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FACULTY OF FISHERIES AND PROTECTION OF WATERS

Effects of residual pharmaceuticals present in aquatic environment on fish

Vliv reziduí farmak přítomných ve vodním prostředí na ryby

Zhi-Hua Li

Czech Republic, Vodňany, 2011

I thereby declare that I wrote the Ph.D. thesis myself using results of my own work or collaborative work of me and colleagues and with help of other publication resources which are properly cited.

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Content

Chapter 1

An Introduction of residual pharmaceuticals in aquatic environment

1.1. General Introduction

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1.1. General Introduction

Pharmaceuticals are complex compounds with different functions, physiological and biochemical properties. Nowadays, these compounds become a class of emerging environmental contaminants that are extensively and increasingly being used in human and veterinary medicine. Awareness of residual pharmaceuticals in aquatic ecosystems is growing as research into these pollutants increase and analytical detection techniques improve.

1.1.1 Occurrence and fate of residual pharmaceuticals in aquatic environment

It is estimated that worldwide consumption of pharmaceuticals amounts to some 100000 tons or more per annum. Use may vary from country to country. After administration, pharmaceuticals or their metabolites are excreted and discarded into urban wastewaters and eventually make their way to wastewater treatment plants. Since metabolic stability is necessary for pharmacological action, these compounds are often resistant to biodegradation in sewage treatment and are persistent and highly mobile in the aquatic environment, which can be tracked from municipal sewage water to drinking water.

The related investigation about occurrence of pharmaceuticals in the aquatic environment are now available for Canada, England, Germany, Greece, Italy, Spain, Switzerland, U.S. and other countries. Till now, more than 80 different pharmaceuticals were detected in sewage treatment plants effluent, surface water and groundwater and even in drinking water. Polar compounds such as antiepileptic drug carbamazepine, the antiphlogistics diclofenac and ibuprofen, which are relatively easy to measure, are generally among the compounds analyzed in most detail and used for environmental fate investigations. The concentrations in surface waters and effluent from sewage treatment plants have been shown to lie in the ng/L to pg/L range. Otherwise, pharmaceuticals used in veterinary medicine, livestock farming and aquaculture or therapeutic purposes, has become a considerable pollution source and been analyzed in manure and soil. Evidence of a wide variety of different pharmaceutical active substances in the aquatic environment, in liquid manure, and in the soil also shows that the active substances are at the very least not completely eliminated during sewage treatment nor are they biodegraded in the environment.

The investigations deal explicitly with the fate of residual pharmaceuticals is very limited. To our knowledge, the elimination processes for pharmaceuticals in the different

environmental compartments are sorption and bio-degradation, as well as photo-degradation and hydrolysis. Sorption of pharmaceuticals depends on the extent of neutral and ionic species present and the characteristics of the target particles. Sorption may have an impact on the spread and bioavailability of pharmaceuticals in the environment and their removal during wastewater treatment.

1.1.2 Ecotoxicological effects of residual pharmaceuticals in aquatic environment

Pharmaceuticals are of major concern because they are designed to retain or to prevent health of humans and animals by impacting very efficiently physiological processes, but they often have substantial adverse effects. When introduced into the aquatic environment, it can be expected that pharmaceuticals might have biological effects in lower animals (such as fish) with identical or similar target organs, tissues, cells or biomolecules, because certain receptors in lower animals resemble those in humans. It is important in this respect to recognize that for many pharmaceuticals, their specific modes of actions are not well known and often not only one, but many different modes of actions occur. In the past, pharmaceuticals have been studied only for their toxicological properties in their targets but they have never been requested for determining their for ecotoxicological risk. Therefore OECD, EU, and several Environmental Agencies started because of the evolving major concern about pharmaceuticals to request their testing for ecotoxicological risk assessment.

The assessment of potential biological effects of pharmaceuticals has been started to emerge from single reports in phytoplankton, zooplankton and bacteria to become recently more abundant especially for aquatic vertebrates and invertebrates. Actually, mostly acute tests are used to evaluate the risk of pharmaceuticals for the environment, which are often obtained at non-environmentally relevant concentrations. Furthermore, it is well known that acute toxicity to aquatic organisms is unlikely to occur at the lower measured environmental concentrations. However, prolonged exposure to low concentrations of the residual pharmaceuticals, the obvious toxic effects would be caused.

Aquatic vertebrates, especially fish, are always used as experimental models in environmental toxicology, in genetics, in cancer research, in biomedicine, in neurobiology, in endocrinology, in ecology, in gerontology, in developmental biology, in aquaculture and in general as a tool to obtain basic information in biological sciences. In fact, fish have been used in scientific research for a long time, less so than other animals such as rats and mice but at an increasing rate since the 1960s. Among their peculiar intraspecies characteristics it must be underlined that they often have a short life cycle, they produce eggs in large quantities and costs of rearing and maintenance are low. Though there is little evidence of any direct adverse effects of residual pharmaceuticals in the aquatic environment on fish at environmentally realistic concentrations, the ecotoxicological effects on fish should not be ignored.

Detailed information on residual pharmaceuticals in aquatic environment is given in the Chapter 1.2. Residual pharmaceutically active compounds (PhACs) in aquatic environment: status, toxicity and kinetics – a review.

IN THIS THESIS

The overall aim of present thesis was to investigate the effects of several pharmaceuticals present in aquatic environment on fish, including Verapamil, Propiconazole and Carbamazepine.

The specific objectives were to:

- Get the basic toxicity data and investigate the acute and chronic effects of Verapamil on behavioral, biochemical and physiological responses in fish.
- Evaluate the effects of environmental related levels of Verapamil on fish through the proteomics approach and try to find the influence mechanism.
- Investigate the effects of Propiconazole on the digestive system in fish, including the intestinal enzyme activity and the RNA/DNA ratio.
- Find a novel and efficiently means for monitoring residual pharmaceutical Cabarmazepine in aquatic environment by using fish spermatozoa in vitro assays.

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Záhlaví liché stránky

An Introduction of residual pharmaceuticals in aquatic environment

Záhlaví sudé
Chapter 1.2.

Chapter 2

Ecotoxicological effects of short-term exposure to a human pharmaceutical Verapamil in juvenile rainbow trout (*Oncorhynchus mykiss*)

Li, Z.H., Li, P., Randak, T., 2010. Ecotoxocological effects of short-term exposure to a human pharmaceutical Verapamil in juvenile rainbow trout (*Oncorhynchus mykiss*). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 152 (3), 385–391.

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Záhlaví liché stránky

Ecotoxocological effects of short-term exposure to a human pharmaceutical Verapamil in juvenile rainbow trout (*Oncorhynchus mykiss*)

Záhlaví sudé

Chapter 2

Chapter 3

Chronic toxicity of Verapamil on juvenile rainbow trout (*Oncorhynchus mykiss*): effects on morphological indices; hematological parameters and antioxidant responses

Li, Z.H., Velisek, J., Zlabek, V., Grabic, R., Machova, J., Kolarova, J., Li, P., Randak, T., 2011. Chronic toxicity of Verapamil on juvenile rainbow trout (*Oncorhynchus mykiss*): effects on morphological indices; hematological parameters and antioxidant responses. Journal of Hazardous Materials 185 (2–3), 870–880.

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Záhlaví liché stránky
Chronic toxicity of Verapamil on juvenile rainbow trout (*Oncorhynchus mykiss*): effects on morphological indices; hematological parameters and antioxidant responses

Záhlaví sudé stránky
Chapter 3

Chapter 4

Hepatic proteome sensitivity in rainbow trout after chronically exposed to a human pharmaceutical Verapamil

Li Z.H., Li P., Sulc M., Hulak M., Randak T., 2011. Hepatic proteome sensitivity in rainbow trout after chronically exposed to a human pharmaceutical Verapamil. Molecular & Cellular Proteomics. (submitted)

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Zahlavi sudé stránky

Chapter 4

Hepatic proteome sensitivity in rainbow trout after chronically exposed to a human pharmaceutical Verapamil

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Abbreviations list:

VRP, Verapamil; EDTA, Ethylene diamine tetraacetic acid ; DTT, Dithiothreitol; PMSF, Phenylmethylsulfonyl fluoride; SDS, Sodium dodecyl sulphate; 2-DE, Two-dimensional polyacrylamide gel electrophoresis; MALDI-TOF/TOF, Matrix-associated laser desorption/ionization time-of-flight/mass spectrometry; MW, Molecular weight; p*I*, Iisoelectric point; HSP, heat shock proteins; GRP78, Glucose-regulated protein 78; GRP94, Glucose-regulated protein 94; ER, endoplasmic reticulum; BiP, immunoglobulin chain-binding protein; ROS, reactive oxygen species; PDI, Protein disulfide isomerase; PDIA3, protein disulfide isomerase associated 3

Abstract

Verapamil (VRP), a cardiovascular pharmaceutical widely distributed and persistent in aquatic environment, has the potential toxicity to fish and other aquatic organisms. However, the molecular mechanisms that lead to these toxic effects are not well known. In the present study, proteomic analysis has been performed to investigate the protein patterns that are differentially expressed in liver of rainbow trout exposed to sublethal concentrations of VRP (0.5, 27.0 and 270 μg/L) for 42 d. Two-dimensional electrophoresis coupled with MALDI-TOF/TOF mass spectrometry was employed to detect and identify the protein profiles. The analysis revealed that the expression of 6 hepatic acidic proteins showed markedly altered in the treatment groups compared to the control group, especially 3 proteins were significantly decreased in liver of fish exposed to VRP at environmental related concentration (0.5 μg/L). The results suggest that VRP induce mechanisms against oxidative stress (Glucose-regulated protein 78 and 94, Protein disulfide-isomerase A3) and adaptive changes in ion-transference regulation (Calreticulin, Hyperosmotic glycine rich protein). Furthermore, for the first time, protein Canopy-1 was found to be significantly down-regulated in fish by chronic exposure to VRP at environmental related levels. Overall, our work supports that fish hepatic proteomics analysis serves as an *in vivo* model for

monitoring the residual pharmaceuticals in aquatic environment and provides insight into the molecular events in VRP-induced toxicity in fish and other organisms.

Keywords: residual pharmaceutical, fish, proteomics, chronic toxicity

Introduction

Over the past few decades, potential risks are associated with the pharmaceutically-derived chemicals in the aquatic environment. Developments to promote human health and well-being, certain pharmaceuticals are now attracting attention as a potentially new class of water pollutants (Fent et al., 2006). Such drugs as antibiotics, anti-depressants or birth control pills have been detected in varied water sources (Li and Randak, 2009). Verapamil (VRP), is an L-type calcium channel blocker of the Phenylalkylamine class. It has been used in the treatment of hypertension, cardiac arrhythmia, and most recently, cluster headaches. During the last decade the occurrence of VRP has also been reported in aquatic environment at concentrations ranged from 0.058 to 0.9 μg/L (Khan and Ongerth, 2004; Al-Rifai et al., 2007). Moreover, it has already been reported that VRP induced toxic effects on aquatic animal (e.g. *Daphnia magna*) even after treatment at low doses (Villegas-Navarro et al., 2003). However, the lack of information exists about toxic effects in fish. Fish have proven to be a better medium than flowing water to detect the impact and mechanism of action of various contaminants on aquatic animals and human being (Arai et al., 2004).

In our previous studies, rainbow trout (*Oncorhynchus mykiss*) was used as a model system to study the toxic effects of VRP on biochemical and physiological responses, which showed that treatment with sublethal concentrations of VRP significantly changed the physiological and biochemical responses (including behavior changes, morphological indices, hematological parameters and antioxidant responses) (Li et al., 2010a; Li et al., 2011). However, there was no significant change in all parameters measured in fish exposed to VRP at environmental related concentration (Li et al., 2011).

Environmental stress has been shown to affect patterns of protein expression in fish (Dowling and Sheehan, 2006), thus we hypothesize that protein expression profiles of VRP-exposed rainbow trout will differ from unexposed individuals. To do this we have used proteomic approaches to have an insight on protein expression profiles in the liver of rainbow trout. The rainbow trout is one of the most extensively researched and characterized species of fish. In addition its low tolerance to poor water quality has established it as one of the most useful sentinel species for aquatic toxicology. The liver is the central organ in detoxifying of xenobiotics and processing metabolic products for degradation (Li et al., 2010b). Therefore, to increase our understanding about the biological response of rainbow trout liver, we studied the changes in hepatic proteome after exposure to VRP.

Proteomic methods have recently been employed to get deep knowledge's about toxicity and mechanism of action of several toxicants, such as pentachlorophenol in rare minnow (*Gobiocypris rarus*) (Fang et al., 2010), microcystin in medaka (*Oryzias latipes*) (Mezhoud et al., 2008), and in zebrafish (*Danio rerio*) liver treated with Microcystin-LR (Wang et al., 2010). In contrast to conventional biochemical methods, the proteomic approach offers great potential in identifying proteins involved in the response of organisms to contaminants

through massive comparison of protein expression profiles and can help to identify novel and unbiased biomarkers related to toxicity (Dowling and Sheehan, 2006).

The main objective of this study was to identify differentially expressed proteins in the livers of rainbow trout chronically exposed at different concentrations of VRP, which could be used as biomarkers for monitoring levels of exposure to VRP in contaminated water resources. We anticipate that this information will provide novel information about the chronic toxicity of VRP to rainbow trout and identify new protein biomarkers that can help us to increase our understanding about toxicity mechanisms in aquatic organisms and human being.

Experimental procedures

Chemicals

Verapamil and other chemicals were obtained from Sigma-Aldrich Corporation (USA). The VRP was dissolved in pure distilled water to make a stock solution at a concentration of 50 mg/ml.

Animal and exposure

Juvenile rainbow trout, weighing 40.43 ± 2.55 g (mean ± S.D.), were obtained from a local commercial hatchery (Husinec, Czech Republic). They were held in aquaria containing 250 L of freshwater continuously aerated to maintain dissolved oxygen values at 7.5–8.0 mg/L. Temperature was 15 ± 1 °C and pH was 7.4 ± 0.2. Photoperiod was a 12:12 light-dark cycle. Fish were acclimatized for 14 d before the beginning of the experiment and were fed with commercial fish food (47% protein and 26% fat; BioMar, Denmark). The fish were starved for 24 h prior to experimentation to avoid prandial effects during the assay.

Prior to experiment the semi-static system composed of eight aquaria (200 L each) was used. The 40 fish were randomly distributed per each aquarium.

The nominal concentrations of VRP used were

- E1 group, environmental related concentration, 0.5 μg/L;
- E2 group, 1% 96 h LC 50 of VRP in rainbow trout (Li et al., 2010a), 27.0 μg/L ;
- E3 group, 10% 96 h LC 50 of VRP in rainbow trout, 270 μg/L

A control group exposed to clean freshwater. Each experimental condition was duplicated. The fish were fed daily with commercial fish pellets at 1% total body weight at a fixed time and the extra food was removed. The exposed solution was renewed each day after 2 h of feeding to maintain the appropriate concentration of VRP and water quality. The treatment period in all experimental groups was 42 d. At the end of treatment, 6 fish from each aquarium were randomly sampled and the livers were immediately frozen and stored at -80 ºC.

To ensure agreement between nominal and actual compound concentrations in the aquaria, water samples were analyzed during the experimental period by LC-MS/MS. Water samples were collected from the test aquaria after 1 h and 24 h of renewing the test solutions. The mean concentration of VRP in the water samples was always within 20% of the intended

concentration (the measured concentration of VRP in the water samples was 0.47 ± 0.05, 26.18 ± 1.36, 251.33 ± 19.81 μg/L corresponding to the nominal concentration 0.5, 27.0, 270 μg/L).

Protein sample preparation

The 0.05–0.1 g of frozen tissue was immersed in lysis buffer (40 mM Tris, 8 M Urea, 2 M thiourea, 2% Chaps, 3 mM Ethylene diamine tetraacetic acid (EDTA), 50 mM Dithiothreitol (DTT), 1 mM Phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml Leupeptin) and treated by ultrasonication using (BANDELIN Sonopuls, HD2070). Then the suspension was centrifugated at 16000 × *g* for 25 min to remove the cellular debris. After that, the protein supernatants were collected and stored at -20 ºC for proteomics analysis. Protein levels were estimated spectrophotometrically by the method of Bradford (1976) using bovine serum albumin as a standard.

Two-dimensional polyacrylamide gel electrophoresis (2DE)

Isoelectric focusing was performed using PROTEAN® IEF (Bio-Rad, USA). For liver samples, 100 μg of protein in a total volume of 125 μl of rehydration buffer (7 M Urea, 2 M thiourea, 2% Chaps, 50 mM DTT, 0.4% IPG buffer) was applied to 7 cm IPG strips (pH range 4 to 7 and 7 to 10, respectively). Electrical current conditions for the separation were set up as follows: passive rehydration for 14 h; isolelectric focusing: 200 V for 1 h (gradient); 500 V for 1 h (gradient); 1000 V for 1 h (gradient); 4000 V for 1 h (gradient) and 4000 V for 2 h (rapid). After isoelectric focusing, the IPG strips were equilibrated with a solution containing 6 M urea, 29.3% glycerol, 2% sodium dodecyl sulphate (SDS), 75 mM Tris-HCl pH 8.8 and 2% (w/v) DTT for 15 min, and in the second step with a solution containing 2.5% (w/v) iodacetamide instead of DTT for another 15 min. Each IPG strip was laid onto a 12% SDS-PAGE gel for second dimension electrophoresis. Protein spots were visualized by Coomassie Brilliant Blue R-250 (Applichem, Germany) staining.

The stained gels were scanned and analyzed by Nonlinear's 2D software (USA). Average gels for each group were derived from three replicates. Protein spots were detected using automated routines from the software combined with manual editing to remove the artefacts. Spot abundance was determined by the area of the spot multiplied by the density and referred to as the spot volume. For each gel, normalized spot volumes were calculated as the ratio of each spot volume to total spot volume in the gel. The criteria for determination of differential expression of proteins in each group were as follow, 1) the protein spots were up- or down regulated significantly ($p < 0.05$, ANOVA) with the statistic analysis and the changes were consistent in the three replicate analyses; 2) protein spots appeared or disappeared in experimental groups compared with control. In addition, spots were analyzed with adjusted spot filtration setting, by which spots with normalized volumes smaller than 0.05 were excluded.

In-gel digestion and mass spectrometry

Spots of interest were excised from gels stained by Coomassie Brilliant Blue R-250, cut into small cubes (approx. 1 mm^3), and destained using 0.1 M 4-ethylmorpholine acetate (pH 8.1) in 50% acetonitrile. After complete destaining, the gel was washed with water, dehydrated in acetonitrile, and rehydrated in water. The gel was partially dried using a SpeedVac concentrator and subsequently reconstituted with cleavage buffer containing 25 mM 4-ethylmorpholine acetate, 10% acetonitrile, and sequencing grade trypsin (Promega, 50 ng/μl). Digestion was carried out overnight at 37 °C. The resulting peptides were extracted with 40% MeCN/0.4% acetic acid. Following extraction, the peptides were subjected directly to mass spectrometric analysis.

Mass spectra were measured on a MALDI-TOF/TOF (matrix-associated laser desorption/ionization time-of-flight/mass spectrometry) mass spectrometer ultraFLEX III (Bruker-Daltonics, Bremen, Germany) equipped with a nitrogen laser (337 nm). Spectra were calibrated externally using the monoisotopic $[M+H]^+$ ion of peptide standards PepMix II (Bruker-Daltonics). A 5 mg/ml solution of α-cyano-4-hydroxy-cinnamic acid in 50% MeCN/0.3% acetic acid was used as a MALDI matrix. A 0.5 μl droplet of the sample was loaded onto the target. The droplet was allowed to dry at ambient temperature and overlaid with 0.4 μl of matrix solution. The positive MALDI-TOF and MS/MS LIFT spectra of selected ions were collected and interpreted using the MASCOT program (http://www.matrixscience.com/) in conjunction with the MW (molecular weight) and p*I* (isoelectric point) of proteins in the gels.

Statistical tests

All measurements were replicated at least three times and the data were expressed as mean values ± standard deviation. Statistical analysis was carried out using one-way ANOVA to evaluate whether the means were significantly different among the groups. Significant differences were indicated at $p < 0.05$. Prior to one-way ANOVA, data were log transformed to meet ANOVA assumptions of normality and homoscedasticity of variance.

Results

Protein expression

The 2-DE gels of VRP-exposed and nonexposed rainbow trout livers are shown in Figure 1, and quantitative spot comparisons were made by image analysis software. On average, over 800 acidic protein spots (*pI* 4–7) and over 300 basic protein spots (*pI* 7–10) were detected in gels. Compared with the control, a total of 6 acidic protein spots from the VRP-exposed fish were observed to significantly alter in abundance. Moreover, none of the basic protein spots were significantly changed in accordance with the criterion.

The all altered acidic proteins (Pro. 3, 4, 5, 7, 12 and 23) were significantly down-regulated ($p < 0.01$) in fish exposed to VRP with high concentrations (27.0 and 270 μg/L). Interestingly, the environmental related concentration (0.5 μg/L), significantly altered expression pattern of 3 protein spots (Pro. 4, 7 and 23). The quantitative comparisons of detected spots were made by image analysis software and the summary of significant differences observed between

experimental groups is shown in Figure. 2. Protein spots that showed significant differences ($p < 0.05$) between treatment groups and control were selected for identification.

[Lukáši, sem prosím vlož obrázky s popisky 1, 2, co jsou na konci tohoto článku]

Protein identification

The chosen hepatic acidic protein spots were cut from the gels, digested using trypsin protease and resulting mixtures of peptides were directly subjected to MALDI-TOF MS. Four of the 6 proteins were identified by Mass Fingerprinting approach (Table 1). These proteins were identified by their sequence similarities to the following proteins: Glucose-regulated protein 78 (Pro. 3), Calreticulin (Pro. 4), Glucose-regulated protein 94 (Pro. 5), Protein disulfide-isomerase A3 precursor (Pro. 7).

Moreover, the MS/MS Ion Search approach has been used in cases Pro. 3, 4 and 7 to assign un-matched m/z base peak signals to corroborate previous identification or to find the another protein masked under the first significant protein identification by MS Fingerprinting method. The positive results, corroborated previous identification, are shown in Table 2A. A further 2 proteins not identified by Mass Fingerprinting were examined by MS/MS Ion search MASCOT tool from selected m/z signals (Table 2B). Pro. 12 and 23 were identified as Hyperosmotic glycine rich protein (Pro. 12) and Canopy-1 precursor (Pro. 23), respectively. Among all the indentified protein spots, Pro. 3, 4 and 5 are present in *Oncorhynchus mykiss*, Pro. 7 is present in *Salmo salar* or *Gallus gallus* and the other two proteins are found in *Salmo salar*.

[Tabulky 1, 2]

Discussion

The exposure of organisms to sublethal levels of environmental pollution has been shown to trigger the cellular accumulation of proteins that mainly act as molecular chaperones (Ellis and Vandervies, 1991; Feder and Hofmann, 1999). Recent proteomic studies in the environmental monitoring field have aimed to decipher changes in protein expression patterns and to identify proteins governing the mechanism of toxicity of environmental xenobiotics. To our knowledge, this is the first study to apply a proteomics approach for identifications of fish liver proteins which are regulated in response to the exposure of Ca^{2+}-antagonist (VRP). Results of the present study suggest that the fish hepatic proteome was significantly altered after the exposition to VRP at environmental related concentrations. Moreover, the proteins that have been significantly affected in VRP-treatment groups (see Table 1 and 2) are involved in various processes such as immunity, redox signaling and ion-transference regulation.

Among stress proteins, the heat shock proteins (HSP), in particular HSP70, have been proposed to be ubiquitous biomarkers of exposure to extrinsic stress (Efremova et al., 2002), since it is regularly altered in response to a wide variety of natural, experimental or anthropogenic stress, such as heat shock, oxidative stress, heavy metals and organic contaminants (Sanders and Martin, 1993; Fulladosa et al., 2002; Delaney and Klesius, 2004). In this study, the abundances of Glucose-regulated protein 78 (GRP78) and Glucose-regulated protein 94 (GRP94) were significantly decreased in the liver of fish that were chronically exposed to higher concentrations of VRP.

The GRP78/HSPA5 and GRP94/HSP90B1, are the chaperone proteins located in the endoplasmic reticulum (ER) and encoded by the *HSP70* and *HSP90B1*gene, respectively. The GRP78, referred as a immunoglobulin heavy chain-binding protein (BiP), is involved in the folding and assembly of proteins in the ER (Hendershot et al., 1994). And GRP94 is essential immune chaperones that play a critical role in regulation of both innate and adaptive immunity (Baker-LePain et al., 2002). Our previous results showed that long-term exposure of VRP could induce oxidative stress in fish liver and other tissues (Li et al., 2011). Therefore, the possible explanation for the depression of these two proteins is that VRP-induced reactive oxygen species (ROS) causes alterations in ER homeostasis and serves as a stress signal activating ER stress genes such as GRP78/BiP and GRP94, which is consistent with other reports (Cohen et al., 1997; Martin et al., 2003; Schroder et al., 2006).

Proteins that traverse the secretory pathway typically depend on disulfide bonds for their maturation and function and these bonds are often crucial for the stability of a final protein structure (Tu and Weissman, 2004). Protein disulfide isomerase (PDI) is an enzyme in the endoplasmic reticulum in eukaryotes that catalyzes the formation and breakage of disulfide bonds. Among this PDI family, protein disulfide isomerase associated 3 (PDIA3/ERP57) can catalyze the oxidation, reduction and isomerization of intra- and intermolecular disulfide bonds to ensure the correct folding of secretory proteins prior to their further modification and transport in the ER (Huang et al., 2009).

In the present study, the inhibition of PDIA3 expression in all VRP-treated groups shows that the oxidative stress is present in fish liver. In particularly, this protein was down-regulated in fish exposed to VRP at environmental related concentrations indicated that hepatic PDIA3 plays a role in an early adaptive response in VRP-induced stress. Moreover, the observed dose-depended alterations of PDIA3 expression suggest that PDIA3 may have a protective function in the cellular stress response to VRP, in addition to its isomerase activities (Kim-Han and O'Malley, 2007; Huang et al., 2009). According to other reports, the PDI and GRP78 are induced by ER stress caused by interference with Ca^{2+} homeostasis, inhibition of disulfide bond formation of protein glycosylation and oxidative stress to rescue the accumulation of misfolded or unfolded proteins with the ER (Hendershot, 2004). These results support our finding that the expression of PDIA3 and GRP78 was significantly induced by VRP treatment, suggesting that ER is the target for oxidative stress.

Calreticulin, located in the ER, is a multifunctional protein, binding Ca^{2+} ions (a second messenger in signal transduction), and participating in transcription regulation. Decreased expression of calreticulin should lead to altered levels of intracellular calcium (Merchant et al., 2009). These changes in intracellular calcium stores would surely affect the expression of many proteins that are dependent on protein kinase C activity (Merchant et al., 2009). Poli (1998) found that the suppression of calreticulin expression is linked to increased expression of C/EBP proteins, which are critical transcription factors for the inflammatory response in organism to the external stress. As VRP, the chemical used in this study, is a calcium channel blocker, it is not surprised to find that the calreticulin expression was significantly inhibited in liver of all VRP-treated fish, especially at the lovest concentration (the environmental related level).

A hyperosmotic glycine rich protein was identified as significantly down-regulated by 42-d exposure to higher concentrations of VRP. Osmoregulation is physiologically controlled by

chemical messages from the endocrine system, along with cell signaling and nerve transmission (Connon et al., 2009). The Ca^{2+}-antagonists have been suggested to induce osmotic imbalances in fish (Zhang and Wang, 2007) which are linked to effects on ATPase activity responsible for maintaining the Sodium transmembrane electrochemical gradient (Pedersen and Bjerregaard, 1995). In this study, the decreased expression of this hyperosmotic glycine rich protein may be directly caused by conditions affecting endocrine regulation in fish after VRP exposure.

Canopy-1 is a novel regulator of fibroblast growth factor (Hirate and Okamoto, 2006). In the present study, this protein was significantly depressed in fish liver under VRP- induced stress. To our knowledge, it is the first evidence that Canopy-1 could be altered in organism by the external toxicant, and the reason is not clear, which need more detailed investigation in future.

In summary, the present study demonstrated that environmentally relevant exposure to Verapamil can significantly modify the liver proteome of rainbow trout. Alterations in protein expression in fish liver can provide some novel information of VRP toxicity, but the exact mechanisms behind the cellular stress responses yet remain to be identified and hence require further investigation. However, the changes observed in this study have to be taken into account while estimating the toxicological hazard of pharmaceutically-derived chemicals in the aquatic environment. Our study also indicates that fish hepatic proteome can be used as a good model for monitoring the residual pharmaceuticals in aquatic environment.

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Table 1. Protein identification by Mass Fingerprinting approach.

Spot no.	Protein Name (organisms)	Accession no.	MW (kDa)/pI	*pI*	Mascot score	Significant hit	Sequence coverage (%)	Matched/searched peptides
3	Glucose-regulated protein 78 (*Oncorhynchus mykiss*)	gi|60223019	70	5.02	234	73<	36	21/29
4	Calreticulin precursor (*Salmo salar*)	gi|209148412	48	4.33	205	73<	49	16/23
	Calreticulin (*Oncorhynchus mykiss*)	gi|185134556	48	4.39	89	73<	33	9/23
5	Glucose-regulated protein 94 (*Oncorhynchus mykiss*)	gi|303324549	91	4.69	206	73<	28	20/21
7	Protein disulfide-isomerase A3 precursor (*Salmo salar*)	gi|209153384	55	5.46	98	73<	23	12/19

Table 2. Protein identification by MS/MS Ion Search approach.

Spot no.	Protein Name (organisms)	Accession no.	MW (kDa)	*pI*	Mascot Score	Significant hit	Sequence	MS/MS m/z
TABLE 2A								
3	Glucose-regulated protein 78 (*Oncorhynchus mykiss*)	gi|60223019	69	5.02	210	41<	K.VMEDSDLKKTDIDEIVLVGGSTR.I	2520.290
4	Calreticulin (*Oncorhynchus mykiss*)	gi|185134556	48	4.39	75	40<	K.KPEDWDDRPK.I	1285.619
					139	52<	A.TVYFKEQFQDGDAWK.S	1861.875
7	Protein disulfide-isomerase A3 precursor (*Gallus gallus*)	gi|45383890	56	5076	117	52<	R.GFPTIYFAPAGKK.Q	1396.721
TABLE 2B								
12	Hyperosmotic glycine rich protein (*Salmo salar*)	gi|185133178	21	8.88	65	36<	R.SYGGGGGGR.S	767.343
					160	33<	K.YDNPEDAKDAMDAMNGQSLDGR.T	2413.011
					142	39<	K.LFVGGLSFDTTEQSLAEAFSK.Y	2247.117
23	Canopy-1 precursor (*Salmo salar*)	gi|209737392	21	4.93	62	44<	K.TIHVGGFR.L	886.503
					243	41<	R.SSDAGDFPDFNNFKFDGPEGSNALK.F	2676.192

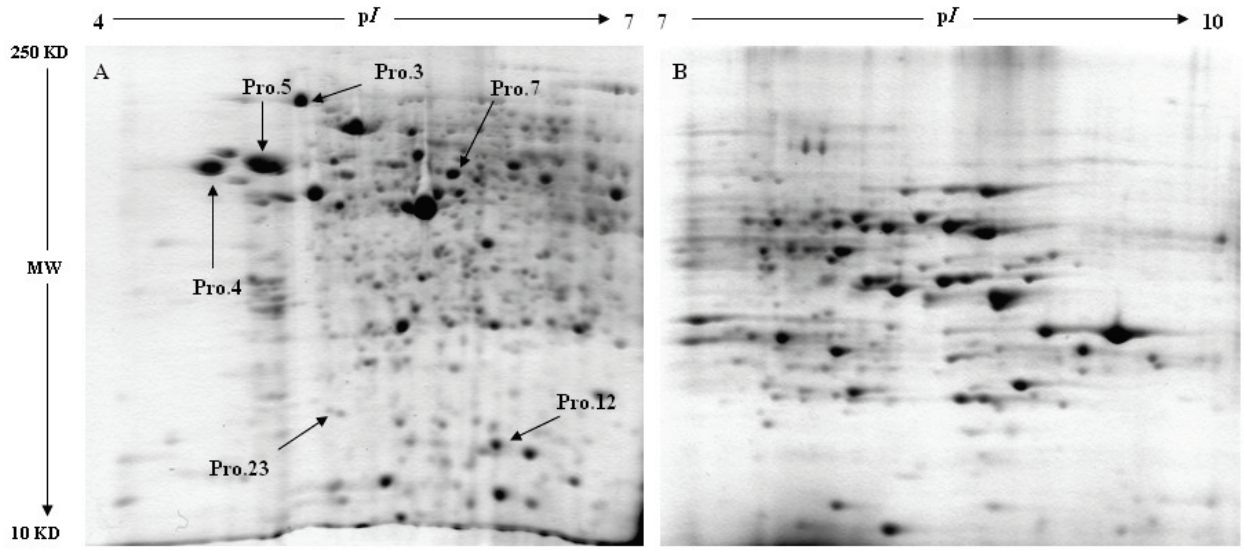

Figure 1. Representative 2-D gels of rainbow trout liver protein. Protein spots marked by arrows were found to be differentially expressed as a result of verapamil (VRP) treatment. The molecular weights (MW) and isoelectric point (p*I*) scales are indicated. The corresponding number is the spot reference number.

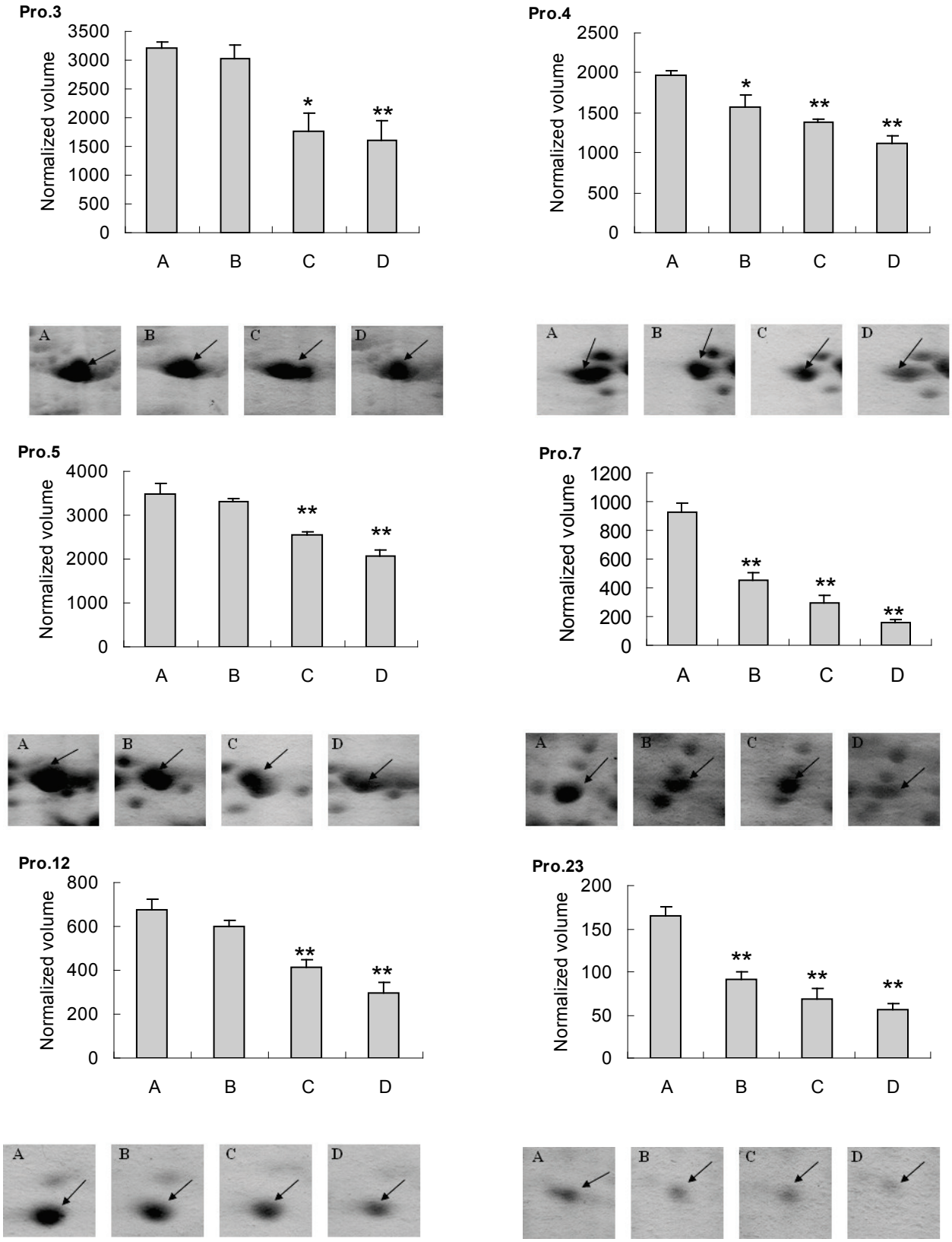

Figure 2 Changes in abundance of hepatic protein spots in fish among test groups. Protein identification is in table 1 and 2. A-control group; B-VRP treatment group at 0.5 μg/L; C-VRP treatment group at 27.0 μg/L; D-VRP treatment group at 270 μg/L. *, Significant difference was $p < 0.01$ with one-way ANOVA; **, Significant differences was $p < 0.001$ with one-way ANOVA.

Chapter 5

Effects of exposure to sublethal propiconazole on intestine-related biochemical responses in rainbow trout, *Oncorhynchus mykiss*

Li, Z.H., Zlabek, V., Grabic, R., Li, P., Machova, J., Velisek, J., Randak, T., 2010. Effects of exposure to sublethal propiconazole on intestine-related biochemical responses in rainbow trout, *Oncorhynchus mykiss*. Chemico-Biological Interactions 185 (3), 241–246.

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Záhlaví liché stránky

Effects of exposure to sublethal propiconazole on intestine-related biochemical responses in rainbow trout, *Oncorhynchus mykiss*

Záhlaví sudé stránky

Chapter 5

Chapter 6

Effect of human pharmaceutical Carbamazepine on the quality parameters and oxidative stress in common carp (*Cyprinus carpio* L.) spermatozoa

Li, Z.H., Li, P, Rodina, M, Randak, T., 2010. Effect of human pharmaceutical Carbamazepine on the quality parameters and oxidative stress in common carp (*Cyprinus carpio* L.) spermatozoa. Chemosphere 80 (5), 530–534.

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Záhlaví liché stránky
Effect of human pharmaceutical Carbamazepine on the quality parameters and oxidative stress in common carp (*Cyprinus carpio* L.) spermatozoa

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Chapter 7

General Discussion

English Summary, Czech Summary,

Acknowledgements

List of Publications

Training and Supervision Plan during study

Curriculum Vitae

General Discussion

In recent years, awareness of residual pharmaceuticals present in the aquatic environment is growing as investigations into these pollutants increase and analytical detection techniques improve (Li and Randak, 2009). Fish exposed to environmental pharmaceuticals exhibit a variety of biochemical responses, including behavioral responses, morphological paramenters, physiological and biochemical indices (Egaas et al., 1999; Hinfray et al., 2006). Laboratory studies on analyzing biological responses in fish exposed to environmental pharmaceuticals can help to understand the influence mechanism and hence the results can be used to explain the impact of residual pharmaceuticals on fish in field.

Acute toxicity test of Verapamil in fish

The 96 h LC50 is one of the most important parameters in acute toxicity testing. The calculated 96 h LC50 of Verapamil (VRP) using a static bioassay test in *O. mykiss* was 2.72 mg/L. Compared with the previous results of our department, this value is lower than 96 h LC50 of other pharmaceuticals on rainbow trout with the same size, such as Carbamazepine (19.9 mg/L) (Li et al., 2011a) and Propiconazole (5.04 mg/L) (Li et al., 2011b), which indicates that VRP has more toxic effects on the fish and is a potent toxicant even at lower concentrations demanding a proper attention be paid to stop it from becoming a potential environmental pollutant.

Behavioral responses of fish exposed to Verapamil

Behavior is considered a promising tool in ecotoxicology and these studies are becoming prominent in toxicity assessments in many various species, including fish (Jensen et al., 1997; Tahedl and Hader, 2001). Since behavior is not a random process, but rather a selective response that is constantly adapting through direct interaction with physical, chemical, social and physiological aspects of the environment, behavioral endpoints serve as valuable tools to discern and evaluate effects of exposure to environmental stressors and fish behavioral alterations can provide important indices for ecosystem assessment (Robinson, 2009). In the present study, the abnormal behavioral responses (loss of balance, hanging vertically in the water, rapid gill movement, erratic swimming, gather at the water surface, and capsize in water) in juvenile rainbow trout was observed in the VRP treated groups. Similar behavioral responses determined in this study have been observed with the guppy, freshwater catfish and sea bass exposed to various concentrations of the synthetic xenobiotica (Saha and Kaviraj, 2003; Viran et al., 2003; Calta and Ural, 2004). Additionally, some studies found that more mature bluegill sunfish were less susceptible than the juveniles (Koprucu et al., 2006), providing further evidence that juvenile fish are more easily affected by toxins. The possible reason for the fish behavioral alterations may attribute to xenobiotica-induced inhibition of acetylcholinesterase activity leading to accumulation of acetylcholine in cholinergic synapses ending up with hyperstimulation (Mushigeri and David, 2005), which lack direct evidences and need further studies.

Mophological indices

Morphological indices, especially CF and HSI, have been proposed as an "exposure index" to environmental contaminants (Li et al., 2010e). CF, which assumes that heavier fish of a given length is in better condition, is able to indicate fish fitness under stress of pollution as metabolic trade-off is required to deal with detoxification and the energy available for growth may thus be reduced (Fang et al., 2009). HSI reflects the relative liver size and is linked to the hepatic enzyme activity for detoxification of compounds (Fang et al., 2009). In the acute study, environmental stress-related effects were indicated by differences in CF after acute exposure of VRP when compared to the control group, although effects were not significant, which indicates a decrease in fish growth and overall condition caused by direct metabolic effect on fish and a depletion of energy resouces (Goede and Barton, 1990). In the chronic study, significant lower CF and HSI were observed in the VRP treated group with the highest concentration, indicating a decrease in fish growth and overall condition caused by direct metabolic effect on fish.

Hematological and biochemical blood plasma parameters

Blood is known to exhibit pathological changes before the onset of any external symptoms of toxicity (Blaxhall and Daisley, 1973). Hematological and plasma biochemical parameters are important for toxicological research, environmental monitoring and as indicators of disease and environmental stress (Talas and Gulhan, 2009; Li et al., 2010b). Many studies have demonstrated changes in blood variables as a result of environmental conditions and presence of contaminants. Fish exposed to environmental pollutants exhibit a variety of physiological responses, including blood balance disturbances, ion regulatory disturbances, oxygen uptake and transport inhibition (Booth et al., 1988). Therefore, hematological analysis and assessment of biochemical parameters of blood plasma are useful in monitoring the physiological status of fish and as indicators of the health of the aquatic environment, although they are not routinely used in fish disease diagnosis (Pimpao et al., 2007). Our results showed that VRP at various concentrations exerted a certain influence on some of the blood indices in fish.

In the chronic study, the main hematological response of rainbow trout to the effect of VRP was a significantly lower PCV, Hb concentration, RBC count and Leuko count after exposure at the highest VRP concentration (E3 group). Decreases in Hb concentration, RBC count and PCV levels may be an indicator of anemia. Changes in differential leukocyte count are recognized as a sensitive indicator of environmental stress (Cole et al., 2001). There were no prominent changes in MCV, MCH and MCHC of fish in all groups, indicating that VRP-induced anemia in fish is characterized as normocytic type (Zhang et al., 2008b). Additional, the hematological parameters changes can be interpreted as a compensatory responses that improves the O_2 carrying capacity to maintain the gas transfer and a change in water blood barrier for gas exchange in gill lamellae, which were also reported in previous results (Sweilum, 2006).

Long-term exposure to higher concentrations of VRP resulted in increases of levels of GLU, NH_3, TP and activities of LDH, ALT and AST, but decrease in LAC. In our study, VRP

showed elevated levels of plasma ammonia concentration, indicating that detoxifying mechanisms were unable to convert the toxic ammonia to less harmful substances. Moreover, significantly higher levels of blood glucose were observed in exposed fish than in controls, indicating metabolic stress (Li et al., 2010b). The increasing plasma protein content indicates physiological adaption to overcome stress situation. Lactate is the end product of glycolysis under hypoxic conditions. In this study, the significant lower level of LAC in plasma of fish exposed to the highest concentration of VRP, indicates a decrease in the glycolytic process due to the lower metabolic rate as a result of the effect of VRP.

Activities of plasma enzymes LDH and the transaminases (ALT and AST) are relevant stress indicators (Ishikawa et al., 2007). A significant increase in activity of the above mentioned enzymes indicates stress-based tissue impairment (Svoboda et al., 2001). LDH is a tetrameric enzyme recognized as a potential marker for assessing the toxicity of a chemical. The elevated levels of LDH in the haemolymph might be due to the release of isozymes from the destroyed tissues (Mishra and Shukla, 2003). Increased activities of transaminases (ALT and AST) indicate amplified transamination processes. An increase in transamination occurs with amino acid input into the TCA cycle to cope with the energy crisis during pesticide stress (Philip et al., 1995).

Oxidative stress and antioxidant responses in fish exposed to Verapamil

Oxidative stress has been defined as an imbalance of oxidants and antioxidants in favour of the oxidants, potentially leading to cell damage (Azzi et al., 2004). It is well known that oxidative stress is caused by the formation of ROS, e.g. hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), and superoxide anion radical (O_2^{-}), mainly as byproducts of oxidative metabolism (Zhang et al., 2003; Bagnyukova et al., 2005). In order to cope with the oxidative damage, organisms have evolved multiple systems of antioxidant defence. Endogenous enzymatic and non-enzymatic antioxidants are essential for the conversion of ROS to harmless substances and for maintenance of cellular metabolism and function (Mates, 2000; Li et al., 2003; Zhang et al., 2008a). The activity of enzymes such as GPx, GR and SOD prevents adverse effects of oxidative stress in cells. Oxidative stress will occur if the activities of the antioxidant defense systems decrease or ROS production increase (Zhang et al., 2004; Li et al., 2009a).

Lipid peroxidation and protein carbonylation are widely used as oxidative stress indicators for aquatic animals, and they have been reported as a major contributor to the loss of cell function under oxidative stress conditions (Storey, 1996). Considering that the typical reaction during ROS-induced damage involves the peroxidation of unsaturated fatty acids, our results clearly showed that both acute and chronic exposure to VRP led to oxidative stress, with higher levels of LPO and CP in all tissues, when compared with the control. The increased LPO and CP production in the present study suggests that ROS-induced oxidative damage can be one of the main toxic effects of VRP.

Oxidative substances in cells may cause an elevation of antioxidant enzymes as a defense mechanism. Due to the inhibitory effects on oxyradical formation, the SOD provides the first defense line against oxygen toxicity and usually used as a biomarker indicating ROS production (Ballesteros et al., 2009). GPx act cooperatively as scavengers of hydrogen

peroxidae and other hydroperoxides (Gate et al., 1999). GR plays an important role in cellular antioxidant protection and adjustment processes of metabolic pathways (Li et al., 2010c). In this study, after exposure to VRP, activities of SOD, GR, and GPx in *O. mykiss* were higher in intestine, muscle, and especially in liver. These results agree with other reports of increased antioxidant enzymes in fish exposed to environmental pollutants (Sturve et al., 2008). But in the chronic test, all antioxidant enzymes activities in muscle and intestine of E3 group showed a significantly inhibited after 42 days exposure, indicating the serious damage caused by accumulation of ROS in these tissues.

The range of antioxidant activity was higher in liver compared to muscular and intestinal tissues. Antioxidant defenses are typically developed preferentially in liver as a result of the central role of this organ in detoxifying xenobiotics and processing metabolic products for degradation (Lushchak et al., 2005). The muscle analyzed in the present study was white muscle, which provides burst power output for short-term intensive swimming (Hochachka et al., 1996). This type of muscle dominates fish musculature, whereas the lateral red muscle represents a very small fraction of the total. Both white muscle and intestine have a low content of mitochondria and low intensity of oxidative metabolism; hence, it is not surprising that the activities of all antioxidant enzymes in fish white muscle and intestine were much lower than in liver.

However, we also found that all antioxidant enzymes activities in fish brain were strongly inhibited when exposed to a higher VRP concentration, which could be due to the flux of superoxide radicals, resulting in H_2O_2 increase in the cell (Zhang et al., 2008a; Li et al., 2010d). In the gill, the activities of all antioxidant enzymes decreased at higher concentrations of VRP, leading to accumulation of oxidative substances, suggesting inadequate compensation for the presence of environmental pollution (Cazenave et al., 2006). The observation, together with some previous results (Li et al., 2009b), further demonstrated that gill, the first organ which contact with environmental pollutants, becomes the prime target to toxic chemicals because of not only its large surface area facilitates greater toxicant interaction but also its weak detoxification system (Pandey et al., 2008).

RNA/DNA ratio in fish exposed to Verapamil

In fish, energy metabolism is related to various environmental stress factors including toxicant exposure. Toxic pollutants that interfere with energy-yielding reactions indirectly inhibit the synthesis of RNA, DNA and protein (Kim and Kang, 2004). Therefore, RNA to DNA ratio provides a measure of synthetic capacity of cell and it has been extensively used to estimate growth and energy metabolic conditions (James and Sampath, 1999; Varo et al., 2007).

In the present study, a significant reduction in the RNA/DNA ratio was found in the VRP treatment group with the higher concentration. These results are in agreement with the previous studies carried out in fish that found a depressed RNA/DNA ratio after exposure to metal and organic contaminants (Varo et al., 2007).But the use of RNA/DNA ratio as a biomarker of environmental pollutants exposure in fish has not been confirmed. Some other studies have also suggested the application of RAN/DNA ratio to evaluate the effects of toxicants on fish is questionable, because they did not found any relation between the

RNA/DNA ratio and the changes of environmental contaminants (Mckee et al., 1989; DeBoeck et al., 1997). Therefore, more studies should be carried out to investigate the variation of this index in fish before it is applied in the field study.

Proteomics study in fish exposed to Verapamil

Recent proteomic studies in the environmental monitoring field have aimed to decipher changes in protein expression patterns and to identify proteins governing the mechanism of toxicity of environmental xenobiotics. Results of the present study suggest that the fish hepatic proteome was significantly altered after the exposition to VRP at environmental related concentrations. Moreover, the proteins that have been significantly affected in VRP-treatment groups are involved in various processes such as immunity, redox signaling and ion-transference regulation.

Among stress proteins, the heat shock proteins (HSP), in particular HSP70, have been proposed to be ubiquitous biomarkers of exposure to extrinsic stress (Efremova et al., 2002). In this study, the abundances of Glucose-regulated protein 78 (GRP78) and Glucose-regulated protein 94 (GRP94) were significantly decreased in the liver of fish that were chronically exposed to higher concentrations of VRP. Protein disulfide isomerase (PDI) is an enzyme in the endoplasmic reticulum in eukaryotes that catalyzes the formation and breakage of disulfide bonds. Among this PDI family, protein disulfide isomerase associated 3 (PDIA3/ERP57) can catalyze the oxidation, reduction and isomerization of intra- and intermolecular disulfide bonds to ensure the correct folding of secretory proteins prior to their further modification and transport in the endoplasmic reticulum **(**ER) (Huang et al., 2009). In the present study, the inhibition of PDIA3 expression in all VRP-treated groups shows that the oxidative stress is present in fish liver. Calreticulin, located in the ER, is a multifunctional protein, binding Ca^{2+} ions (a second messenger in signal transduction), and participating in transcription regulation. Decreased expression of calreticulin should lead to altered levels of intracellular calcium (Merchant et al., 2009). As VRP, the chemical used in this study, is a calcium channel blocker, it is not surprised to find that the calreticulin expression was significantly inhibited in liver of all VRP-treated fish, especially at the lovest concentration (the environmental related level). A hyperosmotic glycine rich protein was identified as significantly down-regulated by 42-d exposure to higher concentrations of VRP. Osmoregulation is physiologically controlled by chemical messages from the endocrine system, along with cell signaling and nerve transmission (Connon et al., 2009). In this study, the decreased expression of this hyperosmotic glycine rich protein may be directly caused by conditions affecting endocrine regulation in fish after VRP exposure. Canopy-1 is a novel regulator of fibroblast growth factor (Hirate and Okamoto, 2006). In the present study, this protein was significantly depressed in fish liver under VRP- induced stress. To our knowledge, it is the first evidence that Canopy-1 could be altered in organism by the external toxicant, and the reason is not clear, which need more detailed investigation in future.

Digestive enzymes in fish exposed to Propiconazole

Digestive enzyme patterns can reflect the feeding rate and digestive capacity of fish; hence, digestive enzyme activity can be used as bio-indicators of growth and health status of fish (Suarez et al., 1995; Debnath et al., 2007). In the present study, the activities of proteolytic enzymes and amylase in the intestine were significantly inhibited with higher levels of Propiconazole (PCZ) exposure. This indicated that long-term exposure to PCZ could have negative effects on digestive enzyme activity in intestine. This is in agreement with Quistad and Casida (2000), who found three digestive enzymes (α-chymotrypsin, elastase and trypsin) to be highly sensitive to organophosphorus pesticides. In addition, studies have found that the activity of various digestive enzymes is inhibited in fish exposed to heavy metals (Sastry et al., 1979; Gupta and Sastry, 1981). However, activity of maltase and lactase remained unchanged in intestine of *Heteropneustes fossilis* exposed to lead nitrate (Sastry and Gupta, 1979a), and activity of amylase, trypsin, and pepsin were induced in intestine of *Channa punctaus* after 20 days exposure to mercuric chloride (Sastry and Gupta, 1979b). Therefore, the influence of pollutants on digestive enzymes in fish is unclear, and requires further study.

Quality parameters of fish sperm exposed to Cabarmazepine

Due to the conservative nature in molecular and biochemical processes, the rationale is probably most likely to be true when environmental effects of human pharmaceuticals on vertebrates are under investigation (Fent et al., 2006). Toxicity screening *in vitro* using animal spermatozoa has developed rapidly as a simple and valid model during the past decades since its use does not require expensive sterile cell culture conditions (Rurangwa et al., 2002). Although aquatic pollution assessment traditionally relied on the use of fish for toxicity tests (including acute and chronic tests) (Li et al., 2009b), the need for sensitive, short-term tests has led to the increasing use of gametes in aquatic toxicology. Results of the present study suggest that fish spermatozoa quality and antioxidant defense system can be impaired by Cabarmazepine (CBZ)-mediated oxidative stress.

Reproductive success is one of the key factors in determining species survival. Environmental pollution could impair reproductive success of adult organisms through decreasing the quality of gametes, which in turn may affect fertilization success, hatching of embryos and subsequent survival of offspring (Au et al., 2001; Li et al., 2010a). Because the male germ cell of the most kinds of fish are discharged into water and directly exposed to pollutants prior to fertilization, it is significant to assess the adverse effects of environmental heavy metals on the male reproductive system of fish (Gage et al., 2004). In the present study, fish spermatozoa motility and velocity were significantly reduced after *in vitro* exposure to higher concentration of CBZ, which may be due to increased oxidative damage and/or direct cytotoxicty. The possible reason of decreasing of spermatozoa motility affected by CBZ-induced oxidative stress would be a rapid loss of intracellular ATP and an increase in mid-piece morphology defects (Rao and Gangadharan, 2008).

The spermatozoa viability was determined by analyzing the integrity ratio of spermatozoa plasma membrane in the current study. Loss of integrity can also lead to an increase in membrane permeability and a loss in the capacity to regulate the intracellular concentrations of ions involved in the control of spermatozoa movement (Baumber et al., 2000). Surprisingly, an unchanged spermatozoa viability was found in the current study, associated

with significant decreasing of motility and velocity of fish spermatozoa exposed to CBZ. It is known that an increase in the intracellular Ca^{2+} is a prerequisite for the initiation of spermatozoa motility and cAMP has long been implicated as an activator or intracellular messenger of spermatozoa motility through its stimulating effect on the motile apparatus (Rao and Gangadharan, 2008). Thus, there is a possibility that although fish spermatozoa membrane remaining integrity under CBZ-induced stress, the alien chemical might inhibit intracellular ion concentration and cAMP content, leading to impaired motility of spermatozoa in the present study. Thus, measuring Ca^{2+} and cAMP levels should be given more attention in the future, which can give more information to know the exact mechanism of CBZ-induced alteration in fish spermatozoa quality.

Conclusion

In conclusion, sublethal concentrations of pharmaceuticals could lead to multiple biomarkers changes, including morphological indices, hematological parameters, antioxidant responses in fish different tissues and so on. Based on the results, the trout fish, *O. mykiss*, has enough tolerances to pharmaceuticals-induced changes in surrounding condition, but long-term exposure to pharmaceuticals at higher concentrations had a distinct effect on fish. Moreover, alterations in protein expression in fish liver can provide some novel information of pharmaceuticals-induced toxicity, but the exact mechanisms behind the cellular stress responses yet remain to be identified and hence require further investigation. However, the changes observed in this study have to be taken into account while estimating the toxicological hazard of pharmaceutically-derived chemicals in the aquatic environment. It is evident that, from an ecophysiological point of view, the release of pharmaceuticals into the aquatic environment is a matter for concern. The parameters analyzed including various physiological and biochemical parameters, especially proteomics study and fish sperm *in vitro* study, could provide useful information for understanding the impacts of residual pharmaceuticals on fish. But the application of these findings will need more detailed laboratory study before they can be established as special indicators for monitoring residual pharmaceuticals in aquatic environment.

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English Summary

Effects of residual pharmaceuticals present in aquatic environment on fish

Zhi-Hua Li

Residual pharmaceuticals widely distributed and persistent in aquatic environment has the potential toxicity to fish and other aquatic organisms. However, the ecotoxicological effects and influence mechanisms that lead to these toxic effects have not been fully documented. The aim of present thesis was to investigate the effects of several pharmaceuticals (Verapamil, Propiconazole and Carbamazepine) present in aquatic environment on fish, through acute and chronic test, as well as using proteomics approach and analyzing fish sperm *in vitro* study.

Chapter 2: In this study, the toxic effects of Verapamil (VRP) were studied in juvenile rainbow trout, *Oncorhynchus mykiss*, by acute static bioassay. In the acute test, the median lethal concentration (LC50, 2.72 mg/L) was evaluated and the behavioral changes were obviously intensified with increasing VRP concentrations. Compared to the control, oxidative stress was observed in fish tissues with different levels after short-term exposure to sublethal concentrations (0.27 and 1.35 mg/L) of VRP. Activities of SOD and GPx in fish brain were induced at 0.27 mg/L VRP, but all the antioxidant enzymes (SOD, GPx and GR) in fish brain were decreased at 1.35 mg/L VRP. When compared to the control, all the antioxidant enzymes in gill were decreased in both treated groups, but there was no significant change in muscle. Additional, muscle DNA/RNA ratio in fish exposed at 1.35 mg/L VRP was significantly lower than that in the control. In short, the physiological and biochemical responses in of fish indicated that VRP-induced environmental stress.

Chapter 3: In this study, the toxic effects of Verapamil (VRP) were studied in juvenile rainbow trout, *Oncorhynchus mykiss*, by chronic semi-static bioassay. Fish were exposed at sublethal concentrations of VRP (0.5, 27 and 270 μg/L) for 0, 21 and 42 d. Multiple biomarkers were measured, including morphological indices, hematological parameters and antioxidant responses in fish different tissues (brain, gill, liver, muscle and intestine). Based on the results, there was no significant change in all parameters measured in fish exposed to VRP at environmental related concentration, but the effects of VRP-induced stress in fish chronically exposed to higher concentrations were reflected in the significant changes of physiological and biochemical responses. In short, the multiple responses in of fish indicated that VRP-induced physiological stress and could be used as potential biomarkers for monitoring residual VRP in aquatic environment; but molecular and genetic mechanisms of these physiological responses in fish are not clear, which need to be further studied.

Chapter 4: In the present study, proteomic analysis has been performed to investigate the protein patterns that are differentially expressed in liver of rainbow trout exposed to sublethal concentrations of VRP (0.5, 27.0 and 270 μg/L) for 42 d. Two-dimensional electrophoresis coupled with MALDI-TOF/TOF mass spectrometry was employed to detect and identify the protein profiles. The analysis revealed that the expression of 6 hepatic acidic proteins showed

markedly altered in the treatment groups compared to the control group, especially 3 proteins were significantly decreased in liver of fish exposed to VRP at environmental related concentration (0.5 μg/L). The results suggest that VRP induce mechanisms against oxidative stress (Glucose-regulated protein 78 and 94, Protein disulfide-isomerase A3) and adaptive changes in ion-transference regulation (Calreticulin, Hyperosmotic glycine rich protein). Furthermore, for the first time, protein Canopy-1 was found to be significantly down-regulated in fish by chronic exposure to VRP at environmental related levels. Overall, our work supports that fish hepatic proteomics analysis serves as an *in vivo* model for monitoring the residual pharmaceuticals in aquatic environment and provides insight into the molecular events in VRP-induced toxicity in fish and other organisms.

Chapter 5: The effect of long-term (30 days) exposure to Propiconazole (PCZ) (0.2, 50, and 500 μg/L) on intestine-related biochemical markers in rainbow trout was investigated. Multiple biomarkers were measured, including digestive enzymes (proteolytic enzymes and amylase), antioxidant responses (TBARS, CP, SOD, CAT, GR and GPx) and energy metabolic parameters (RNA/DNA ratio, Na^+-K^+-ATPase). Exposure to 500 μg/L PCZ led to significantly inhibited ($p < 0.01$) proteolytic enzyme and amylase activity. Activities of the antioxidant enzymes SOD, CAT, and GPx gradually increased at lower PCZ concentrations (0.2 and 50 μg/L). At the highest concentration (500 μg/L), oxidative stress was apparent as significant higher ($p < 0.05$) lipid peroxidation and protein carbonyls, associated with an inhibition of antioxidant enzymes activity. Moreover, energy metabolic parameters (RNA/DNA ratio, Na^+-K^+-ATPase) were significantly inhibited ($p < 0.01$) in the intestines of fish exposed to 500 μg/L PCZ, compared with controls. We suggest that long-term exposure to PCZ could result in several responses in intestine-related biochemical markers, which potentially could be used as indicators for monitoring residual PCZ present in the aquatic environment.

Chapter 6: The effects of Cabamazepine (CBZ) on the quality parameters and oxidative stress of common carp spermatozoa were investigated *in vitro*. Fish spermatozoa were incubated with different concentration of CBZ (0.2, 2.0 and 20 mg/L) for 2 h. Results revealed that the percentage of spermatozoa motile and velocity were decreased significantly at higher concentration of CBZ (2.0 and 20 mg/L) and a dose-dependent reduction was observed. But the viability of fish spermatozoa was not affected significantly in all CBZ treatment groups. After 2 h exposure of CBZ at higher test concentration (2.0 or 20 mg/L), oxidative stress was apparent as reflected by the significant higher LPO and CP levels in fish spermatozoa, as well as the significant inhibition of antioxidant enzymes activities including SOD, GR and GPx. In short, CBZ can induce ROS stress in fish spermatozoa, which could impair the sperm quality and antioxidant defense system. Our results suggested that the use of fish spermatozoa *in vitro* assays may provide a novel and efficiently means for monitoring residual pharmaceutical in aquatic environment.

Czech Summary

Vliv reziduí farmak přítomných ve vodním prostředí na ryby

Zhi-Hua Li

Rezidua farmak, která jsou široce přítomna ve vodním prostředí, přičemž řada z nich má perzistentní charakter, představují potenciální toxikologické riziko pro ryby a ostatní vodní organismy. Nicméně, v současné době nejsou toxikologické mechanismy účinku těchto sloučenin na organismy dostatečně prozkoumány. Cílem této předložené práce bylo zjišťovat vliv vybraných farmak (Verapamil, Propiconazol and Carbamazepin) přítomných ve vodním prostředí na ryby, a to prostřednictvím akutních a chronických testů toxicity využitím proteomických technik a analýzou rybího spermatu v testech *in vitro*.

Kapitola 2: V rámci této studie byl zjišťován toxický vliv Verapamilu (VRP) na juvenilní jedince pstruha duhového, *Oncorhynchus mykiss*, a to pomocí akutních testů toxicity. V rámci akutního testu byla zjištěna letální koncentrace VRP (LC50, 2,72 mg/L) a byly pozorovány změny v chování exponovaných ryb, které byly nejvýznamnější v nejvyšších testovaných koncentracích. V porovnání s kontrolou byly v rybích tkáních pozorovány rozdílné úrovně oxidativního stresu po krátkodobé expozici subletálním koncentracím (0,27 and 1,35 mg/L) VRP. Parametry SOD a GPx v rybím mozku byly indukovány v koncentraci 0,27 mg/L VRP, všechny antioxidační enzymy (SOD, GPx a GR) v tomto orgánu však byly v koncentraci 1,35 mg/L VRP inhibovány. Všechny antioxidační enzymy v žábrách byly v porovnání s kontrolou inhibovány v obou testovaných koncentracích, v případě svaloviny však nebyly signifikantní změny oproti kontrole zjištěny. Poměr DNA/RNA ve svalovině ryb exponovaných 1,35 mg/L VRP byl signifikantně nižší než u kontrolní skupiny. Obecně lze říci, že expozice ryb VRP vyvolává environmentální stres.

Kapitola 3: V této studii byl prostřednictvím chronického semistatického testu sledován chronický vliv verapamilu (VRP) na juvenilního pstruha duhového. Ryby byly vystaveny subletálním koncentracím VRP (0,5; 27 a 270 ug/l) po dobu 0, 21 a 42 dnů. Byla sledována široká paleta biomarkerů zahrnujících morfologické ukazatele, hematologické parametry a antioxidační ukazatele v různých tkáních (mozek, žábry, játra, svalovina a střevo). Na základě získaných výsledků lze říci, že u žádného sledovaného parametru nebyly u ryb exponovaných environmentálním koncentracím VRP pozorovány statisticky významné změny oproti kontrole. V případě vyšších koncentrací však již byl pozorován vliv na sledované ukazatele. Prostřednictvím statistických analýz byl prokázán negativní vliv vyšších koncentrací VRP na sledované ukazatele. Pomocí dvoufaktorové analýzy rozptylu bylo zjištěno, že jednotlivé sledované ukazatele vykazovaly odlišnou odezvu na koncentrace VRP a dobu expozice. Jelikož byl zjištěn vliv expozice VRP na sledované ukazatele, je možno tyto ukazatele používat jako biomarkery při hodnocení vlivu residuí VRP na vodní organismy. Nicméně molekulární a genetické mechanismy nejsou zcela známy a je zapotřebí je dále studovat.

Kapitola 4: V rámci této studie byly realizovány proteomické analýzy za účelem posouzení proteinových vazeb v játrech pstruhů duhových, které jsou různě ovlivňovány v důsledku expozice ryb subletálním koncentracím VRP (0,5; 27,0 and 270 μg/L) po dobu 42 dnů. Pro detekci a identifikaci proteinových profilů byla použita dvoudimensionální elektroforéza spojená s MALDI-TOF/TOF hmotnostním spektrometrem. Analýzy prokázaly, že exprese 6 jaterních acidických proteinů byla silně odlišná v exponovaných skupinách v porovnání s kontrolou, a navíc 3 proteiny byly signifikantně sníženy v játrech ryb exponovaných environmentální koncentraci VRP (0,5 μg/L). Výsledky ukazují, že VRP indukuje mechanismy působící proti vzniku oxidativního stresu (glukosu-regulující protein 78 a 94, protein disulfid-isomerasa A3) a adaptivní změny v regulaci transportu iontů (calreticulin, hyperosmotický glycinový protein). Dále bylo poprvé zjištěno, že protein Canopy-1 byl signifikantně inhibován u ryb vystavených chronické expozici VRP na úrovni environmentálních koncentrací. Práce ověřila, že jaterní proteomické analýzy použité při testech *in vivo* jsou použitelné v rámci monitoringu zatížení vodního prostředí rezidui farmak a umožňují zaměřit se na molekulární úroveň studia toxicity VRP v rybách i v dalších organismech.

Kapitola 5: V rámci studie byl testován vliv dlouhodobé (30denní) expozice Propiconazolu (PCZ) (0,2; 50 a 500 μg/L) na střevní biomarkery u pstruha duhového. Byla sledována řada biomarkerů zahrnující trávicí enzymy (proteolytické enzymy, amylázy), antioxidační ukazatele (TBARS, CP, SOD, CAT, GR a GPx) a metabolické parametry (RNA/DNA, Na^+-K^+-ATPase). Expozice 500 μg/L PCZ vedla k signifikantní inhibici ($p < 0,01$) proteolytických enzymů a amylázové aktivity. Aktivity antioxidačních enzmymů SOD, CAT, a GPx významně vzrostla v nižších koncentracích PCZ (0,2 a 50 μg/L). V nejvyšší koncentraci (500 μg/L) bylo patrné zvýšení úrovně oxidativního stresu projevující se signifikantně zvýšenou ($p < 0,05$) lipid peroxidázou a proteinovými carbonyly současně s inhibicí antioxidačních enzymů. Dále byly v porovnání s kontrolou signifikantně ($p < 0,01$) inhibovány metabolické parametry (RNA/DNA ratio, Na^+-K^+-ATPase) ve střevech ryb exponovaných 500 μg/L PCZ. Bylo zjištěno, že dlouhodobá expozice PCZ může vést k řadě změn biomarkerů zjišťovaných ve střevech ryb. Tyto markery mohou být používány jako indikátory kontaminace vodního prostředí PCZ.

Kapitola 6: Vliv Carbamazepinu (CBZ) (humánní farmakum běžně přítomné ve vodním prostředí) na kvalitatativní parametry a oxidativní stres spermií kapra obecného byl sledován pomocí *in vitro* testu. Rybí spermie byly inkubovány v rozdílných koncentracích CBZ (0,2; 2,0 and 20 mg/L) po dobu 2 hodin. Bylo zjištěno, že procento pohybujících se spermií statisticky významně klesalo v nejvyšších koncentracích CBZ (2,0 a 20 mg/L) a bylo závislé na velikosti dávky. Životaschopnost spermií však významně ovlivněna nebyla v žádné koncentraci. Po dvouhodinové expozici vyšším testovaným koncentracím (2,0 nebo 20 mg/L) byl zřejmý vliv na parametry indikující oxidativní stres – byly signifikantně zvýšeny hodnoty LPO a CP v rybích spermiích a také byla zjištěna signifikantní inhibice enzymové aktivity SOD, GR a GPx. V krátkosti je tedy možno říci, že CBZ může aktivovat ROS stres v rybích spermiích, což může mít vliv na jejich kvalitu a antioxidační systém. Naše výsledky potvrzují myšlenku využívat rybí spermie při studiu výskytu farmak ve vodním prostředí a hodnocení jejich vlivu na exponované organismy.

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Li Z.H., Li P., Randak T., 2011. Protective roles of calcium channel blocker against cadmium-induced physiological stress in freshwater teleost *Oncorhynchus mykiss*. Water, Air & Soil Pollution DOI: 10.1007/s11270-011-0754-4.

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