Vitellogenin assay by enzyme-linked immunosorbent assay (ELISA) as a biomarker of endocrine disruptor chemicals (EDCs) pollution

Ebrahimi, M.

Department of Physiology, Green Research Center, University of Qom, Qom, Iran

Correspondence: M. Ebrahimi, Department of Physiology, Green Research Center, University of Qom, Qom, Iran. E-mail: ebrahimi@qom.ac.ir

Summary

There are increasing evidences that show many xenobiotic chemicals (called as endocrine disruptor chemicals EDCs) through interfering with endocrine system, have the capability to induce developmental and reproductive abnormalities in humans and animals. The yolk protein precursor vitellogenin (VTG) has proved to be a simple and sensitive biomarker for assessing exposure of fish to EDCs especially the estrogenic compounds. Work is ongoing to develop screening and testing programmes for endocrine disrupting effects of new chemicals, and in the focus of this development are the fish test species common carp (*Cyprinous carpio*). In this study we have developed quantitative enzyme-linked immunosorbent assays (ELISA) for VTG in common carp. The working range of the ELISA was 11.25 to 2,000 ng/ml (25-75% specific binding/maximum antibody binding [B/B0]) with a 50% B/B0 intra- and interassay variation of 3.9% (n = 10) and 12.5% (n = 30), respectively. This ELISA is capable of detecting VTG as low as 6 ng/ml, and can accurately detect VTG in even 10 µl of plasma. The ELISA was applied to measurement of VTG production by male carp (*Cyprinous carpio*, Cyprinidae) fish exposed to ethynylestradiol. The results showed that the amount of VTG produced in plasma of exposed fish increased in logarithmic order comparing to the control group and the ELISA described here could be used as an indicator of water pollution to estrogenic pollutants.

Key words: Immunoassay, ELISA, Endocrine disruptor chemicals, VTG

Introduction

The last two decades have witnessed growing scientific concerns and public debate over the potential adverse effects that may result from exposure to a group of chemicals that have the potential to alter the normal functioning of the endocrine system in wildlife and humans (Tsutsumi, 2000). According to WHO definition, an endocrine disruptor chemical (EDC) is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations (Damstra *et al*., 2002).

Mammalian studies have focused on the male since it is much easier to determine changes in the quality and quantity of sperm than of eggs, since eggs are few and are retained internally (Lathers, 2002). Fish have a major advantage over mammals in studies of endocrine disruptors in that they produce large numbers of both eggs and sperm and release them to the external environment. An assessment of pollution requires a simple biomarker and vitellogenin (VTG) can be the easiest choice.

In very simple terms, vitellogenesis involves ovarian estradiol stimulating the liver to produce VTG, which in turn is incorporated into the yolk of developing oocytes. Estrogenic xenobiotics can also act on the hepatic receptors to induce synthesis of VTG (Nakari, 2004). Both male and female fish, as well as immature juveniles have hepatic estrogen receptors, but only the livers of female fish will normally be exposed to estrogens (Versonnen and Janssen, 2004). Production of VTG by males and juveniles, or non-vitellogenic females can therefore provide a bioindicator of exposure to environmental estrogens

(Hornung *et al*., 2004).

Trout exposed to sewage effluent in English rivers had up to 100,000-fold increase in plasma VTG compared to fish upstream of the effluent (Aerni *et al*., 2004). A follow-up study showed VTG levels of over 20 mg/ml 5 km downstream of the effluent (Sharpe *et al*., 2004). Elevated plasma VTG was also found in eels below the Paris outfall (Versonnen *et al*., 2004). Since eels may spend up to 20 years in the river before their spawning migration consequent reproductive disruption may account for some of the decrease in the European eel population (Versonnen *et al*., 2004). The flounder, as a bottom dwelling estuarine species, also provides a useful biomonitor of effluents in the rivers which may potentially affect marine fish stocks (Madsen *et al*., 2003). In another study, male flounder in a number of British rivers had elevated plasma VTG and, in the River Mersey, the concentrations were around 100 mg/ml (10 million times higher than controls) (George *et al*., 2004).

Enzyme-linked immunosorbent assay (ELISA) using microtiter plates have been described as an alternative to radio-immunoassay for the mammalian and non-mammalian assays (Yamada *et al*., 2002) and to monitor the effects of endocrine disrupting chemicals on their reproductive function (Wade *et al*., 2002). A report (Tattersfield *et al*., 1997) has highlighted the need for a simple, rapid and reliable method for the measurement of hormones. In this communication we describe a general preparation procedure for the enzyme label of carp VTG which is applicable to other species. The method was applied to the determination of VTG production by ethynylestradiol, a potent xenoestrogen, in carp (*Cyprinous carpio*) plasma.

Materials and Methods

Chemicals and equipment

Cyprinous carpio VTG and anti-VTG antibodies were a kindly gift from: Professor Oliana Carnevali, Dipartimento di Biologia MCA, University of Camerino, 62032 Camerino, Italy. The purified VTG is diluted to 10 and 50 mg/ml with VTG storage buffer [PPBS, 0.1 M, pH = 7.4; potassium chloride, 27 mM; Tween-20, 0.05%; bovine serum albumin, 0.2%; sodium azide, 5 mM; aprotinin, 20 TIU/ml, 1% (Sigma)], divided into various volumes in eppendorf tubes and stored at -20°C. Goat F (ab') 2 fragment of affinity isolated rabbit antigen specific antibody conjugated to alkaline phosphatase was obtained from Sigma Immuno Chemicals (Catalogue No. A-3937). 4-Nitrophenyl-phosphate (pNPP) obtained from Boehringer Mannheim GmbH (Cat. No. 107 905).

Ninety-six well polystyrene high-binding microtiter plates (Costar Cat. No. 3590) were used for the ELISA. Plates were sealed for storage with Anachem sealplate sealing film. Plates were processed using a Chelsea Instruments shaker, an Anthos Model AW-1 plate washer and read with an Anthos Model HTII plate reader. 96-well low binding microtiter plates (Costar Cat. No. 2501) were used for sample dilution and preparation of standard curves and AnachemÒ sealplate plate sealing film, (PK/100) purchased from Anachem (UK).

Fish exposure

Four groups of six male common carp (initial mean weight: 268.9 ± 52.5g, mean ± SD) originating from a local fish breeder farm (Mahyar company) kept in four separate tanks. They were exposed to 0, 5, 10 and 50 ng/ml ethynylestradiol up to 30 days. Blood samples were taken at 0, 15 and 30 days of exposure and plasma kept at -20°C until VTG assay.

Plate coating with VTG

ELISA plates were coated with 50 ng of purified VTG. 100 µl of carbonate buffer (0.05 M, pH = 9.6) containing 500 ng per ml of purified standard VTG added to each well of the plate and incubated for 1 hr at room temperature in a humid container and then overnight at 4°C and washed once with nanopure water. The plate was blocked by addition of a 200 µl per well of fresh VTG blocking buffer (potassium phosphate buffer (PPB) 0.1 M, pH = 7.4; bovine serum albumin, 2%; sodium azide, 5 mM), washed three times with wash buffer (potassium phosphate buffered saline (PPBS), 0.02 M,

pH = 7.4; Tween-20, 0.05%), sealed with plate cover and stored at -20°C.

Standard assay procedure

Serial dilutions of purified standard VTG (3.9 to 2000 ng/ml) in 100 µl VTG assay buffer were made in the first 10 wells of rows A and B of the coated plate. The remaining 2 wells in these rows were reserved for B0 (0 pg) and non-specific bound (NSB). Ten microliters of the diluted samples from sample dilution plate (2, 200 and 2000 times) was pipetted into the remaining 6 rows in duplicate. Twenty microliters of anti-VTG antisera diluted 1/15,000 (carp) in VTG assay buffer was added to all wells apart from NSB wells and made up the wells to 150 µl by VTG assay buffer. The plates sealed and incubated 2 hrs at room temperature in a humid chamber. Plates were then washed three times with wash buffer and 150 µl of anti-rabbit IgG alkaline phosphatase conjugate diluted 1/15,000 in VTG assay buffer added to each well and incubated overnight at 4°C temperature. Plate washed with wash buffer once and then twice with pNPP Substrate buffer (diethanolamine, 0.5 M, pH = 9.8; MgCl, 0.5 mM), 175 m1 per well of 1 mg per ml 4-nitrophenylphosphate in pNPP Substrate buffer added and then incubated dark at room temperature until colour developed to around 1.5 ODs (normally approx. 45 min but can be left up to 4 hr). The reaction stopped by adding 50 ml of 3 M NaOH and read at 405 nm. Nanograms per ml were calculated for the samples from the standard curve using Stingray software (Dazdaq, Ringmer, UK).

Assay validation

A pool of male common carp (*Cyprinous carpio*) plasma (500 µl) placed in 100°C water bath for 1 hr to denature proteins from the plasma and then centrifuged. One hundred microliter aliquots were pipetted in duplicate into wells on two rows of a microtiter plate. Pure VTG was added to one pair of wells to give a concentration of 2000 ng/well, serial dilutions (x2) performed and the samples assayed according to standard procedure. The same procedure was used to assay VTG in carp exposed to ethynylestradiol (0, 5, 10 and 50 ng/ml). SPSS 10 for Windows software (SPSS Inc., 444 N. Michigan Avenue, Chicago, Illinois 60611, USA) was used for the statistical analysis (t-test) of VTG production in plasma of control fish group (0 ng/ml ethynylestradiol) with other experimental groups (5, 10 and 50 ng/ml ethynylestradiol) and duration of fish exposure (15 and 30 days).

Results

Standard curves and assay sensitivity

Typical standard curves for VTG showed the working range of the ELISA was 11.25 to 2,000 ng/ml (Fig. 1) (25-75% specific binding/maximum antibody binding [B/B0]) with a 50% B/B0 intra- and interassay variation of 3.9% (n = 10) (Fig. 2) and 12.5% (n = 30), respectively. This ELISA is capable of detecting VTG as low as 6 ng/ml.

Assay specificity

There was a strong positive correlation ($r>0.999$) between the amount of VTG added to deproteinated plasma and the amount found (Fig. 3).

Application to measuring VTG production by carp exposed to ethynylestradiol

VTG was measured in duplicate by ELISA using 10 µl of the diluted plasma (2, 200 and 2000 times) from fish exposed to different concentrations of ethynylestradiol. The amounts of VTG detected by this method for fish exposed to 0, 5, 10 and 50 ng/ml of ethynylestradiol was 0,189 ± 25.9, 3154 ± 684 and 24569 ± 7698 (mean ± SE of mean, for fish exposed for 15 days) and 0,897 ± 50.6, 12857 ± 1235 and 86354 ± 10423 µg/ml (mean ± SE of mean, for fish exposed for 30 days), respectively (Fig. 4). A clear

Fig. 1: Typical standard curves for ELISA of (VTG). Values are means of duplicate assays

Fig. 2: Intra-assay coefficient of variance (CV) for ELISA of VTG determined from a standard curve with 10 replicates at each concentration

Fig. 3: Assay accuracy for VTG in deproteinized plasma. A known amount of VTG was added to an aliquot of a protein free carp plasma and 2 x serial dilutions made with it. Assays were performed in duplicate

and potent estrogenic acitivity in inducing VTG by fish liver was found with increase in ethynylestradiol concentration exposure and the amount of VTG induced by different concentrations of ethynyles-tradiol between control (0 ng/ml) and experimental (5, 10 and 50 ng/ml) groups and between experiment groups were different significantly ($P<0.05$).

Discussion

Examination for the effects of potential EDCs begins only at levels at which there is no apparent stress or discomfort to the animal, so that it is potentially equally able to reproduce (Ackermann *et al*., 2002). Reproductive dysfunction at such low concentrations can be caused either by direct action on the gametes, or indirectly by modulation of the endocrine system so that gamete development takes place during an imbalance hormonal environment (Okai *et al*., 2004).

Fig. 4: The amount of VTG assayed in fish plasma exposed to different concentrations of ethynylestradiol for 30 days (vertical bars indicate SE and * means significant differences in VTG induction)

In female fish, endocrine modulators can affect hypothalamic gonadotrophin releasing hormone (GnRH) secretion, pituitary gonadotrophin (GtH) release, estrogen biosynthesis and catabolism, estrogen binding to hepatic receptors, hepatic VTG production, or the feedback by steroids and VTG (Berg *et al*., 2004). Although hepatic production of VTG in male fish could be initiated by estrogen hormone, but no estrogenic hormones secret in male fish and no VTG production can be expected in normal life (Aerni *et al*., 2004). The use of in vitro fish hepatocyte cultures or in vivo fish exposure to estrogenic chemicals is shown to provide a simple and effective way to screen estrogenic activities of EDCs (Tyler *et al*., 1996). VTG assay in cell

culture or fish plasma needs a quick, cheap, reliable and efficient method to fulfil such a duty (Fujita *et al*., 2004) and many researchers have already introduced different techniques to measure VTG (Anderson *et al*., 2003; Aerni *et al*., 2004), an ELISA method for assaying VTG has been described here.

Assay validation

Sensitivity: sensitivity of the assays, which has been taken as 90% of the binding with 0 pg is just over 10 µg for VTG. Using identical conditions for label preparation and assay we also obtained a sensitivity of 2 µg for VTG comparable to that obtained by others (Volz and Chandler, 2004). As with radioimmunoassay, the sensitivity is a function of both the specific activity of the label and the quality of the antiserum, and will therefore vary with the material available to the user. So in this study, we used the antiserum that were available to us but have little reason to expect that any antiserum which gives good results with RIA will not give similar results to those that we describe for ELISA.

Previous reports of ELISA for VTG have used different enzyme labels (Martin-Diaz *et al*., 2004) and in this communication a method described previously (Cuisset *et al*., 1994) and the results showed it is sufficiently sensitive for the rapid measurement of VTG in common fish species such as cyprinids, and present an example of such an application was described.

Accuracy and reproducibility: a major advantage of ELISA is the ability to minimise errors resulting from multiple pipetting by use of multichannel pipettes. Inter- and intra-assay coefficient of variations (CV) reported in RIA are frequently 15-20% even when measured at the steepest part of the curve. For the assays reported here we obtain intra-assay CVs around 3.9% in the steep part of the curve, rising to 15-20% at the flatter end regions (Fig. 2), while interassay variation (at 50% displacement) was around 12%, comparable to that reported by Cuisset *et al*., (1994). There was a highly significant correlation between the VTG added to and that measured in deproteinized plasma (Fig. 3). A further advantage of ELISA is that sample handling can also be made more rapid and reproducible by the use of low binding microtiter plates for sample storage and dispensing with multichannel pipettes.

Application to the measurement of VTG production in fish

To demonstrate an application of the ELISA method we have measured the amount of VTG produced by carp exposed to ethynylestradiol. The results confirmed previous findings that ethynylestradiol is a potent estrogenic chemical (Craft *et al*., 2004) (Fig. 4) and a clear and potent estrogenic activity in inducing VTG by fish was found with increase in ethynylestradiol concentration and the time of exposure (15 vs 30 days) (Fig. 4). The amounts of VTG production in fish exposed increased dramatically so the logarithmic scales used to demonstrate it in Fig 4. Such a potent induction of VTG in other studies have already been discussed (Versonnen *et al*., 2003).

The rapid determination of VTG from the very small volumes of blood allows the examination of water pollution with EDCs and this methodology will therefore be of great interest to workers screening the estrogenic of new chemicals and phytoestrogens.

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