Application of Molecular Biological Biomarkers to Endocrine Disruption Studies

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Introduction

Endocrine Disrupting Chemicals (EDCs), also called environmental hormones, were first proposed in the book "Our Stolen Future" by a journalist Dianne Dumanoski [1]. EDCs are a group of xenobiotics greatly impacting the environment. As substances similar to endocrine hormones in living organisms, EDCs disrupt the normal secretion of hormones in bodies when entering animal and human bodies. This way, physiological disorders will occur, impacting the reproductive, nervous and endocrine systems of animals and humans or even causing carcinogenicity. Specifically, aquatic organisms are directly exposed to pollutants, and the endocrine interference impact on them is even greater [2-4]. EDCs feature various types, continuous production, diversified forms and bioaccumulation. In recent years, EDCs have become the most severe pollution issue after ozone depletion and global warming and attracted attention of many countries and international organizations [5].

Fish is often considered one of the model organisms for endocrine disruptor evaluation because endocrine disruptors may interfere with reproduction hormones in fish. First, sexual differentiation is unstable in fish, implying that during the sexual differentiation phase of this vertebrate, it may be disrupted by external hormones, or its sex may be even changed [6]. Second, aquatic ecosystem is under attack from various pollutants, and fish is directly exposed to industrial, agricultural and municipal wastewater and pollutants. Current evidence showed that long-term exposure to EDCs would affect reproduction and population of fishes [7]. Endocrine disruptors may affect important hormones or receptors by disrupting the endocrine pathway along the...
Hypothalamus-Pituitary-Gonad (HPG) axis of fish, which finally has an impact on animal reproduction. EDCs may also disrupt the Hypothalamic-Pituitary-Thyroid (HPT) axis of fish, interfere with thyroid hormone synthesis, transport and combination, destabilize the thyroid hormone environment, and are therefore harmful to the growth and development of fish. Environmental pollutants can affect the synthesis of steroid hormones and serve as an endocrine disruptor through a receptor independent pathway. Molecular biological technologies, such as -omics and transgenic techniques, provide a reliable approach to evaluate the threats from endocrine disruptors in environmental pollutants. They may also reveal the pollutant mechanism. In addition, specific genes in fish also help determine biomarkers as endocrine disruptors [8].

Biomarkers can be defined as an indicator for the level of exposure to and/or toxic effects of one or more chemical pollutants, which affect organisms at biological levels such as molecular, cell, and individual levels and cause measurable changes in tissues, biological fluids, or biochemical, cell, physiological, or behavioral features of organisms [9]. Some biomarkers can predict the effects of reproductive or other endocrine disruption based on the linkage between endocrine disruption and endpoints of molecular biological experiments. Therefore, biomarkers for EDCs now become a study focus after the establishment of highly efficient experimental methods reflecting the actual situation in organisms with a low cost and little or no damage. Biomarkers are widely applied to and play a significant role in endocrine disruption researches. Due to the diversified functions of EDCs, the types of biomarkers become increasingly abundant, with continuous emergence of a large number of new biomarkers.

**Estrogenic Biomarkers**

A distinctive feature of EDCs is their estrogenic effect. By emulating endogenous estrogens, EDCs lead to feminization of male individuals. Many synthetic compounds with different structures have estrogen or antiestrogen effects [10]. According to multiple studies, Vitellogenin (VTG) in adult fish may function as an ideal biomarker. VTG is a precursor protein of egg yolk and is usually synthesized as a result of endogenous estrogen stimulation. VTG generally exists in the plasma of female fish. Though male fish also has VTG genes, VTG proteins cannot be autonomously synthesized in male fish in the absence of estrogen. However, stimulated by exogenous estrogen, VTG proteins may be synthesized in male fish. VTG is specific and sensitive to estrogen stimuli and has a high expression level in a number of organisms. Therefore, it can be used as an excellent estrogenic biomarker [11]. Recently, VTG expression was employed to evaluate the estrogenic effect of environmental chemicals. As compared to protein-based detection, VTG-based detection has a comparable sensitivity. Therefore, VTG can be employed as a biomarker to detect estrogenic effect at an early phase [12,13]. At present, VTG genes in nearly 20 kinds of fish have been successfully cloned. Therefore, VTG may promote the applications of this biomarker in a wider range.

Estrogen plays a critical role in regulating the reproductive, developmental, and neuroendocrine systems of fish. In the study of the estrogenic effect, some other biomarkers have been discovered. Estrogenic Receptors (ERs) and aromatase are essential for synthesis, secretion and physiological functions of estrogen. Estrogenic effect is produced when compounds simulate estrogen to combine with ERs and activate the ER genes. In bony fish, there are mainly two estrogenic receptor subtypes ERα and ERβ, which activate gene expression by binding to specific response elements [14,15]. In addition, estrogenic effect may be indicated by an increase in the level of free endogenous estrogens in plasma caused by the competitive binding of exogenous chemical hormones to globulin [16]. The antiestrogen effect implies that compounds have no or little estrogen activity but could perform competitive binding with ERs. In addition, the antiestrogenic effect influence, the expression and activity of the cytochrome p450a19 enzyme (aromatase).

Villeneuve et al. studied the impact of fadrozole on the expression of genes related to reproduction in the HPG axis and liver of fathead minnow. In the perspective of systems biology, they established an evaluation system for the impact of pollutants on the HPG and determined the association between gene expression and experimental endpoints of reproductive biology. This method aimed at the functions of multiple important organs instead of only one organ such as the gonad, which helped understand the influence and interaction between pollutants and the entire endocrine system of fish [17]. Moreover, this approach depicted the molecular function model and helped establish a relationship reflecting the influence of molecular biological changes on organism reproduction. Zhang et al. explored the impacts of prochloraz and ketoconazole on the expression of reproduction-related genes in the HPG axis and liver of medaka. They found a linear relationship between the expressions of six genes in the liver and spawning amounts. Therefore, gene expression amounts could be used to speculate on the biological experiment endpoint of fish in the view of ecology [18].

Dongmei et al. [19] studied the estrogen-related genes in the HPG axis of the zebrafish embryo, or more specifically estrogen-associated biomarkers including VTG1, VTG2, ERα, ERβ1, ERβ2, CYP19a1a and CYP19a1b, based on which the joint effect of compound estrogen involving cypermethrin, malathion and prochloraz on zebrafish could be determined. The test results demonstrated that within a period under a certain drug amount, the estrogenic effect was effectively enhanced with binary pesticides of cypermethrin and malathion or malathion and prochloraz when compared with the treatment of a single chemical. In addition, the expression amounts of ERα, ERβ1, ERβ2, CYP19b and CYP19a were significantly changed.

Jinhua et al. [20] used molecular biomarkers such as estrogen and thyroid hormone to study the endocrine disruption of acetochlor at the early life stage of zebrafish. After zebrafish was exposed to acetochlor at the concentrations of 50, 100 and 200 µg/L, the HPG/
HPT-associated genes in zebrafish, such as VTG1, ERβ1, CYP19a and TRα, and many key genes in the apoptosis pathway, including Bcl2, Bax, Bcl2, and Cas8, underwent significant changes. The results demonstrated that acetylcholine could induce oxidative stress and apoptosis at the developmental stage of zebrafish and affect the immune and endocrine systems of zebrafish. Yingying C. et al. [21] reported the estrogenic effects of 2,3,7,8-Tetrachlorodibenzo-Para-Dioxin (TCDD) on the gene expressions of the Zebrafish Liver (ZFL) cell lines, and zebrafish embryo, larva, juvenile and adult zebrafish livers, both independently and when combined with Cd 2+. In ZFL cells, mRNA expression of VTG1 was significantly suppressed by Cd 2+ but not affected by TCDD. In ZFL cells, the expressions of ERα, ERβ1, ERβ2 and GPER were insignificantly changed. They also conducted a study on the VTG1 promoter deletion mutant and found no reaction in the presence of TCDD or Cd 2+. However, following co-transfection with a VTG1 promoter-luciferase construct to the ERα, ERβ1, ERβ2 and GPER expression vectors, decreased luciferase activity was observed in the ERα co-transfection group after treatment with Cd 2+, suggesting that ERα participated in VTG1 transcriptional regulation and was affected by Cd 2+. The regulation of these genes at the mRNA level varied in male and female zebrafish livers at different developmental phases.

**Thyroid Hormone Biomarkers**

Another prominent feature of EDCs lies in the thyroid effect. Thyroid hormone helps maintain the normal physiological conditions of vertebrae and is considered the most important factor in controlling their growth, development, and behaviors. In bony fish, thyroid hormone also plays a vital role in regulating metabolism. The synthesis and release of thyroid hormones are controlled by the HPT axis. Chemicals that disrupt one or more points in the HPT axis may affect the thyroid hormone function and finally impact the growth and development of animals. Thyroid-disrupting chemicals may interfere with different regulation pathways, especially synthesis and metabolism (binding) of thyroid hormones, for example, synthesis through iodinated thyroglobulin or changing T4 into T3 with higher physiological activity. They may also disrupt plasma protein binding or combine with Thyroid Receptors (TRs) [22]. After combining with ligands, inhibitory factors activated proteins and TR homo-/hetero-dimers or retinoic X to induce the expression of target genes of thyroid response elements, such as phosphoenolpyruvate carboxykinase or 5'-diodinase [23]. In bony fish, there were two TR isoforms (α and β) and splice variants [23]. It was difficult to identify insignificant impacts of thyroid hormone on organisms in laboratories. However, molecular markers could serve as an indicator and prediction signal to overcome the difficulty in short-period experiments. Molecular biomarkers, which are similar to estrogen regulation genes such as VTG, could be used as potential sensitive biomarkers for thyroid hormones.

Wenqing et al. [24] studied the disruption of Synthetic Pyrethroids (SPs) on the HPT axis of zebrafish embryo under acute exposure conditions. SPs might damage the thyroid endocrine system of mammals. 1, 3, and 10 µg/L of bifenthrin or cyhalothrin might influence the levels of T4 or T3. In addition, the genes such as CRH, TSHβ, TTR, UGT1ab, Pax8, Dio2 and TRα in the HPT axis of zebrafish were significantly upregulated as induced by bifenthrin. Cyhalothrin could influence different test genes and significantly induce the expressions of TTR, Pax8, Dio2 and TRα. However, the Dio1 gene was greatly suppressed. At the atomic level, compared with cyhalothrin, the combined protein of bifenthrin and TRα had more powerful influence on the HPT signal conduction. According to Shi et al. [25], PFOS had apparent development toxicity effect on zebrafish embryo. The expressions of marker genes hhex and Pax8 for early thyroid development were significantly upregulated, indicating possible thyroid development toxicity. The study on the thyroid toxicity of PCB126 to salmon [26] revealed that the levels of T4 in the salmon plasma and T4 glycosylation significantly rose, resulting in swelling of the thyroid epithelial cells. As the herbicides used with the largest amounts in China, acetochlor decreased the Thyroid Hormone (TH) level according to the Crum’s study on xenopus laevis, which impacted the thyroid functions. The author explained the mechanism at the molecular level [27].

Xuesong et al. [28] studied the thyroid endocrine toxicity of 2,2'-4,4'-tetrabromodiphenyl ether (BDE-47) and Perchlorate (PER) based on the TH-mediated pathway after zebrafish embryo was exposed to them for 14 days. The test results indicated that when compared with exposure to only BDE-47, combined exposure to BDE-47 (10 µg/L) and PER (3.5 mg/L) could significantly upregulate the genes NIS and Nkx2.1a that participated in TH synthesis, which highly downregulated HPT axis (CRH and TSH) mediated by expression of regulation-related genes. Compared to single BDE-47 exposure, combined exposure to BDE-47 and PER could greatly lift the TG gene and protein levels, while significantly downregulated the TTR gene and protein levels. In addition, exposure to the compounds of BDE-47 and PER greatly decreased the T4 level, indicating that the damage to the BDE-47 thyroid was increased by PER. The results helped explain the complicated chemical interactions and the molecular mechanisms of the two disruptors.

**Reproduction Hormone Biomarkers**

The HPG axis controls the reproduction of fish species among all vertebrates. During the fish spawning season, external factors such as light and temperature of water affect the gonad recovery and brain maturation. Signals from the brain control the Gonadotropin Releasing Hormone (GNRH), and GNRH stimulates the hypothalamus-pituitary, which secretes gonadotropin, including Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH). Then, LH and FSH, in return, control the production and secretion of steroid hormones from the gonad. Hormones, including the main hormones and sex inducing hormones of 17β-estradiol and testosterone in female individuals, as well as testosterone and 11-keto-testosterone in male individuals, initiate changes in secondary sexual characteristics, in addition to the development and maturation of sperm and eggs. In different physiological
stages, reproduction hormones regulated the secretion of pituitary gonadotropin hormones according to the positive or negative feedback [29]. The HPG axis provided several potential modes, which affected the synthesis and secretion of EDCs. By binding to hormone receptors or changing the usability of endogenous hormones, EDCs exerted their disruption function through the mediation of steroid hormones or similar elements. Therefore, biomarkers directly reflected the level of steroid hormones and could function as a widely applied molecular indicator of endocrine disruptors. VTG synthesis and secretion were induced in livers by cyclopropen estrogens, and 17β-estradiol was dedicated to the identification of exogenous estrogen [30].

Wei Zh et al. [31] studied the chronic reproductive toxicity and possible mechanism of Perfluoroalkyl Acids (PFAAs) after adult zebrafish (Danio rerio) was exposed to different concentrations (0.01, 0.1 and 1 mg/L) of PFAAs for 180 days. The disrupted expression of genes, such as ESR, ERβ, FSHR, LHR, StAR and 17βHSD, indicated possible interference of PFNA on the HPGL axis function and sex hormone synthesis. PFNA interfered with the HPGL axis function and sex hormone synthesis by disturbing the expression of genes in the HPGL axis, which led to estrogenc effects such as significantly increased VTG content in males and increased E2 levels in both genders. The results of this study provided a basis for research on the potential risks of this ubiquitous and persistent contaminant in aquatic ecosystems worldwide.

Kyunghee J et al. [32] carried out a study on the impact of non-steroidal anti-inflammatory drugs (NSAIDs) on the PHG genes of zebrafish and their reproduction toxicity. This study found that ibuprofen and mefenamic acids significantly increased the concentrations of 17β-estradiol and testosterone in females, while decreased those of testosterone among male fish after the adult zebrafish was exposed to the drugs for 14 days. Significant upregulation of FSHβ, LHβ, FSHR and LHR was observed in females, whereas downregulation was observed in males exposed to NSAIDs. After adult zebrafish pairs were exposed to ibuprofen for 21 days, and the egg production was significantly decreased at 1 µg/L ibuprofen, parental exposure resulted in delayed hatching even if they were transferred to clean water for hatching. The results demonstrated that ibuprofen could modulate hormone production and related gene transcription of the HPG axis in a sex-dependent way, which could cause adverse effects on the reproduction and development of offspring.

Qun-Fang Z et al. [33] conducted research on the reproductive toxicity of zebrafish under exposure to inorganic mercury (Hg). After adult zebrafish was exposed to 0 (control), 15 and 30 µg of Hg L (added as mercuric chloride, HgCl2) for 30 days, the activities and mRNA levels of antioxidant enzymes (catalase (CAT), Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPx)) as well as the contents of Glutathione (GSH) and Malondialdehyde (MDA) were changed. In females, although the ovarian 17β-estradiol (E2) content remained relatively stable, significant downregulation of LHR, GNRH2, GNRH3, LHR and EAR were observed. In males, Testosterone (T) levels in the testis significantly decreased after Hg exposure, accompanied by down-regulated expression of GNRH2, GNRH3, FSHβ and LHβ in the brain as well as FSHβ, LHβ, ARβ, CYP17 and CYP11b in the testis. Thus, our study indicated that waterborne inorganic Hg exposure altered sex hormone levels by disrupting the transcription of related HPG-axis genes, which could subsequently impair fish reproduction.

Conclusion

EDCs may bring about great impact on the ecological environment and human health, and people have begun to understand the importance of studying the potential risks resulted from the pollution of EDCs. It is important to perform researches on the toxicity of EDCs, especially the joint toxic effects caused by long-term exposure to endocrine disruptors at low dosage. The biological screening methods of EDCs, either in vivo or in vitro, require a high cost. Moreover, damages will be present in in-vivo biological tests, and significantly different results will occur in in-vitro tests when compared to in-vivo tests. Molecular biomarkers provide an innovative technique for the screening and research of EDCs. One of the advantages of this method is the low cost and effective reflection of actual conditions inside organisms. Another advantage lies in its sensitivity to the molecular endpoints in aromatase inhibitors, hormone levels and expressions of various genes. After fish is exposed for two to three weeks to chemicals at the concentration that can influence the reproduction, the molecular endpoints of the tests can be sensitively influenced. Using molecular endpoints is a relatively simple screening method for the study of endocrine disruption effects. Therefore, it is extremely important to use molecular biological technologies to establish a fast, reliable, specific and sensitive screening method for effective screening and detection of contaminants in EDCs.

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