Invited Review

PLASMA VITELLOGENIN – A BLOOD PARAMETER TO EVALUATE EXPOSURE OF FISH TO XENOESTROGENS

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Abstract

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Besides natural and synthetic estrogens, a variety of industrial chemicals and pesticides are suspected to mimic the natural estrogen 17ß-estradiol, thereby disrupting the animal endocrine system. Based on observations, such as the occurrence of hermaphroditism and feminization of male fish, many investigations focus on fish as indicator organisms for xenoestrogenic compounds within the aquatic environment. Both *in vitro* assays and *in vivo* approaches have been developed to evaluate estrogenic effects of these toxicants. The occurrence of the female specific egg yolk precursor protein vitellogenin (Vtg) in the plasma of male fish has widely been used as an indicator or biomarker of xenoestrogen exposure. This paper briefly reviews potential xenoestrogens known so far, physiological aspects of vitellogenesis, current applications of Vtg plasma levels in fish as a biomarker for estrogenic compounds, and various aspects concerning the possible biological significance of this parameter.

Xenoestrogens, vitellogenin, fish

Since several years there is increasing concern on anthropogenic chemicals within the aquatic environment which might have the potential to interfere with hormonal regulatory pathways. A so-called endocrine disruptor has been defined as an exogenous substance, that cause adverse health effects in an intact organism, or ist progeny, secondary to changes in the endocrine function (European Community, 1997). Although the above mentioned definition includes a broad spectrum of chemicals inducing a variety of endocrine effects (Colborn 1993), most emphasis has been layed on substances, which mimic the natural female steroid hormone 17ß-estradiol and might disturb reproductive functions. Physiological consequences on wildlife due to xenoestrogens detected so far, include abnormal gonadal development and reduced reproduction in reptiles such as alligators (Guillette et al. 1994), and turtles (Bergeron et al. 1994), feminisation in birds (Fry and Toone 1981), and hermaphroditism as well as the induction of the female specific egg yolk protein precursor vitellogenin (Vtg) in male fish (Purdom et al. 1994).

The present paper provides a short overview on the aquatic xenoestrogens known so far and the mechanisms by which they may act. Special attention is given to the egg yolk precursor vitellogenin (Vtg), which is synthesized by the liver of female fish during sexual maturation (Copeland et al. 1986). Under the influence of estrogenic substances, also male fish can produce Vtg (Mommsen and Walsh 1988). Besides physiological aspects of vitellogenesis, the use of plasma Vtg levels in male fish as a biomarker for estrogenic aquatic toxicants (Purdom et al. 1994; Sumpter and Jobling 1995; Ankley et al.

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1998; Hansen et al. 1998) are emphasized. The bibliographic databases "Biological Abstracts", "Current Contents" and "Medline" have been screened in order to obtain recent data on the topic of this short review.

Aquatic Xenoestrogens

Besides the natural oestrogen 17ß-estradiol, synthetic estrogens (e.g. ethinylestradiol), and phytoestrogens (Fawell and Wilkinson 1994; Pelissero et al. 1991), a variety of chemical compounds with potential estrogenic effects reach the aquatic environment (Colborn 1993). In principle, xenoestrogens may act directly by binding to estrogen receptors or indirectly by altering/influencing metabolic processes, transport proteins, or the endocrine activity of the hormonal regulatory organs hypothalamus and pituitary (Thierfelder et al. 1995). Many of the xenoestrogenic compounds, known so far, bind directly to the estrogen receptor including organochlorine pesticides, such as o,p'-DDT (Nelson 1974) and Kepone (Chlordecone) (Thomas and Smith 1993), Bisphenol A (Krishnan et al. 1993), PCBs (Arcochlor 1221) (Nelson 1974), or alkylphenolic substances (White et al. 1994; Jobling and Sumpter 1993).

As has been determined *in vitro* by the E-screen test, using the proliferation of estrogen sensitive MCF-7 human breast cancer cells as a parameter for estrogenic activity (Soto et al. 1992), the relative estrogenic activity of most of these xenoestrogens seems to be weak in comparison to the natural female hormone 17ß-estradiol (Soto et al. 1995). Another *in vitro* bioassay with rainbow trout hepatocytes, which is based on the fact, that the synthesis of the egg yolk protein precursor vitellogenin (Vtg) is estrogen dependent, revealed, that the estrogenic potency of alkylphenol compounds, for example, is in the range of 1×10^{-4} to 1×10^{-6} of the activity of 17ß-estradiol (Jobling and Sumpter 1993). Due to the fact, that *in vitro* systems do not take into account bioaccumulation and metabolic processes that may occur in intact organisms, it can not be excluded however, that chemicals which have been shown to be weak estrogens in *in vitro* assays, might be more potent *in vivo* (Harries et al. 1997). Besides the ability of many xenoestrogens to bind to the estrogen receptor, other factors, such as a possible lack of an inherent affinity to protein carriers within the blood (Vom Saal et al. 1992, 1995), or interactions with metabolic pathways, such as the cytochrome P450 dependent monooxygenase system (McKinney and Walker 1994) might also enhance estrogenic activity, thereby influencing the dose at which xenoestrogens exert adverse effects *in vivo* (Gimeno 1997). Furthermore, additive effects have been achieved with mixtures of estrogenic compounds (Sumpter and Jobling 1995), probably due to their action via the same receptor (Arnold et al. 1996; Harries et al. 1997).

Vitellogenin in fish under physiological conditions

Vitellogenin is a high molecular weight phospholipoprotein, which is synthesized by the liver of females of oviparous vertebrates including teleosts under the control of estrogen (Mommsen and Walsh 1988; Bon et al. 1997). The production of estrogen (17ß-estradiol), which takes place within the follicular epithelium of the ovaries (Nagahama 1983), is regulated by gonadotropin releasing hormone (GnRH) from the hypothalamus and the gonadotropic hormones in the pituitary (Mommsen and Walsh 1988). Swanson et al. (1987) demonstrated two different forms of gonadotropins (GTH I and II) in a variety of salmonids. In sexually immature fish only GTH I is present (Feist and Schreck 1996), which in females seems to control 17ß-estradiol production (Swanson 1991). In mature animals also GTH II can be detected (Nozaki et al. 1990), which regulates the production of maturation-inducing steroids during the final stages of follicular development (Swanson 1991).

The synthesis of Vtg is initiated by binding of estrogen to a highly specific estrogen receptor in the hepatocytes. The interaction of the hormone-receptor-complex with specific nucleotide sequences (nuclear estrogen response elements) leads to the transcription of Vtg mRNA followed by the translation and secretion of the Vtg protein (Mommsen and Walsh 1988). These processes are temperature-sensitive showing greater efficiency at higher temperatures (MacKay and Lazier 1993). After synthesis, Vtg is not stored in the liver but is secreted into the blood stream (Copeland et al. 1986), from which it is sequestered by the developing oocyte and proteolytically processed as yolk protein to supply fish embryos and larvae during their development (Mommsen and Walsh 1988).

The Vtg content of the plasma of female fish is dependent on the age and the stage of the reproductive cycle. Investigations in rainbow trout (Copeland et al. 1986) demonstrated that Vtg is already present in small amounts in juvenile females long before spawning and before it is sequestered by developing oocytes. Accordingly, sex specific differences concerning the body content of sex steroids such as 17ß-estradiol have been detected already 78 and 90 days post fertilization (Feist and Schreck 1996). In this context it should be mentioned, that sex steroids seem to be of maternal origin during early developmental stages of fish (Yeoh et al. 1996). Up to spawning the plasma Vtg levels increased one million-fold in fish females (Copeland et al. 1986). Interestingly, the latter authors described occasional low levels of Vtg also in the plasma of male individuals, whereas in a study of Bon et al. (1997) plasma of untreated males did not contain Vtg. Whether or not this is due to higher detection levels seems not clear. Treatment of males and juvenile females with exogenous estradiol resulted in an increased Vtg synthesis (Idler and Campbell 1980; Copeland et al. 1986). It has been demonstrated in Atlantic salmon (*S. salar*), that also the hepatocytes of male or nonvitellogenic fish contain specific high-affinity estrogen receptors (Lazier et al. 1985), and that the synthesis of Vtg in these fish is relatively or completely inactive only due to the absence of endogenous estradiol stimulation (Copeland et al. 1986).

Plasma vitellogenin as a biomarker for xenoestrogen exposure

For aquacultural purposes, the administration of natural or synthetic estrogens to manipulate the reproductive system of fish has long been described (Goetz et al. 1979; Nakamura 1984; Hunter and Donaldson 1983; Patiño 1997). Dependent on the fish species, a complete gonadal feminization of genotypic males was achieved due to the application of sex hormones during the period of gonadal sex differentiation (Yamamoto 1969), or after testicular differentiation (Nakamura 1984).

Even though, a complete sex reversal in wild fish populations has not been reported so far, recent field observations, e.g. the occurrence of hermaphroditic fish living in effluent water from sewage treatment works (Purdom et al. 1994), or changes in the sex ratios within urban waterways (Hansen et al. 1998), clearly show the need for a sensitive and reliable *in vivo* assay to detect early estrogenic effects of aquatic toxicants in fish. The most frequently applied *in vivo* parameter to evaluate exposure of fish to estrogenic compounds seems to be the blood Vtg levels in male fish.

For the quantification of Vtg in the plasma of fish, various methods have been developed, including the determination of indirect indicators such as plasma phosphoprotein, total protein and calcium levels (Tinsley 1985), or direct measurement of Vtg by immunochemical methods such as RIA (radio-immunoassay) (Sumpter 1985; Tyler and Sumpter 1990) or ELISA (enzyme-linked immunosorbent assay) (Nuñez et al. 1989; Mañanos et al. 1994; Bon et al. 1997; Islinger et al. 1997). Because of the different antigenic properties of the Vtgs between fish species, an universal assay to quantify Vtg in the blood of different fish species is difficult to develop (Tyler et al. 1996). Salmonid fish

have been shown to reveal similar antigenic determinants on the Vtg molecule within a family, but not between families (Benfey et al. 1989; Norberg and Haux 1988). In contrast to salmonids, a variety of cyprinid fish showed cross-reactivity in a RIA (Tyler et al. 1996), which was originally developed for carp Vtg (Tyler and Sumpter 1990). Folmar et al. (1995) presented a highly conserved N-terminal amino acid sequence for Vtg, which might be of potential value to detect Vtg from phylogenetically diverse teleost fish in the future.

The induction of Vtg synthesis in male fish has widely been used as a biomarker to detect estrogenic effects of aquatic xenoestrogens in the field. Within the frame of a nationwide survey in the United Kingdom, caged rainbow trout have been exposed to numerous effluent sites of sewage-treatment works, which, in most cases, caused a rapid and pronounced increase in their plasma Vtg concentrations (Purdom et al. 1994; Harries et. al. 1997). It has been suggested, that ethinylestradiol, most probably deriving from oral contraceptive pills, and natural estrogens (Desbrow et al. 1998), as well as degradation products of alkylphenol polyethoxylates, such as nonylphenol (Purdom et al. 1994; Harries et. al. 1997), might be responsible for these effects. Male carp, deriving from a site near a major metropolitan treatment plant in the United States revealed elevated plasma Vtg levels as well (Folmar et al. 1996), which is suggested to be a consequence of estrogenic components in the sewage. Most studies, which focused on the induction of vitellogenin in marine fish species, have been conducted with flounders (*Platichthys flesus*) as indicator organism. Investigations on wild flounder populations exposed to sewage effluents revealed elevated Vtg levels (Lye et al. 1997). Individuals, which were kept for three years in mesocosm systems containing a variety of PCBs, PAHs, and other organic chemical compounds revealed premature vitellogenesis (Janssen 1995). In Germany, Hansen et al. (1998) reported elevated plasma Vtg levels in male rainbow trout exposed to several dilutions of municipal effluents which are known to contain natural and synthetic hormones, such as 17ß-estradiol and ethinylestradiol and low concentrations of various xenoestrogens, e.g. alkylphenols and Bisphenol A.

Besides its use as a biomarker for estrogenicity in the field, plasma Vtg has also been applied as a parameter to determine threshold levels for estrogenic effects of chemicals under laboratory conditions. An extensive study of Jobling et al. (1996) on the estrogenic potential of various alkylphenols in rainbow trout revealed that threshold concentrations above which a significant elevation in the Vtg synthesis was observed, were in the range of 10 µg/l and 3 µg/l for nonylphenol and octylphenol, respectively. Our studies on the estrogenic effects of nonylphenol in male rainbow trout revealed a threshold value for elevated Vtg plasma levels, which was almost in the same order of magnitude (unpublished data). As discussed in detail by Jobling et al. (1996), the concentrations of nonylphenole, which occasionally can be reached in the aquatic environment (Blackburn and Waldock 1995), might potentially stimulate Vtg synthesis in fish. Besides these, some pesticides such as ß-hexachlorocyclohexane, which constitutes 5 to 10% of the organochlorine insecticide lindane, was shown to induce vitellogenesis in juvenile guppies (*Poecilia reticulata*) (Wester et al. 1985). Investigations of Donohoe and Curtis (1996) on the estrogenic activity of chlordecone, o,p'-DDT, and o,p'-DDE suggested, that plasma Vtg was the most sensitive marker of estrogen exposure.

Conclusions

A variety of anthropogenic compounds may reach the aquatic environment, which can be considered as environmental estrogens (Colborn et al. 1993). Most of the studies, conducted so far, to identify estrogenic chemicals are based upon estrogen receptor-mediated mechanisms *in vitro*, e.g. receptor binding assays (Nelson 1974; Krishnan et

al. 1993; Thomas and Smith 1993; White et al. 1994). However, the fact, that a chemical compound can bind to an estrogen receptor, does not necessarily imply, that it can stimulate estrogenic activity. Or conversely, other mechanisms than binding to the estrogen receptor may lead to estrogenic effects. There is a need to link biomarkers of estrogenicity to a biological response (Arcand-Hoy and Benson 1998). The expression of the Vtg gene has been shown to depend on the interaction of estrogen with estrogen receptors in the liver (Chen 1983). The induction of Vtg mRNA in the fish liver is a rapid and sensitive process. For example, a primary acute stimulation with estradiol resulted in a rise of Vtg mRNA in the liver of male rainbow trout 2 days after hormone administration, and secondary stimulation resulted in a more rapid accumulation of Vtg mRNA (Le Guellec et al. 1988). In male rainbow trout exposed to nonylphenol, Lech et al. (1996) detected an induction of Vtg mRNA already after 1 day of exposure by using an RT-PCR technique. An increase of Vtg mRNA levels has been shown to be paralleled by an increase of blood Vtg levels in the liver of male fish during chronic estradiol stimulation (Le Guellec et al. 1988). Therefore, the stimulation of the vitellogenin synthesis *in vitro* (Jobling and Sumpter 1993) and *in vivo* (Purdom et al. 1994; Jobling et al. 1996; Lye et al. 1997; Hansen et al. 1998) appears to be a sensitive biomarker for exposure to estrogenic chemicals. However, there are few data available which clarify the biological significance of elevated Vtg plasma levels due to xenoestrogen exposure for individual fish as well as for fish populations. Jobling et al. (1996) showed a coincidence between elevated Vtg levels and inhibited spermatogenesis in developing male rainbow trout exposed to alkylphenolic compounds. Histopathological kidney lesions have been associated with an accumulation of Vtg in guppies (*Poecilia reticulata*) (Wester et al. 1985), and in rainbow trout (Herman and Kincaid 1988), after exposure to estrogenic compounds. Recent investigations revealed a decline in plasma calcium levels (Schwaiger et al. 1997) in association with elevated plasma Vtg levels in rainbow trout exposed to the xenoestrogen nonylphenol (unpublished data). As discussed by Herman and Kincaid (1988), a causal relationship between the calcium deficiency and the accumulation of Vtg may be suggested due to the fact, that teleost vitellogenin is known to bind calcium.

It can be concluded, that for the risk assessment of xenoestrogens, combined investigations have to be performed. Apart from *in vitro* assays, which may be useful to identify xenoestrogens and to assess their mechanisms of action (Gimeno 1997), *in vivo* studies have to be performed to evaluate adverse effects of these compounds on organisms (Arcand-Hoy and Benson 1998; Ankley et al. 1998). As recommended in general for the risk assessment of endocrine-disrupting chemicals (Arcand-Hoy and Benson 1998), these should focus on relationships between exposure to xenoestrogens and effects on the population level. Vtg levels in the plasma of male fish seem to be a suitable indicator of exposure to estrogenic compounds. In addition, however, population relevant endpoints such as reproduction success or sex differentiation of offspring should be considered.

So far, existing international guidelines do not consider specific endpoints to evaluate endocrine disrupting effects of aquatic toxicants. As discussed in detail by Gimeno (1997), guidelines such as the OECD guidelines 204 "fish, prolonged toxicity test:14 days study" (OECD 1984), the "fish, juvenile growth test-28 days" (OECD, 1994), or complete life cycle tests (U.S. Environmental Protection Agency, 1982), could be adapted for this purpose. Several endpoints, which are routinely used in these tests, such as behaviour, development, and reproduction might be suitable to reflect estrogenic effects. However, because of the relative nonspecifity of these parameters in terms of mode of action (Ankley et al. 1998), specific endpoints such as elevated plasma Vtg levels in male fish have to be included.

Vitellogenin v plasmě - krevní ukazatel hodnotící expozici ryb xenoestrogenům

Vedle přirozených a syntetických estrogenů celá řada průmyslových chemikálií a pesticidů napodobuje přírodní estrogen 17ß-estradiol, a narušuje živočišný endokrinní systém. Na základě pozorování např. výskytu hermafroditismu a feminizace samců ryb, je řada výzkumů zaměřena na ryby jako na indikátorový organismus pro xenoestrogenní komponenty ve vodním prostředí. Rozpracovávány jsou testy *in vitro* i *in vivo* pro hodnocení estrogenního vlivu těchto toxikantů. Výskyt specifického samičího prekursoru proteinu vitellogenin (Vtg) v plazmě samců je široce využíván jako indikátor nebo biomarker xenoestrogenního působení. Předložená práce podává krátký přehled potenciálních xenoestrogenů, fyziologických aspektů vitellogeneze, současného využití plazmového Vtg jako biomarkeru pro estrogenní komponenty a různé aspekty týkající se možné biologické významnosti tohoto parametru.

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