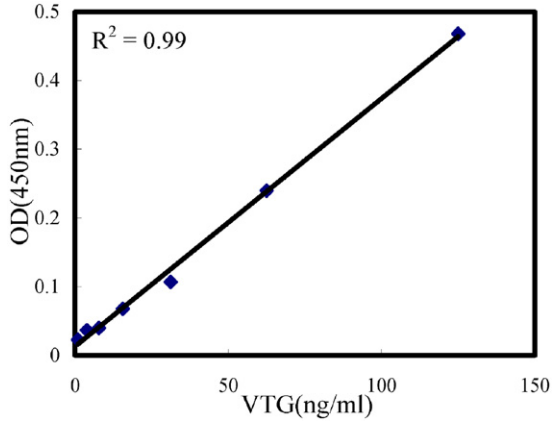

Fig. 4. Optimized sandwich ELISA for detecting VTG from 0.98 to 125 ng/mL. The polyclonal and monoclonal antibodies were diluted at 1:500 and 1:1000, respectively.

concentration in secondary effluent was 5051.1 ± 3673.1 ng/mL after exposure for 8 weeks, while it was 1892.6 ± 718.65 ng/mL in reclaimed effluent after exposure for the same time. Compared with 4-week exposure, VTG concentrations in secondary effluent and reclaimed effluent

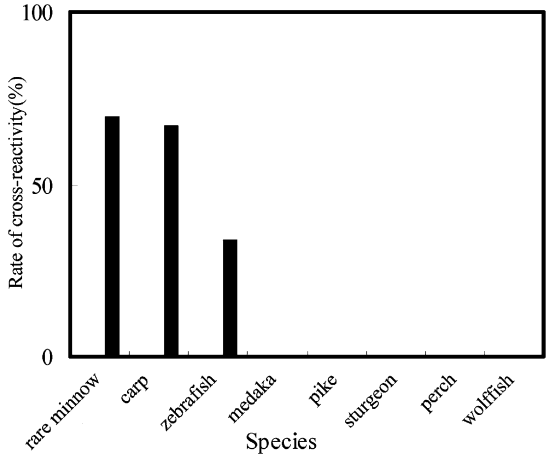

Fig. 5. Cross-reactivities between teleost VTGs and crucian carp anti-VTG.

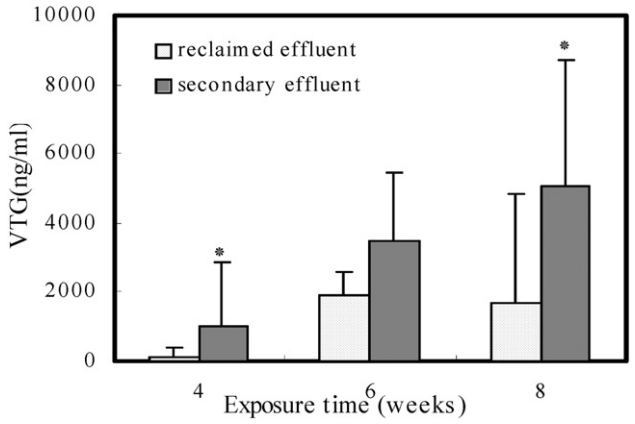

Fig. 6. Mean plasma VTG concentrations in juvenile crucian carp exposed to secondary effluent and reclaimed effluent from Gaobeidian STP for 4, 6, and 8 weeks. Values are mean ± SE ($n = 5$). Error bar indicates standard deviation of mean. *, $P < 0.05$.

after 8-week exposure were increased 4.8 times ($P > 0.05$) and 13 times ($P < 0.05$), respectively. Rodgers-Gray et al. (2000, 2001) also found similar results.

The plasma VTG of crucian carp captured in the field was also detected with the sandwich ELISA. The plasma VTG in males at the control site, Yuyuantan Lake, was under the detection limit, and the VTG concentrations in male fish at Gaobeidian Lake ranged from 51.5 to 2440 μg/mL with a mean concentration of 888.62 ± 827.73 μg/mL. In addition, the VTG concentrations in females from the control and research sites were 473.33 ± 530.26 and 2329.06 ± 462.62 μg/mL ($P < 0.05$), respectively (Table 1).

4. Discussion

ELISA using polyclonal and monoclonal antibodies is useful for assessing VTG production in ecotoxicological tests of the endocrine-disrupting activity of chemicals. In this study, a McAb against crucian carp VTG was

Table 1
VTG concentrations in female and male crucian carp collected from Gaobeidian Lake and Yuyuantan Lake

Site	VTG (μg/mL)	
	Female	Male
Yuyuantan Lake	473.33±530.32 ($n=6$)	ND ($n=7$)
Gaobeidian Lake	2329.06±462.62 ($n=4$)	888.62±827.73 ($n=8$)

Note: Gaobeidian Lake: downstream of Gaobeidian STP; Yuyuantan Lake: upstream of Gaobeidian STP.

developed, and the establishment and validation of a sandwich ELISA was used to determine VTG in crucian carp. This ELISA method was sensitive, with a detection limit of VTG 98 ng/mL plasma.

In this study, the Sp2/0 cell line was chosen because the cell line has been well established for antibody development. It is known that IgM induction is part of the initial immune response following exposure to a novel antigen and upon subsequent exposure to the same antigen; there is typically a class shift toward synthesis of IgGs. For preparing the McAbs, the subclass of IgG McAbs is often targeted due to its specificity, and it can be expected to reduce the high background. Heppell et al. (1995) made an attempt to develop the IgG McAbs against trout VTG but failed. The authors attributed the failure to the weak antigenicity of the conserved VTG epitopes. In fact, there are natural autoantibodies present in most healthy individuals; in mice, the vast majority of these are IgM class (Kaushik et al., 1988). It should be noted that the IgG1 subclass of the McAb was obtained in this study, suggesting an antibody subclass shift during the immunization processes for VTG. Comparing this work with that reported by Heppell et al., we found that there were some differences, such as the species of fish. Moreover, as described in the Experimental section, a direct spleen immunization on the basis of the standard immunization process was adopted in this experiment, which enhanced IgG McAb induction. According to our previous experiences, such as immunization process can strengthen the immunization.

The IgG subclass showed higher affinity (7.0×10^8 L/mol) and was used to develop the sandwich ELISA, quantifying antigen by using monoclonal and polyclonal antibody. This ELISA method shows higher specificity for analyzing the VTG in plasma. The interference of plasma protein in the measurement of ELISA was eliminated by plasma dilution, and it yielded a working range of 0.98–125 ng/mL. The crucian carp anti-VTG was found to be specific to the fish VTGs from the cyprinid families. No cross-reactivity was detected with VTG from other fish families. It is highly possible that the antibody recognized an epitope of the VTG molecule conserved throughout the cyprinid family. Previous studies have shown that the VTG molecule is highly conserved within the same family but not in different families (Tyler et al., 1996). Thus, a sensitive sandwich ELISA method for analyzing VTG with a quantification limit of 98 ng/mL plasma was established, which is sensitive enough to monitor the EDCs according to the dose–response of VTG for several chemicals such as nonylphenol (Seki et al., 2003).

In the exposure experiment, the juvenile crucian carp VTG was detected after exposure to effluents for 8 weeks, and the VTG level increased with increasing exposure time in secondary effluents. It was found that the VTG induction in secondary effluents after exposure for 8 weeks was higher than after exposure for 4 weeks ($P<0.05$). On the other hand, the VTG concentrations in reclaimed effluents were lower than those induced in secondary effluents. The lowest VTG concentration (637.5 ng/mL) was detected in fish exposed to reclaimed effluent for 4 weeks and the VTG level did not increase after 6- and 8-week exposure ($P>0.05$). In Fig. 6, it was found that the standard error for VTG in males is relatively high because VTG was not detected in some males. A similar phenomenon was also reported with rainbow trout (Pickering and Sumpter, 2003), which may be due to the effects of fish sex on VTG production. These results suggested that the estrogenic compounds were difficult to eliminate thoroughly with traditional treatment processes, but can be reduced through coagulation with $AlCL_3$ to some extent with regard to VTG induction.

To assess the impacts of discharging of STP effluents on the ecological environment of the receiving river, females and males were collected from sites downstream and upstream of the STP. While the VTG concentration upstream was lower than the quantification limit (98 ng/mL), high VTG concentrations ranging from 33.75 to 2440 μg/mL were monitored in males collected from the downstream area (''polluted'' lake); these concentrations were even higher than those in females from the upstream site (''nonpolluted'' lake). On the other hand, the mean VTG concentrations in females from downstream of the STP were also higher than those in females collected from upstream of the STP. The above results indicated the presence of estrogenic compounds in the secondary effluents leading to the induction of VTG in wild crucian carp. Thus, the sandwich ELISA method developed in this study is an effective way to evaluate estrogenicity in the field and its variation with exposure time and during STP treatment processes.

This study proved that the VTG in plasma could be detected with ELISA of crucian carp anti-VTG. This makes it possible to evaluate the estrogenic effects in highly polluted aquatic environments in the future by using experimental fish such as rare minnows in the lab and using crucian carp as field fish.

Acknowledgments

We are grateful to Prof. Zhenquan Guo, Peking University, Beijing, for providing SP2/0 cells. Financial support by the National Natural Science Foundation of

China (20377002 and 40021101) is gratefully acknowledged.

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