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Physiological and molecular responses of aquatic organisms to chemicals exposure

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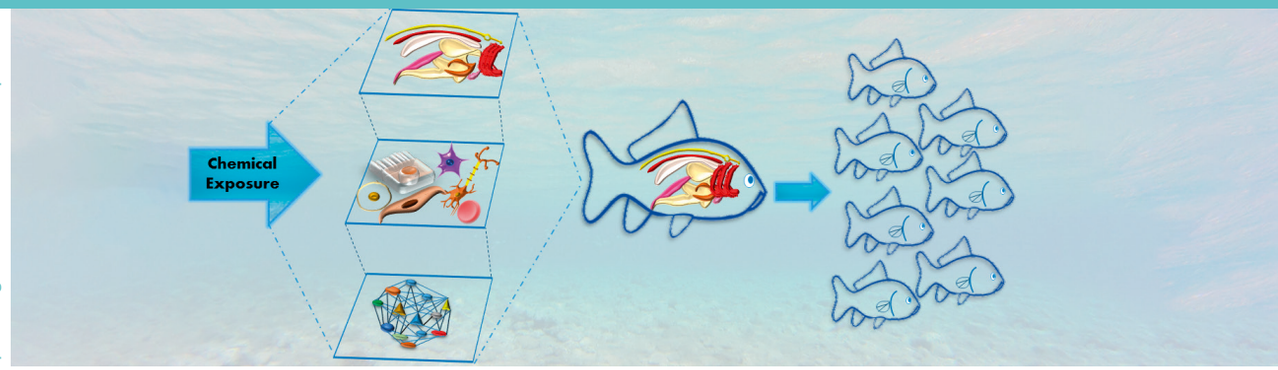

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CONTENT

List of Abbreviations

AAS	Atomic absorption spectroscopy
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CAT	Catalase
CCT	Chaperonin containing TCP-1
cf	Fold change
CHOL	Cholesterol
CK	Creatine kinase
CRM	Certified reference materials
eIF	Eukaryotic translation initiation
FRD	False discovery rate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLU	Glucose
GO	Gene ontology
GR	Glutathione reductase
GRAS	Generally recognized as safe
GST	Glutathione transferase
H&E	Haematoxylin and eosin
Hb	Haemoglobin
IPA	Ingenuity pathways analysis
LC	Liquid chromatography
LDH	Lactate dehydrogenase
LXR	Liver X receptors
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MDA	Malondialdehyde
MF	Molecular function
MHC	Major histocompatibility complex
MS	Mass spectrometry
NBT	Nitro-blue tetrazolium
NPs	Nanoparticles
PAA	Peracetic acid
PCV	Haematocrit
PMS	Phenazine methosulfate
PSMs	Peptide spectrum matches
RBC	Erythrocyte count
ROS	Reactive oxygen species
RXR	Retinoid X receptors
SDH	Succinate dehydrogenase
SOD	Superoxide dismutase

STRAP	Rapid annotation of proteins
SUCLG2	Succinate-coa ligase, subunit beta
TAG	Triacylglycerol
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TEM	Transmission electron microscopy
TP	Total proteins
TRAP1	Tumor necrosis factor receptor-associated protein 1
WBC	White blood cell
ZnO	Zinc oxide

CHAPTER 1

GENERAL INTRODUCTION

Toxicology

Toxicology is the science dealing with adverse effects that occur in living organisms due to exposure of chemicals, either environmental agents or chemical compounds found in our habitats or pharmaceutical compounds synthesized for medical purpose. It focuses on observing and quantifying harmful or undesirable effects of chemicals on biological systems as well as attempting to provide information associated with the degree of chemical safeness (Loomis and Hayes, 1996). Therefore, toxicology exploits various branches of science in a field commonly known as risk assessment, where the potential adverse effects of different kinds of chemical exposure are considered relative to human and environmental health (Eaton and Klaassen, 1996). Modern toxicology is a multidisciplinary and highly evolved field which develops its own conceptual framework by extracting many of principals and techniques from other related sciences, such as physiology, biochemistry, pathology, pharmacology, chemistry and medicine (Burcham, 2014). Because of its diverse nature, toxicology has evolved into a number of specialized areas as a result of the different applications of toxicological information. The professional activities of toxicologists could be classified in three major categories: descriptive, research/mechanistic and applied toxicology (Williams et al., 2000).

Descriptive toxicology focuses on toxicity testing of chemicals and drugs, usually on animals and then extends to human conditions. It provides basic toxicity data that identifies target organs which tested agent induced hazard or undesired effects as well as dose-response information upon exposure to a chemical. The results are typically applied to approval of product use, regulating allowable concentrations in the environment (James et al., 2000).

Research/mechanistic toxicology conducts in-depth studies of how the chemicals or drugs initiate their biochemical or physiologic effect in cells or tissues which subsequently results in toxicity or adverse effects in the living organisms. Mechanistic experiments are carried out at different biological levels, including molecular, biochemical, cellular, and tissue levels to exposed organisms (Loomis and Hayes, 1996). The results of mechanistic studies are very important in risk assessment, where adverse effects observed in laboratory conditions may be relevant to humans. In addition, the results can be beneficial to design and manufacture safer chemicals and in design of new therapeutic agents (Eaton and Klaassen, 1996).

Regulatory toxicity establishes risk assessment and exposure guidelines based on results obtained from descriptive and mechanistic studies and decides whether a drug or chemical has a sufficiently low risk to be marketed (Benford et al., 2000).

Biomarkers in toxicology

Organisms often respond to intentional or unintentional chemical exposure in some measurable and predictable way across different levels of biological organization. In any field of toxicology, it is crucial to be able to measure the exposure of a toxic chemical, the extent of response to that agent, as well as to predict the expected response. Biomarkers are the tools which provide the possibility of these measurements (Timbrell, 1998).

A biomarker generally refers to a measurable indicator or signal in biological systems at molecular, biochemical, cellular, physiological, pathological, or individual levels in response to chemical or toxin exposure (Gupta, 2014). However, the term “bioindicator” refers to individual level and describes the presence or absent of a species as a metric of environmental conditions (Gardner and Oberdorster, 2016). Response of organism to chemical exposure triggers at molecular levels. If the effect of a chemical is great enough to deviate chemical-responsive signals from the normal condition,, then its effects will be manifested at higher

hierarchical levels of biological systems e. g., cells, tissue, organ, individual and ultimately at population (Tryphonas et al., 2005). A broad variety of biomarkers have been used for several years in ecotoxicology in order to evaluate environmental risks as well as in Phase-1 clinical trials to assess safety of new drugs, determine a safe dosage range, and identify side effects. Biomarkers can be subdivided into biomarker of exposure, susceptibility, and effect.

Biomarkers of exposure

A biomarker of exposure gives an assessment at the level of chemical, its metabolites, and products of an interaction between chemical and target molecule or cell in exposed organism (Martın-Dıaz et al., 2004). Biomarkers of exposure can be categorised into two markers of internal dose and markers of effective dose. Biomarkers of internal dose demonstrate that exposure to a particular chemical has occurred by measuring the chemical or its metabolite(s) in body fluids or tissues (Timbrell, 1998). Biomarkers of effective dose which measured in target or surrogate tissues and generally quantify exposure to a particular chemical which has reached the target tissue and have to toxicologically significant effects (Hu and Hou, 2015).

Biomarker of susceptibility

Biomarker of susceptibility indicates an inherent or acquired ability of an individual to respond to a challenge of exposure of a specific substance (Nordberg, 2010). These markers are used to explain individual variation associated with biomarkers of exposure and effects. The difference in individual response to a specific chemical may arise from genetic and non-genetic elements. Genetically-based factors include polymorphism (genetic variants within the population) which may influence expression and activation of genes related to detoxification enzyme, DNA repair processes, target toxicological molecules, biologically effective dose, and pathogenic state and disease in exposed individuals (Luttrell et al., 2008). Non-genetic factors may affect an individual's susceptibility to chemical exposure and could be related to nutritional status, health status, life stage, sex, and exposure to other chemicals (Tarcher, 2013).

Biomarker of response or effect

Biomarker of effect measures the effect of chemical on physiological process in an organism which indicates potential adverse effect on health status (Lowry, 1995). Many biomarkers have been used in toxicological research in order to assess the effects of chemical exposure in living organisms. They range from simple markers such as monitoring of body and organ weights to more complex, such as enzyme activities, histology, and molecular biomarkers. Below we consider some examples of widely used biomarkers in toxicological research.

Haematological indices and blood chemistry

Haematological and biochemical parameters provide valuable information about physiological reactions occurring in response to chemical exposure and changes in environmental conditions to predict health status. Haematological and plasma biochemical parameters are considered as effective and sensitive biomarkers of response to chemical exposure in toxicological research as well as indicators of disease (Li et al., 2011). They provide important knowledge on metabolic disorders, deficiencies and chronic stress status (Talas and Gulhan, 2009). A survey of literature shows that chemical exposure can be reflected

in altering haematological parameters such as various blood cell counts, haematocrit, red blood indices (mean corpuscular volume; mean corpuscular haemoglobin; mean corpuscular haemoglobin concentration) as well as in blood chemistry indices including enzymes, total protein level, nutrients, metabolites, waste products, and inorganic ions (Christensen et al., 1972; Folmar, 1993; Jung et al., 2003; Pereira et al., 2013; Singh and Reddy, 1990; Williams and Wootten, 1981).

Oxidative stress biomarkers

Reactive oxygen species (ROS) are continuously generated in living cells. Aerobic organisms have developed through evolutionary processes antioxidant defence, including adequate enzymatic and non-enzymatic antioxidant to protect cells from damage induced by ROS (Benzie, 2000). Exposure to chemicals can produce ROS which may result in an imbalance between endogenous and exogenous ROS and subsequently a decrease in antioxidant capacity of cells (Valavanidis et al., 2006). Both non-enzymatic antioxidant levels (vitamin E, vitamin C, glutathione, etc.) and enzymatic antioxidant activities (superoxide dismutase, catalase, and glutathione peroxidase) and ROS-induced modifications in macromolecules (nucleic acids, proteins and lipids) are general oxidative stress biomarkers (Lushchak, 2011). Oxidative stress has become an interesting research area in toxicology. They are one of the most investigated biomarkers for assessment of the potential undesired or adverse effects of chemical substances on antioxidant defence in aquatic organisms (Bainy et al., 1996; Ferreira et al., 2005; Monteiro et al., 2006; Regoli, 2000).

Histopathological biomarkers

Histopathological biomarkers represent intermediate biomarkers according to the level of biological organization. They provide a powerful tool to manifest the biological endpoints of chemicals exposure, especially a chronic one, in various target tissues and organs (Bernet et al., 1999). They are closely associated with other biomarkers of effect of chemicals. Because many chemicals are subjected to metabolic activation and affect specific enzymes, they result in changes in metabolism and homeostasis disturbance. If the cells are unable to restore homeostasis, it can further lead to cellular intoxication and death. This response at cellular level can be characterised as necrosis which is considered as histopathological biomarker at the tissue level (Velkova-Jordanoska and Kostoski, 2005). Therefore, histopathological changes have been widely applied as biomarkers for assessment of the health status in organisms exposed to contaminants, both in the laboratory and field studies. A number of lesions induced after chemical exposure, have been reported in different organs such as gill, kidney, liver, skin, and intestine (Camargo and Martinez, 2007; Costa et al., 2009; DiMichele and Taylor, 1978; Mondon et al., 2001; Rabitto et al., 2005; Yildirim et al., 2006).

Toxicoproteomics- the next step in the developing of toxicological biomarkers

Traditional toxicity tests may provide some biological endpoints due to chemical exposure but they yield little insight about the physiological consequences of exposure, particularly when attempting to estimate potential adverse effects of exposure to low concentrations. To predict the subtle effects of chemical exposure, it is necessary to shift beyond the existing paradigm of traditional biomarkers. Recent technological advances in the areas of "OMICS" disciplines (genomics, proteomics, and metabolomics) are continually brought to bear on toxicology field to develop novel biomarkers. Particularly, markers that reflect both chemical

exposure and pathways of toxicity (i.e. biological effects), and hence providing a kind of bridge between exposure and effects of chemicals on sentinel organisms (Benninghoff, 2007). Among the "OMICS" approaches, proteomics applying high-throughput methodologies for assessment of changes in proteins, are particularly interesting in biomarker discovery. Because proteins are final products of gene expression, they are known as the molecular effectors of biological processes. Therefore, protein changes are more likely to be linked to physiological outcomes in the exposed organism (Trapp et al., 2014). Proteomics techniques allow researchers to visualize, compare and identify thousands of proteins after a chemical exposure. A direct comparison of proteome which is obtained for control versus an exposed condition, allows identification of toxicant-altered proteins based on subcellular localization which can be used as a biomarkers or/and predict the mode of action of chemicals (Alex Merrick, 2006).

Biomarkers in environmental toxicology

During recent years, release of environmental pollutants into the aquatic ecosystems and associated potential adverse effects on aquatic animals has become an increasingly key issue for environmental regulators and scientists. Various efforts have been made to develop early warning signals or biomarkers that persuasively reflect adverse biological response to anthropogenic environmental chemicals. The biochemical responses in aquatic toxicology were demonstrated during 1970s, when physiologists and toxicologists first attempted to understand the effects and toxicity mechanisms of pollutants in fish. Within a few years, biochemical responses suggested as biological markers show the presence of problem rather tightly linked with presence of specific contaminant (McCarty and Munkittrick, 1996). High-throughput techniques in molecular biology and inventions and application of sophisticated analytical instruments have driven traditional biomarkers into novel, more sensitive and validated biomarkers of exposure, effect, and susceptibility to address the adverse effects of contaminants (Kaviraj et al., 2014).

Zinc oxide nanoparticles

Nanoparticles (NPs) have been widely used in industrial and consumer products because of their physicochemical properties, as well as improving techniques to synthesize and manipulate such materials. The attractive physicochemical properties of NPs make them innovative breakthrough candidates in food processing and packaging so as to improve food taste and texture and enhance nutrient bioavailability (van den Brule et al., 2016). The huge increase in production and application of NPs in broad different areas increase the likelihood of leakage and accumulation of NPs into the environment which ultimately increases the potential toxicity risk to human and a wide variety of organisms at different trophic levels (Nowack and Bucheli, 2007). Perhaps the most environmentally relevant route of NPs exposure is through the gastrointestinal route, either via ingestion of food contained NPs or by consumption of organisms that have already accumulated them (Petersen and Henry, 2012). Among different metal oxide nanoparticles, zinc oxide (ZnO) NPs are one of the prominent versatile and technologically important materials due to their potential applications in diverse areas such as chemical sensor, bio-sensor, cosmetics, personal care products, solar cells, and drug-delivery (Vaseem et al., 2010). Their unique properties have great potential to revolutionize food packaging because of their antimicrobial properties as well as human body's degradation ability (Ben-Slama et al., 2015). Likewise, it is highly possible that these

NPs could leach into aquatic ecosystems from NPs-contained products through widespread human application of such products and may thereafter be ingested by aquatic organisms.

Over recent decades, researches have indicated that ZnO NPs are toxic to many aquatic species (Aruoja et al., 2009; Bai et al., 2010; Hao and Chen, 2012; Heinlaan et al., 2008). The majority of studies have focused on the assessment of toxic effects of waterborne exposure to ZnO NPs; whereas, data about the magnification and potential toxic effects through dietary route are rare and the published studies pointed out the necessity for more knowledge in this field., ZnO NPs are widely used in industry and medicine as well as being considered as a promising candidate in food production and packaging. Thus, understanding their interaction with biological systems, especially at cellular and molecular levels, becomes key for their safe and efficient application. Dietary exposure is perhaps the main route for humans and aquatic animals to nanomaterials. Study of molecular basis of any change in a model fish digestive tract related to exposure to nanoparticles, can also provide insight into potential negative effects of contamination on aquatic environment as well as human health.

Biomarkers in drug development

Adverse effects of drugs are a major issue for drug development and include a broad range of clinical toxic drug reactions (Anadón et al., 2014). Biomarkers in clinical research can be used to expedite the drug development process. They can identify early potential toxicity through safety assessment, both generally and specifically, by monitoring drug toxicity on different target organs. In general, the role of these biomarkers in pre-clinical safety lies in the assessment of an indication of toxicity, the mechanistic characterization of toxicity and defining the maximal tolerated dose (Marrer and Dieterle, 2010). Doses of potential therapeutic chemicals may be high enough to evoke adverse effects in treated animals. Biomarkers can provide insight into mechanism(s) of toxicity and tolerability to therapeutic agents. Therefore, they can help to eliminate a potentially toxic effect of a new therapeutic chemical earlier in the developmental process. In aquaculture, although biomarker based methods are not specially developed, it can also be used for assessment of general health status of treated animals to characterize the potential toxic effects of new therapeutics.

Peracetic acid

Peracetic acid (PAA) is a strong oxidant agent which has long been known for its germicidal properties (Schmidt, 1997). It has been widely used as a disinfectant in laboratories, food and beverage sectors, medical and pharmaceutical industries as well as for the treatment of municipal waste water to inactivate many pathogenic and indicator microbes (Kitis, 2004). PAA has attracted considerable attention as an alternative biocide for malachite green and formaldehyde in aquaculture. Since 2000, the use of malachite green on fish farms has been prohibited in Europe due to concerns about its accumulation in fish (Sudová et al., 2010). The application of formaldehyde has also been recommended to be reduction because of workers' safety and potential toxic effects on water bodies (Pedersen et al., 2013). The most desirable attributes of PAA for aquaculture are its wide spectrum of antimicrobial activity, relative stability in environments with low organic matter, harmless by-products, and the ease of application in water bodies (Pedersen et al., 2009). PAA products are commercially available in the form of quaternary equilibrium aqueous solution containing PAA, hydrogen peroxide, acetic acid, and water (Falsanisi et al., 2006). Recently, use of PAA as a disinfectant in aquaculture has shown promise for treatment against the fish ectoparasite *Ichthyophthirius multifiliis* (Meinelt et al., 2007; Sudová et al., 2010), fungal infections (Straus et al., 2012)

and crayfish plague (Strand et al., 2011). However, there is a paucity of data on the potential toxic impacts of PAA on aquatic organisms. As with any other therapeutic chemicals, PAA may have the potential to induce some adverse response in treated animal at high doses. Likewise, the potential toxic impacts and dose at which those effects are observed should be identified. The assessment of drug safety can provide insight into the potential toxicity on treated animals and assist to the therapeutics developmental process.

Objectives

The overall aim of this thesis was to use integrated biomarkers to evaluate fish response to diets which contain ZnO NPs and assessment of PAA safety for aquaculture application. In order to achieve the aim, biochemical, histological, and OMICS" approaches were applied.

The specific objectives were:

- To assess zinc bioaccumulation, biochemical and histological changes in a dose-dependent manner in fish.
- To explore the effects of ZnO NPs dietary exposure on serum proteome.
- To identify changes in the intestinal proteome and associated pathways which may be disrupted by dietary exposure to ZnO NPs.
- To evaluate the potential adverse impacts of PAA on health of fish and crayfish.

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CHAPTER 2

CHRONIC DIETARY TOXICITY OF ZINC OXIDE NANOPARTICLES IN COMMON CARP (*CYPRINUS CARPIO* L.): TISSUE ACCUMULATION AND PHYSIOLOGICAL RESPONSES

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CHRONIC DIETARY TOXICITY OF ZINC OXIDE NANOPARTICLES IN COMMON CARP (*CYPRINUS CARPIO* L.): TISSUE ACCUMULATION AND PHYSIOLOGICAL RESPONSES

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Abstract

Concerns regarding the potential toxic effects of zinc oxide nanoparticles (ZnO NPs) on aquatic organisms are growing due to the fact that NPs may be released into aquatic ecosystems. This study aimed to investigate the effects of dietary exposure to ZnO NPs on juvenile common carp (Cyprinus carpio). Fish were fed a spiked diets at doses 50 and 500 mg of ZnO NPs per kg of feed for 6 weeks followed by a 2-week recovery period. Fish were sampled every 2 weeks for haematology trends, blood biochemistry measures, histology analyses, and determination of the accumulation of zinc in tissues. At the end of the exposure and post-exposure periods, fish were sampled for an assessment of lipid peroxidation levels. Dietborne ZnO NPs had no effects on haematology, blood biochemistry, and lipid peroxidation levels during the exposure period. After the recovery period, aspartate aminotransferase activity in both treated groups significantly ($p < 0.05$) increased and alanine transferase activity significantly ($p < 0.05$) decreased in the higher exposure group. The level of lipid peroxidation significantly ($p < 0.05$) decreased in liver of treated fish after 2 weeks post-exposure period. A histological examination revealed mild histopathological changes in kidneys during exposure. Our results did not show a significant increase of zinc content at the end of experiment in any of tested organs. However, chronic dietary exposure to ZnO NPs might affect kidney and liver function.

Keywords: *Bioaccumulation; blood biochemistry; fish; histology; haematology; lipid peroxidation.*

Introduction

Nanomaterials with their unique chemical and physical properties are driving industries into a dynamic emerging science of nanotechnology. Nanotechnology addresses the construction and use of matter at the supramolecular, molecular and atomic levels in a range of 1–100 nm (Roco, 2003). Almost all major research fields in biology, physics, engineering science and medicine have been affected by nanotechnology and benefit from access to various nano-size materials such as carbon-based materials, metals, metal oxides and biopolymers (Martin, 1994). Zinc oxide nanoparticles (ZnO NPs) are some of the main nanomaterials used in the field of nanotechnology, and based on an estimation on companies producing and using engineered nanomaterials, global ZnO NP production has reached between 100 and 1,000 t/year (Piccinno et al., 2012).

ZnO NPs with such exciting properties are quickly becoming an indispensable part of our daily lives (e.g., food packaging (due to their antimicrobial nature), drug delivery, personal care products, cosmetics (particularly sunscreens given their UV absorption properties), and textiles with water and stain repellent properties and self-cleaning fibres) (Pandurangan and Kim, 2015; Saad et al., 2016). Furthermore, ZnO NPs have a bright future in agriculture in terms of crop bloom production, as colloidal solutions can be applied as nano-fertilizers (Batsmanova et al., 2013). Bulk forms of ZnO as supplemental zinc are added to feed of various farmed species including fish (EFSA, 2012). However, detailed studies must determine the safety of replacing bulk materials with their nano forms in such organisms and must also assess potential hazards that this may pose for aquatic ecosystems.

Promising uses of ZnO NPs in various fields render them an exciting subject for research studies and advances in this area have been remarkable. However, investigations of the potential health and ecological effects of using of ZnO NPs on various taxa have not been extensive when compared to studies on other widely used NPs, such as titanium oxide nanoparticles (Kahru and Dubourguier, 2010).

Aquatic environments can be heavily exposed to ZnO NPs through widespread human applications of such products. It was reported that modelled ZnO NPs concentrations reach 10 $ng.l^{-1}$ and 430 $ng.l^{-1}$ in European natural surface water and treated wastewater, respectively (Gottschalk et al., 2009). Environmental levels of ZnO NPs are expected to increase to 76–760 $\mu g.l^{-1}$ in water (Boxall et al., 2007), thus necessitating their safe use in aquatic ecosystems. Nanomaterial behaviours and bioavailability levels in aquatic environments can be modulated from their inherent physico-chemical properties and from physical and chemical properties of environments (Lowry et al., 2010). Weak solubility in water (ranges from 1.6 to 5 $mg.L^{-1}$) and significant particle aggregation levels in freshwater have been reported for ZnO NP according to transmission electron microscopy and dynamic light scattering tests (Franklin et al., 2007). According to modelled predicted environmental concentrations, accumulation concentrations (30–4,800 $\mu g.kg^{-1}$) of ZnO NPs have been predicted from freshwater sediments over a 14-year period (2000–2014) (Gottschalk et al., 2015). The accumulation of ZnO NPs may increase probabilities of exposure through dietary routes in fish either through sediments or organic materials containing NPs or through the consumption of organisms that contain accumulated NPs.

Over the last decades, researchers have shown that ZnO NPs are toxic to many aquatic species (Franklin et al., 2007; Heinlaan et al., 2008; Aruoja et al., 2009; Zhu et al., 2009; Bai et al., 2010; Hao and Chen, 2012; Ma et al. 2013; Tomilina et al., 2014; Choi et al., 2016). However, there is a consensus that metal dietary exposure is the main route of chronic exposure of metals to fish in aquatic ecosystems (Shaw and Handy, 2011). Most studies have focused on assessing toxic effects of waterborne exposure to ZnO NPs, whereas data on magnification

and potentially toxic effects through dietary routes are limited and published studies point out the need for more knowledge in this area. For instance, Connolly et al. (2016) reported that rainbow trout (*Oncorhynchus mykiss*) given ZnO NPs for 10 days showed high levels of zinc bioaccumulation in gills and intestinal areas as well as oxidative stress and higher levels of ethoxy-resorufin-O-deethylase activity with higher doses (1000 mg ZnO NPs per kg feed). Reliable ecological risk assessments require access to toxicity data on risks of bioaccumulation and on any potential physiological and biochemical disturbances found in different species.

Interactions between NPs and biological systems may result in disturbances in organism homeostasis and may evoke compensatory mechanisms that maintain homeostasis in affected organisms. These responses can be observed in haematological, biochemical and histological alterations. These biomarkers have been used to explore modes of action of toxicants and overall health conditions of organisms exposed to various toxicants including nanoparticles (Priya et al., 2015). ZnO NPs are known to exhibit toxicity through oxidative stress responses (Ng et al., 2017). However, the underlying mechanisms associated with ZnO NPs toxicity remain to be elucidated. One mechanism of toxicity involves dissolution in biological fluid (Cho et al., 2011). Zinc ions released from ZnO NPs may lead to an increase in local concentrations of toxic ions in exposed tissues (gastrointestinal tract) and in other internal organs, subsequently inducing oxidative damage as described for heavy metal toxicity (Morcillo et al., 2016). Therefore, in the current study, we aimed to investigate whether dietary administration ZnO NPs in common carp (*Cyprinus carpio* L.) can affect concentrations of zinc in internal organs of fish. Furthermore, we assessed negative effects of dietary ZnO NPs on carp health, lipid peroxidation as a biomarker of oxidative damage, and haematological and histological biomarkers. We used common carp as a model species given its omnivorous and bottom feeding characteristics.

Materials and methods

ZnO nanoparticle stock solution

The ZnO – Nanopowder (Average particle size: ca. 25 nm; Specific surface area: 19 ± 5 m^2/g Purity: > 99%) was obtained from PlasmaChem GmbH (Berlin, Germany). The ZnO NPs used in the present study was from the same batch that has been previously characterised and are reported in our earlier study (Chupani et al., 2017). Stock solution (5 g/l) from ZnO NPs was prepared in ultrapure (Millipore) water followed by sonication in a bath type sonicator (35 kHz frequency, DT 255, Bandelin electronic, Sonorex digitec, Berlin, Germany) for 6 hours. To prepare an experimental diet containing 500 mg ZnO NPs, 200 ml of the NPs stock solution was sprayed onto 2 kg of pellets. In preparing 50 mg per kg of feed, 20 ml of stock NPs was added to 180 ml of ultrapure water and was applied to 2 kg of commercial pellets by spraying. Then, 3.3% gelatine solution (Sigma-Aldrich, USA) was prepared and pellets were covered with a thin gelatine layer to prevent ZnO NPs releasing from pellets into the media. The thin gelatine layer was air-dried and was stored in an airtight container. The control diet was prepared using the same procedure through the replacement of the ZnO NPs solution with 200 ml of ultrapure water. Zinc metal concentrations in the pellets were confirmed by atomic absorption spectroscopy (AAS) (as was done for the zinc analysis of tissues described below). The concentration of zinc metal in the prepared diets were 42.11 and 342.08 mg per kg of feed in 50 and 500 $mg.kg^{-1}$ diets, respectively. As Zn metal forms 80.33% of the ZnO, this equates to the recovery of 84.22 and 85.16 % of the nominal ZnO NPs concentration for 50 and 500 mg per kg diet. A leaching trial was applied. Pellets were added to an aquarium and zinc concentrations were assessed after 5 and 10 min. The results show that zinc was not released from the pellets before 10 min of water submergence.

Experimental design

Juvenile carp (average length and weight of 13.2 ± 0.9 cm and 37.2 ± 8.9 g, respectively) were purchased from a local hatchery in Blatna, Czech Republic and were acclimated to lab conditions for four weeks in a 300 l glass aquarium (40 fish/tank) with aged tap water and aerated with air stone. The following parameters were applied to the water: temperature 15.6 ± 1.0 °C; total ammonia 0.02 $mg.l^{-1}$; NO_3– 3.25 $mg.l^{-1}$; NO_2– 0.004 $mg.l^{-1}$; chemical oxygen demand 0.6 $mg.l^{-1}$, $PO4_3^-$, 0.02 $mg.l^{-1}$. The experiments were performed in triplicate and fish were fed one of the following diets for 6 weeks: control (no added ZnO NPs) and 50 and 500 $mg.kg^{-1}$ feed ZnO NPs. The experiment was followed by a 2-week recovery period during which the fish were fed a control diet. Fish were fed twice a day (1.5% of body weight) at 8.00 and 16.00. Fish were monitored during feeding to ensure that all of the pellets were consumed. Any leftover food was removed after 5 min. Water was changed twice daily before feeding. Fish were sampled every two weeks during the experiment for haematology, biochemical analysis, zinc accumulation, and histopathology measures. Lipid peroxidation levels (TBARSs) were measured at the end of the exposure and recovery period. During each sampling period, two fish per aquarium (six fish in each group) were randomly sampled.

Haematological and plasma biochemical analysis

Whole blood was taken from caudal veins using a heparinised syringe (40 IU/1 ml blood) (Heparin inj., Leciva, Czech Republic). Immediately after sampling, erythrocyte count (RBC), haematocrit (PCV), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and leukocyte count (WBC) levels were drawn from the blood samples according to unified methods for the haematological examination of fish (Svobodova et al., 1991).

Blood plasma was separated by centrifugation (10 min at 12000 × g) at 4 °C and stored at -80 °C for subsequent biochemical analysis. Biochemical indices including glucose (GLU), total proteins (TP), aspartate aminotransferase (AST) activity, alanine aminotransferase (ALT) activity, triacylglycerol (TAG) and cholesterol (CHOL) were measured using a VETTEST 8008 Analyser (IDEXX Laboratories, West-brook, ME, USA).

Index of lipid peroxidation

The tissue samples were obtained at the end of the exposure period, immediately frozen, and then stored at -80 °C. The frozen samples were homogenized (1:10, w/v) in 50 mM potassium phosphate buffer (KH_2PO_4 with 0.5 mM EDTA, pH 7.0) using an Ultra Turrax homogenizer (Ika, Germany). The level of lipid peroxidation was estimated from thiobarbituric acid reactive substances (TBARS) assayed based on a malondialdehyde (MDA) reaction with 2-thiobarbituric acid (TBA). Levels of MDA were expressed as nmol MDA per mg of protein. Total protein concentrations in each sample were calculated according to the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

Determination of zinc concentration in tissues

Liver, gill, intestine, kidney and brain zinc concentrations were measured. Prior to conducting the measurements, biological tissues were dissolved in a mixture of H_2SO_4 and H_2O_2 overnight. Determinations of zinc concentrations were carried out via an AAS technique with flame ionization using a ContrAA 300 (Analytik Jena AG, Germany) equipped with a high-

resolution Echelle double monochromator and with a continuum radiation source (xenon lamp). Calibration solutions were prepared from the same composition as those used for decomposition. The assessment of the trueness and precision of the measurement procedure was performed by the analysis of several certified reference materials (CRM) : dogfish liver DOLT-4 (NRC-CNRC, Canada), with a certified value for zinc of 116 $mg.kg^{-1}$; non-defatted lobster hepatopancreas LUST-1 (NRC-CNRC, Canada), with a certified value for zinc of 82.9 $mg.kg^{-1}$; and lobster hepatopancreas TORT-2 (NRC-CNRC, Canada) with a certified value for zinc of 180 $mg.kg^{-1}$. Certified values for all used CRMs are presented along with their expanded uncertainties (k = 2).

Histology

Fish were sampled at weeks 2, 4, 6, and 8 for histopathological analysis (six fish per group). From each fish, the whole brain, second gill arch, liver, intestines, and kidneys were fixed in 10% neutral buffered formalin and the whole brain was fixed in Bouin's solution. The tissues were prepared for histological analysis using routine histological techniques. In brief, the tissues were processed using the Histomaster 2052/1.5 (MDS-group, Germany), embedded in paraffin, cut to 5 μm sections on a rotary microtome, stained by haematoxylin and eosin (H&E) through an automatic slide staining system (Tissue-Tek® DRS™ 2000, Sekura, USA) and observed using a light microscope equipped with camera system type E-600 (Olympus BX51, Japan).

Statistical analysis

Kolmogorov-Smirnov and Bartlett's tests were applied to assess normal distribution data and the homoscedasticity of the variance, respectively. A two-way ANOVA with a subsequent Tukey's test performed to test the main effects of time and concentration and their interaction on the zinc concentration in each organ as well as lipid peroxidation. The significance level was set at $p < 0.05$. Data were expressed as the mean ± SD values. The statistical analysis was performed using STATISTICA version 12.0 for Windows (STATSOFT, Inc.).

Results

No mortality was observed in any group during the experiment. The fish exhibited normal swimming and feeding behaviours without presenting any visible signs of palatability throughout the experiment.

Haematological and biochemical analysis

Exposure to ZnO NPs did not have any significant ($p > 0.05$) effect on the measured haematological indices for the treated groups compared to the controls (Table 1). The blood biochemistry results are given in Table 2. The results do not show any significant ($p > 0.05$) disturbance in levels of glucose, total protein, cholesterol, triacylglycerol, and alanine transferase during the 6 weeks of exposure. Aspartate aminotransferase activity did not change significantly ($p > 0.05$) during the six-week treatment with ZnO NPs though it increased significantly ($p < 0.05$) in both treated groups after 2 weeks post exposure period. Alanine aminotransferase activity levels decreased significantly ($p < 0.05$) in fish treated with higher concentrations relative to the controls after 2 weeks recovery period.

Table 1. *Mean values ± SD of haematological indices in carp plasma of control and treatment groups either 50 or 500 ZnO NPs mg/ kg feed and after recovery period (week 8) where all groups fed on control diet.*

Time	Group	PCV (L/L)	HB (g/L)	RBC (T/L)	WBC (G/L)	MCV (f/L)	MCH (pg)	MCHC (L/L)
Week 2	**Control**	0.32 ± 0.03	71.44 ± 7.93	1.35 ± 0.17	38.83 ± 7.6	237.97 ± 24.79	52.96 ± 3.16	0.22 ± 0.01
	50 mg.kg^{-1}	0.32 ± 0.03	78.36 ± 6.8	1.4 ± 0.27	52.33 ± 13.5	239.31 ± 71.44	58.38 ± 15.80	0.24 ± 0.01
	500 mg.kg^{-1}	0.33 ± 0.02	83.01 ± 9.62	1.6 ± 0.15	45.5 ± 7.66	207.91 ± 31.01	52.28 ± 8.98	0.25 ± 0.01
Week 4	**Control**	0.36 ± 0.03	74.07 ± 11.5	1.45 ± 0.17	38.66 ± 14.55	252.54 ± 16.51	50.81 ± 5.8	0.2 ± 0.02
	50 mg.kg^{-1}	0.31 ± 0.04	68.27 ± 9.25	1.29 ± 0.12	40.83 ± 18.96	246.10 ± 22.57	52.88 ± 6.58	0.21 ± 0.0
	500 mg.kg^{-1}	0.35 ± 0.03	68.75 ± 3.31	1.35 ± 0.17	41.16 ± 21.38	268.48 ± 50.67	51.45 ± 6.85	0.2 ± 0.01
Week 6	**Control**	0.34 ± 0.02	62.96 ± 9.77	1.37 ± 0.23	59.83 ± 20.5	258.46 ± 32.83	46.15 ± 2.53	0.18 ± 0.01
	50 mg.kg^{-1}	0.36 ± 0.04	71.73 ± 10.4	1.43 ± 0.3	61.66 ± 19.67	261.09 ± 36.5	50.48 ± 5.46	0.19 ± 0.01
	500 mg.kg^{-1}	0.31 ± 0.05	65.46 ± 12.3	1.39 ± 0.22	56.66 ± 16.71	230.27 ± 66.78	46.75 ± 3.76	0.22 ± 0.07
Week 8	**Control**	0.31 ± 0.04	47.14 ± 8.55	1.65 ± 0.14	60.58 ± 22.15	267.49 ± 13.09	40.33 ± 4.47	0.15 ± 0.01
	50 mg.kg^{-1}	0.29 ± 0.04	55.2 ± 11.06	1.2 ± 0.17	53.33 ± 16.63	246.94 ± 17.96	45.65 ± 3.19	0.18 ± 0.02
	500 mg.kg^{-1}	0.32 ± 0.05	56.87 ± 14.3	1.39 ± 0.26	78.41 ± 17.78	231.59 ± 34.91	41.01 ± 9.54	0.17 ± 0.01

Table 2. *Mean values ± SD of biochemical parameters including glucose, total proteins, triacylglycerol, aspartate aminotransferase activity, alanine aminotransferase activity, and cholesterol in carp plasma of control and treatment groups either 50 or 500 ZnO NPs mg/ kg feed and after recovery period (week 8) where all groups fed on control diet. (Different subscript letters in each row show a significant difference among variables).*

Time	Group	GLU (mmol/L)	TP (g/L)	TAG (mmol/L)	AST (µkat/L)	ALT (µkat/l)	CHOL (mmol/L)
Week 2	**Control**	2.29 ± 0.4	39 ± 2.36	2.57 ± 1.06	2.1 ± 1.71	0.94 ± 0.93	4.15 ± 1.03
	50 mg.kg^{-1}	4.02 ± 0.92	36.5 ± 3.93	1.34 ± 0.38	1.48 ± 0.28	0.59 ± 0.38	3.06 ± 0.39
	500 mg.kg^{-1}	3.28 ± 1.43	41 ± 4	2.09 ± 0.7	2.15 ± 1.35	0.9 ± 0.86	3.46 ± 0.66
Week 4	**Control**	3.11 ± 0.69	41.5 ± 4.18	1.66 ± 1.15	1.38 ± 0.73	0.57 ± 0.16	3.41 ± 1.27
	50 mg.kg^{-1}	2.61 ± 0.56	42.66 ± 3.2	2.05 ± 0.97	2.28 ± 1.75	0.78 ± 0.15	3.41 ± 1.12
	500 mg.kg^{-1}	2.81 ± 0.56	40 ± 4.28	2.63 ± 0.99	1.78 ± 0.63	0.6 ± 0.24	4.28 ± 1.49
Week 6	**Control**	2.66 ± 0.62	39.33 ± 2.58	1.68 ± 0.35	1.85 ± 0.83	0.8 ± 0.53	3.3 ± 0.41
	50 mg.kg^{-1}	4.16 ± 1.57	37.6 ± 3.04	1.54 ± 0.75	2.01 ± 0.53	0.62 ± 0.18	3.05 ± 0.74
	500 mg.kg^{-1}	3.05 ± 1.07	37.16 ± 5.63	1.97 ± 0.76	2.39 ± 1.05	0.82 ± 0.31	3.99 ± 1.74
Week 8	**Control**	3.81 ± 1.52	38.5 ± 5.68	1.69 ± 0.21	1.16 ± 0.58a	1.14 ± 0.44a	3.48 ± 0.4
	50 mg.kg^{-1}	2.6 ± 0.94	41 ± 2.7	1.8 ± 0.6	1.85 ± 0.5b	0.74 ± 0.37a	3.43 ± 0.5
	500 mg.kg^{-1}	3.76 ± 1.42	37.8 ± 2.78	2.21 ± 0.27	3.27 ± 0.41c	0.44 ± 0.1b	4.13 ± 1.84

Index of lipid peroxidation

Levels of lipid peroxidation (TBARS) showed significant ($p < 0.05$) reduction in liver of treated groups after two weeks of recovery. A significant ($p < 0.05$) main effect of time was observed between six weeks of exposure (0.59 ± 0.06 nmol.mg^{-1} protein) and subsequent two weeks of recovery (0.51 ± 0.06 nmol.mg^{-1} protein). Corresponding data are shown in Table 4.

Table 3. *Thiobarbituric acid reactive substances in carp tissues after 6 weeks exposure to ZnO NPs and 2 weeks post exposure period. (Different superscript letters in each column show a significant difference among variables).*

	Group	Gill	Intestine	Liver	Kidney	Brain
Week 6	Control	0.29 ± 0.03	0.47 ± 0.02	0.60 ± 0.05a	0.48 ± 0.06	0.35 ± 0.04
	50 mg.kg^{-1}	0.36 ± 0.08	0.45 ± 0.05	0.58 ± 0.06ab	0.52 ± 0.07	0.38 ± 0.03
	500 mg.kg^{-1}	0.30 ± 0.03	0.58 ± 0.22	0.59 ± 0.05ab	0.55 ± 0.16	0.37 ± 0.03
After post exposure period	Control	0.30 ± 0.04	0.45 ± 0.06	0.55 ± 0.07ab	0.45 ± 0.04	0.32 ± 0.05
	50 mg.kg^{-1}	0.31 ± 0.05	0.52 ± 0.05	0.49 ± 0.05b	0.48 ± 0.04	0.33 ± 0.04
	500 mg.kg^{-1}	0.31 ± 0.03	0.43 ± 0.04	0.51 ± 0.02b	0.44 ± 0.02	0.35 ± 0.04

Tissue zinc concentration

Total zinc concentrations found in the tissues are illustrated in Figure 1. Zinc concentrations in the selected tissues varied according to the following sequence: kidney > intestine > gill > liver > brain.

Results of main effects of concentration showed significant ($p < 0.05$) differences in the levels of zinc among different treatments in brain, kidney and gill. No significant ($p > 0.05$) concentration main effect was observed in liver and intestine (Fig. 1A1–E1).

Results of main effect of time showed that despite some transient significant ($p < 0.05$) changes in the zinc levels, no significant ($p > 0.05$) increase in the level of zinc was observed in the tested organs at the end of the experiment (Fig. 1A2–E2).

Significant increase in the level of zinc was not observed in gills of the treated groups compared to control at the end of experiment (Fig. 1A3). Significant increase in the level of zinc was not observed in kidneys after 6 weeks of exposure in the treated groups. However, kidneys from the 50 mg/kg ZnO NPs treatment showed a significant ($p < 0.05$) increase in zinc levels compared to the controls after the post exposure period (Fig. 1B3). Zinc concentration levels did not significantly ($p > 0.05$) increase in fish intestine tissues of the treated groups in comparison with controls (Fig. 1C3). Hepatic zinc concentration did not undergo any significant ($p > 0.05$) increase in the ZnO NPs treatments relative to the controls (Fig. 1D3). Significant increase in the level of zinc was not observed in the brain of the treated groups at the end of experiment (Fig. 1E3).

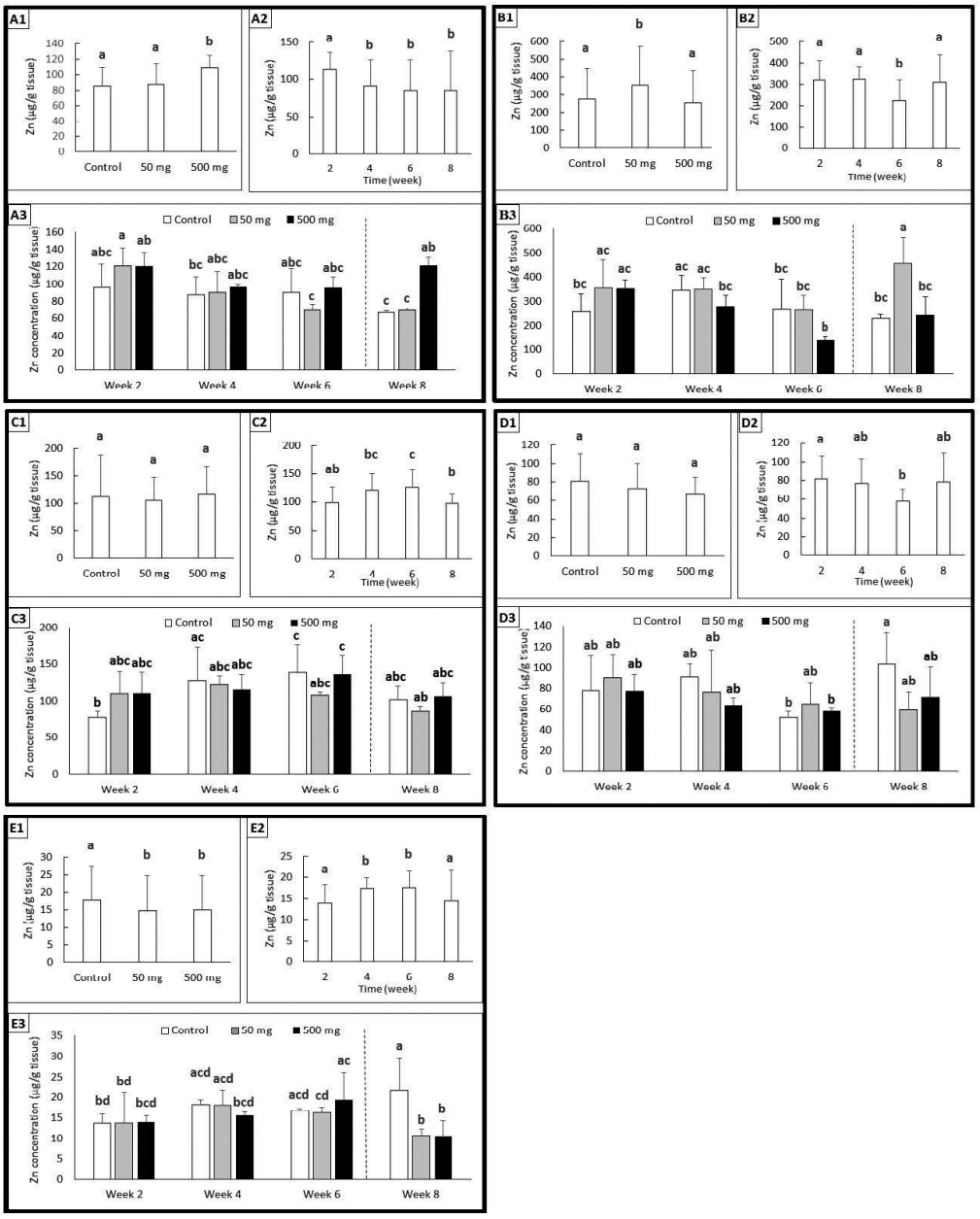

Figure 1. *Effects of concentration, time and their interaction on the zinc levels in the gill (A), kidney (B), intestine (C), liver (D) and brain (E) of carp after exposure to ZnO NPs for 6 weeks, followed by a 2 week recovery period (week 8) where all groups fed on control feed. The dashed line shows the end of exposure and the return of all fish to control diet (recovery period). Commercial diet contained zinc background. Different superscripts indicate a significant difference ($p < 0.05$).*

Histological analysis

Gill tissues in both treated groups exhibited a normal structure similar to that of the controls. The brain, liver and intestine levels did not show any histological changes compared to the controls during exposure and after the post exposure period. The caudal kidneys of fish exposed to the higher concentration treatment showed mild histopathological changes (haemorrhage, congestion, proteinaceous materials in vessels, mild tubular necrosis and tubular degeneration) after 2 weeks of exposure to ZnO NPs (Figs. 2A–D). Intratubular pigment accumulation in a few tubules and tubular degeneration were observed by the end of the exposure period (week 6) in fish exposed to the higher concentration (Fig. 2E). After the recovery period, dark coloured regenerating tubules and necrotic tubular cells were observed in fish exposed to higher treatment concentration (Fig. 2F).

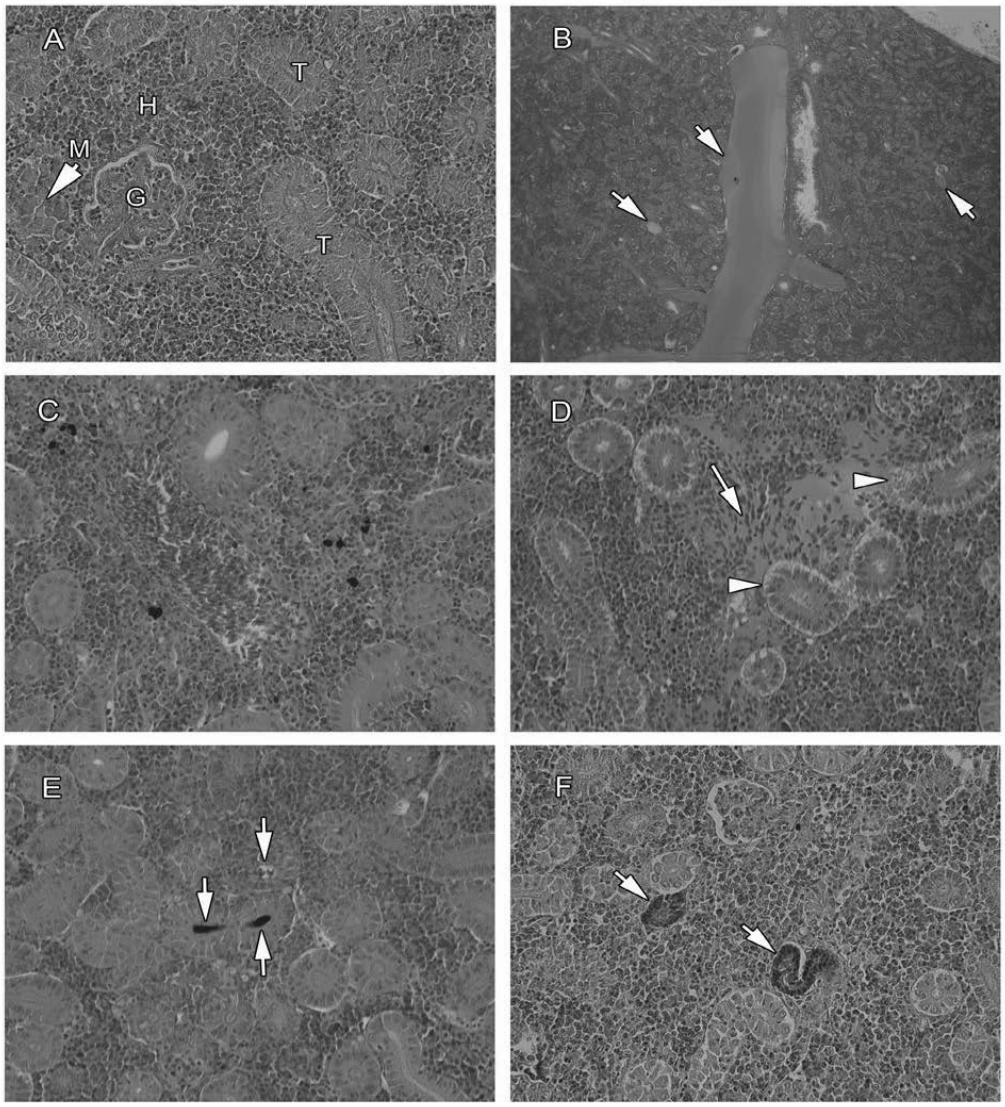

Figure 2. *Histological micrographs of juvenile carp kidneys stained with hematoxylin and eosin. A: Control group showing normal structure: glomerulus (G), hematopoietic tissue (H), renal tubules (T), and melanomacrophage aggregates (M) (magnification 40x). B: After 2 weeks exposure proteinaceous materials in renal vessels (arrows) were observed in both treated groups (50 and 500 mg.kg^{-1} ZnO NPs) (magnification 20x); C: Fish exposed to 500 mg.kg^{-1} ZnO NPs group showed congestion (magnification 40x); D: haemorrhage (arrow) and tubular degeneration (arrowheads) (magnification 40x). E: After six weeks exposure, fish treated with 500 mg.kg^{-1} ZnO NPs showed mild intratubular pigment accumulation (arrows) (magnification 40x); F: After recovery period dark, regenerating tubules (arrows) were observed in higher dose treated group (magnification 40x).*

Discussion

To our knowledge, the present study is one of the first to evaluate zinc tissue distributions and their potentially toxic effects on carp through ZnO NPs chronic dietary exposure. Few studies have considered the bioaccumulation and distribution of waterborne ZnO NPs into internal organs of fish as a result of in vivo exposure. Zinc is an essential trace mineral for human and animals nutrition that is expected to have low toxicity and that is commonly added as supplementary zinc to food (Roney, 2015). In addition, zinc is already present in animal feed ingredients such as fishmeal, and thus it is technically impossible to provide a zinc-free diet. This type of problem is well explained for study on dietary TiO_2 where the natural titania background existed in feed ingredients (Ramsden et al., 2009). The same condition applies to our trial, through which we had limited control over zinc levels in basal diets. Therefore, the results of the present study are considered as effects of the presence of ZnO NPs (combined effects of ZnO chemistry and nano particle size) dietary exposure.

Our results do not show significant disturbances in haematological, blood biochemical, and lipid peroxidation parameters in carp over 6 weeks of ZnO NPs exposure. Our results suggest that ZnO NPs levels in the feed were not potent enough to affect blood in terms of haematological, biochemical and peroxidation parameters via the dietary route. This is in line with findings on dietary rainbow trout (*Oncorhynchus mykiss*) exposure to TiO_2 (Ramsden et al., 2009) but is inconsistent with the results of waterborne treatments applying the same NPs to carp (Hao et al., 2013). These authors studied the bioaccumulation of 50 $mg.L^{-1}$ waterborne ZnO NPs for 30 days in carp and reported high lipid peroxidation levels in the livers and gills of NPs-treated fish. This could be related to differences in trial designs used (i.e., exposure routes and concentrations). Via aqueous exposure, fish are exposed through gills, skin, and the gastrointestinal tract, which may affect fish responses to NPs exposure. As is the case for teleost fish, gills and intestines are considered as two routes of zinc absorption (Bury et al., 2003).

Background zinc levels are found in control carp. In this study, values found in the control fish roughly ranged from 9.6–345.1 $\mu g.g^{-1}$ of wet tissue depending on the tissue and exposure time used, falling within the reported range for common carp (Sun and Jeng, 1998; Hao et al., 2013). Zinc concentration in control tissues also fluctuated during exposure, which could easily be attributed to the fact that the fish were farmed in an outdoor pond with natural feed and then transferred to the lab and fed commercial pellets. Furthermore, inter-individual variations in zinc levels are expected because zinc is an essential nutrient for fish and is the second most common trace mineral found in organisms after iron. In addition, high zinc concentration levels found in almost every tissue have been reported for common carp and its close relative, crucian carp (*Carassius carassius*), relative to those of other fish species (Lin et al., 2011).

Our results did not show accumulation of zinc during the exposure in any of studied organs in treated fish. As it is shown in Fig. 1, some differences were seen in some points of sampling. Those differences might be attributed with the random temporal variations in the measurements of the total zinc in each organ. There is a variability in the control mean values during the experiment that is often as large as any differences observed in the treatments at the end of the exposure. The low and high concentration diets contain about 1.3 and 3.5 times higher zinc content than the control, respectively. That is not a big challenge for fish to keep zinc concentration as normal levels. The maintenance of zinc homeostasis in teleost fish involves a more complex process relative to that employed by mammals given higher levels of variability found in aquatic environments (Jiang et al., 2014). Fish can maintain constant body zinc levels over a broad range of concentrations by either regulating the absorption of

zinc from their diet or through the excretion of extra zinc from the gills or kidneys (Bury et al., 2003).

We observed a mild histological alterations in the kidneys of the higher concentration treated group during the experiment. Tubular regeneration observed after the recovery period may represent a compensatory organism response to nephrotoxicity induced during the exposure period. These findings may support the notion that kidney functioning is affected during ZnO NPs exposure; however, further studies must confirm this hypothesis. Studies related to histopathological changes in the kidneys of fish resulting from nanoparticle exposure are limited in the existing literature. Similar to our observations, histological alterations including deformations of the renal tubule epithelium and necrosis have been observed in the kidneys of tilapia (*Oreochromis niloticus*) following exposure to 1 and 10 $mg.L^{-1}$ of ZnO NPs (Kaya et al., 2016).

We observed a significant decrease in TBARS levels and increase in AST activity in the treated fish relative to those of the controls after the recovery period. Also, ALT activity decreased in the higher concentration group after recovery period. This might be due to an impairment of liver functioning. It is difficult to explain reduction in the levels of lipid peroxidation in liver after recovery period because we did not measure other antioxidant parameters. However, we hypothesize that those reductions might be related to an adaptive process in this organ which enhanced antioxidant defense. There is a kinetic equilibrium between serum and tissue levels of AST; therefore, the elevation in AST serum activity found might be an indicator of hepatotoxicity or might demonstrate an imbalance in biochemical functioning or damage to other tissues such as the kidneys, the heart or to muscles that contain AST. As our results do not show any histopathological changes in the livers of the treated fish after the recovery period, it seems that the observed imbalance in AST and ALT activity could be due to the biochemical dysfunction of the liver or of other tissues.

Conclusion

Our results indicate that long term exposure to ZnO NPs did not results in zinc accumulation in the tested internal organs of treated fish. However, exposure induced mild histological changes in kidney and seems to affect liver functioning in regards to changes in liver enzyme activity after a recovery period. Our observations indicate that the liver and kidneys may be the most sensitive to ZnO NPs exposure through the gastrointestinal route. Whether observed changes in particular organs represents part of an adaptive organismal response to ZnO NPs toxicity or merely a reflection of such toxicity levels must be addressed.

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CHAPTER 3

EFFECTS OF CHRONIC DIETARY EXPOSURE OF ZINC OXIDE NANOPARTICLES ON THE SERUM PROTEIN PROFILE OF JUVENILE COMMON CARP (*CYPRINUS CARPIO* L.)

Chupani, L., Zuskova, L., Niksirat, H., Panacek, A., Lünsmannb, V., Haangeb, S., Bergen M., Jehmlich, N., 2017. Effects of chronic dietary exposure of zinc oxide nanoparticles on the serum protein profile of juvenile common carp (*Cyprinus carpio* L.). Sci. Total Environ. 579, 1504–1511.

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Effects of chronic dietary exposure of zinc oxide nanoparticles on the serum protein profile of juvenile common carp (*Cyprinus carpio* L.)

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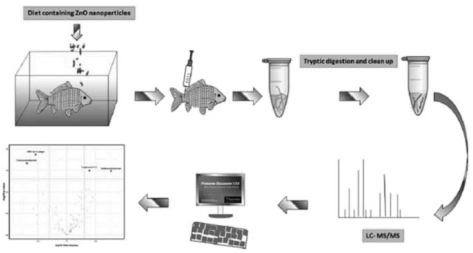
HIGHLIGHTS

- We assessed the effects of dietary exposure of ZnO nanoparticles to carp serum.
- We examined changes in serum proteome after treatment.
- Results suggested that Zno nanoparticles could affect fish homeostasis.
- Homeostasis is disturbed via impairment of coagulation and immune systems.

GRAPHICAL ABSTRACT



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ABSTRACT

Zinc oxide (ZnO) nanoparticles (NPs) have been dramatically used in industry, biology, and medicine. Despite their interesting physico-chemical properties for application in various industrial, medical, and consumer products, safe use of ZnO NPs are under challenges due to the inadequate information related to their toxicological endpoints. Proteomics was applied to evaluate the sub-lethal effects of dietary exposure to ZnO NPs on serum proteome profile of juvenile common carp, (*Cyprinus carpio*). Therefore, ZnO NPs solution (500 mg kg^{-1} of feed) was added to a commercial carp feed for six weeks. We compared the serum proteome profile from 7 controls and 7 treated fish. In addition, zinc accumulation were measured in intestine, liver, gill and brain. In total, we were able to identify 326 proteins from 6845 distinct peptides. As a result of the data analysis, the abundance levels of four proteins were significantly altered (fold change (fc) ≥2 and $p < 0.05$) after dietary exposure to ZnO NPs. The protein levels of the complement component C4-2 (fc 2.5) and the uncharacterised protein encoded by *kng1* (fc 5.8) were increased and major histocompatibility class I (fc 4.9) and the uncharacterised protein encoded by *lum* (fc 3.5) were decreased (fc 2.5). Molecular pathway analysis revealed four canonical pathways including acute-phase response signalling, liver and retinoid X receptors activation, and intrinsic and extrinsic prothrombin activation pathways as significantly regulated in the treated fish. No significant difference was observed for zinc accumulation in exposed fish compared to controls. In summary, despite no apparent

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accumulation, ZnO NPs exposure to common carp probably disturbs the fish homeostasis by affecting proteins of the haematological and the immune systems.

1. Introduction

Nanotechnology, the so-called the next industrial revolution, is a series of multidisciplinary techniques which involve in manipulation of matters at the atoms and molecules scales (Roco et al. 2000). A large number of new engineered nanomaterials have been developed and already hit the market in a wide range of industries and biomedical sciences. Among the different types of nanomaterials, metal oxide nanomaterials have drawn great attentions in the market due to their unique physical and chemical properties. Zinc oxide (ZnO) nanoparticles(NPs) are one of the most widely used metal oxide nanoparticles in a wide range of products including biomedical applications, solar cells, LCDs, electrical, optical, biosensor, ceramics as well as in consumer goods such as foods and cosmetics (Vaseem et al. 2010).

Despite their interesting physico-chemical properties, there is a growing concern about their high adverse toxic effects on biological systems comparted to their microscales form. The widespread production and consumption of ZnO NPs increases their release into the aquatic environments. Hence, the importance of risk assessment for nanomaterials has been highlighted in aquatic toxicology research. Recent studies showed toxic effects of ZnO NPs on different organisms such as fish (Ates et al. 2015; Hao and Chen 2012; Xiong et al. 2011; Zhu et al. 2009), earthworm *Eisenia veneta* (Hooper et al. 2011), microalgae *Pseudokirchneriella subcapitata* (Aruoja et al. 2009; Franklin et al. 2007) and different type of cell lines (Fernández-Cruz et al. 2013; Fernández et al. 2013). However, considering the large size of aquatic ecosystems and the huge diversity of aquatic animals, more studies are needed to assess any possible adverse effects of nanoparticles on the health of aquatic ecosystems. Nanotoxicology research revealed that exposure to ZnO NPs may induce cytotoxicity that is largely mediated by the induction of reactive oxygen species (ROS), causing oxidative injury, release of mediators for inflammation, DNA damage and apoptosis (Sharma et al. 2012). However, the detailed mechanism of action of ZnO NPs is still not fully understood and under debate.

Fish blood parameters are widely used as a pursuing indicator of physiological and pathological changes in abnormal conditions such as disease, stress and chemical exposure (Roche and Bogé 1996; Witeska and Kościuk 2003). Most studies aimed at assessment of biochemical and haematological incidences as monitoring tools whereas OMICs studies on fish plasma/serum are rarely used for assessment of the adverse effects of contaminants exposure.

The label-free protein quantification methods are based on the presumption of linear proportionality between peptide mass peak signal intensity and concentration of a given peptide. The label-free proteomics are promising alternatives to coverage limitations of early methods such as those based on two-dimensional gel electrophoresis and can provide large data bases regarding protein profile of studied targets (Old et al. 2005).

Here, we assess the effects of dietary ZnO NPs on the blood serum protein levels in common carp *Cyprinus carpio*. The findings of our study have broad implications for the understanding of the molecular basis of any biological impairment which is caused by nanoparticles in fish as a human food source and also an important component to maintain aquatic ecosystem stability.

2. Materials and methods

Juvenile common carp (*Cyprinus carpio*) were obtained from a local hatchery (Nove Hrady, Czech Republic). Fish were acclimated to the laboratory condition for four weeks in experimental glass aquaria and fed with commercial carp feed two times daily (1.5% body weight). Fish were randomly distributed in six trial aquaria which were filled with aged tap water and aerated via air–stone using air pumps. Fish mean length and weight were 13.1 ± 0.1 cm and 36.7 ± 8.7 g, respectively. During trial, fish were fed with either control (no added ZnO NPs) or treated (500 mg kg^{-1} dry feed) for six weeks, twice a day (1.5% of body weight) at fixed times (8 a.m. and 4 p.m.).

2.1. Diet formulation

The commercial common carp feed (Aller-Aqua, 2 mm) was used in the current study. The percentage of different compositions of the diets according manufacture's guideline were crude protein 30, crude fat 7, ash 6.5, nitrogen-free extract 43.5, fibre 5, phosphate 1, gross energy (MJ) 18.2, digestible energy (MJ) 13. The ZnO-Nanopowder (Average particle size: ca. 25 nm; Specific surface area: 19 ± 5 m^2 g^{-1} Purity: >99%) was purchased from PlasmaChem GmbH (Berlin, Germany). The size and morphology of ZnO NPs dispersed in distilled water was verified by transmission electron microscopy using JEM 2010 (JEOL, Japan). Stock solution (5 g l^{-1}) from ZnO NPs was prepared in ultrapure (Millipore) water followed by sonication in a bath type sonicator (35 kHz frequency, DT 255, Bandelin electronic, Sonorex digitec, Berlin, Germany) for 6 h. In order to prepare experimental diet, 200 mL of the NPs stock solution was sprayed on 2 kg of pellets with slowly mixing and then covered by 3.3% gelatine solution (Sigma-Aldrich, USA). The thin gelatine cover was air-dried and kept in airtight containers. The control diet was prepared in same procedure except the ZnO NPs solution was replaced by 200 mL of ultrapure water.

2.2. Determination of Zn concentration in pellets and biological tissues

Total amount of zinc in pellets and tissues (liver, gill, intestine, and brain) were determined by atomic absorption spectroscopy (AAS) technique with flame ionization using a ContrAA 300 (Analytik Jena AG, Germany) equipped with a high-resolution Echelle double monochromator and with a continuum radiation source (xenon lamp). All samples were dissolved in the mixture of H_2SO_4 and H_2O_2 overnight. Calibration solutions were prepared with the same composition as that used for decomposition.

2.3. Sample preparation for proteome analysis

The blood from 7 fish in each group were obtained from caudal vein in the end of experiment. Serum was collected through centrifugation of clotted blood at 5000 rpm for 10 min, at 4 °C, and then stored at − 80 °C. Serum protein extracts were prepared. Briefly, the samples were reduced by 2.5 mM dithiothreitol (1 h, 60 °C) and then diluted by 20 mM ammonium bicarbonate to a volume 18 μL. Samples were alkylated with 10 mM Iodacetamid solution (10 min in darkness at 37 °C). Proteolytic digestion was done by sequencing grade modified trypsin (Promega) (16 h, 37 °C). The digestion reaction was stopped by adding 10% formic acid to a final concentration 1% and dried in Speed-Vac. Samples were reconstituted in 15 μL 0.1% formic acid. Peptides were desalted and purified by solid phase extraction using μC-18 ZipTip (Merck Millipore, Darmstadt, Germany). Peptide concentration in the serum samples was determined using a NanoDrop at 280 nm (NanoDrop2000, Thermo Scientific, USA).

2.4. LC-MS/MS measurements

The cleaved peptides (5 μL injection contained 2 μg peptide lysate) were separated on Ultimate 3000 RSLC nano system (Thermo Scientific) over a 100 min linear gradient from 4% B to 55% B (A: 0.1% formic acid in MS-grade water; B: 80% acetonitrile, 0.1% formic acid in MS-grade water) and eluted peptides were online analyzed by a Q Exactive HF mass spectrometer (Thermo Scientific) run at TopN = 20 cycle, with full scans at a resolution of 60,000 and most intense peaks (charge state 2–7) being selected for fragmentation (normalized collision energy at 28%) and subsequent MS/MS (resolution 15,000) measurements. Dynamic exclusion of previously measured precursor ion m/z values was set to 30 s. Thereafter, identification and quantification of proteins were performed by Thermo Scientific Proteome Discoverer software (V 2.1) using the imbedded search algorithm Sequest HT against UniProt/Swiss-Prot protein databases of common carp and Zebrafish, *Danio rerio*, with following parameters: trypsin, up to two missed cleavage sites; carbamidomethlyation as fixed modification; 5 ppm MS tolerance and 0.1 Da MS/MS tolerances, target. Only peptides identified with an FDR ≤1% were considered for further analysis and proteins were inferred from at least one unique peptide. The proteins identified in at least 3 replicates were subjected to proteomics analysis of serum protein profiling in common carp.

2.5. Pathways analysis

Canonical pathway analyses of ZnO NPs treated fish versus normal controls were conducted using the Ingenuity Pathways Analysis (IPA) informatics platform (IPA, QIAGEN Redwood City, CA, USA). All proteomic data set was uploaded for canonical pathways analysis. No value cut-off was selected and all sources of data from human, mouse and rat studies in the Ingenuity Knowledge Base were considered. The probability of each biological function was calculated using Fisher's Exact test and $p < 0.05$ was considered significant.

Gene ontology (GO) analysis was conducted for the identified proteins group using Software Tool for Rapid Annotation of Proteins (STRAP) v1.5.0.0 (Bhatia et al. 2009). The final distribution pie charts were created using Microsoft Excel.

2.6. Statistical analyses for protein quantification

The statistical analysis was conducted using R Statistical Software package (v3.2.2). Protein abundances were determined from peak area of precursor ions from the Full-MS scans. The normalization of these protein intensity values was performed by an in-house written R-script. Briefly, intensity values from each sample were $\log_{10}$ transformed, normalized by the median value of all protein abundance values from the same sample and multiplied by the median of these medians intensity values across all samples. Significant changes in the level of protein abundances between controls and ZnO NPs treated fish were determined using a two-sided independent Student's *t*-test. The threshold for minimum required fold change (fc) was set at ≥2.

3. Results

3.1. ZnO NPs characterization

In the present study the average particle size of ZnO NPs was 28 as it is confirmed by TEM measurements (Fig. 1). The TEM observation demonstrated that ZnO NPs exhibited spherical and oval shape.

3.2. Concentrations of zinc in pellets and tissues

The concentration of zinc in pellets was determined by atomic absorption spectroscopy and was 342.08 mg kg^{-1} feed. Since Zn metal forms 80.33% of the ZnO, it equates the recovery of 85.16% of the nominal ZnO NPs concentration in the 500 mg kg^{-1} ZnO NPs diet. We measured Zn concentration in pellets as triplicate and our results was not vary by more than ± 15% which shows an acceptable homogeneous distribution according OECD guideline (OECD 2012). The leaching trial with pellets showed no release of zinc from the pellets into water at least for the first 10 min.

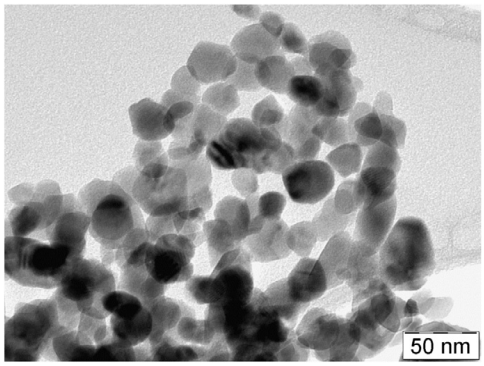

Fig. 1. Transmission electron micrograph of ZnO nanoparticles.

Total zinc concentration in the tissues after six weeks exposure to ZnO NPs is illustrated in Fig. 2. Our results did not show any significant difference in the levels of zinc in none of tissues of treated fish compared to control.

3.3. Proteomics

In total, 327 proteins were identified in serum of common carp (Supplementary Table 1) with 211 of them observed in at least three biological replicates and used for Gene Ontology annotations. There were no unique proteins in ZnO NPs treated group in comparison to the control group. Four proteins showed significant ($p < 0.05$, fc ≥ 2) changes in their abundance after ZnO NPs treatment, with two being up-regulated and two down-regulated (Table 1 and Fig. 3). Canonical enrichment pathway analysis of identified proteins showed that four pathways were significantly ($p < 0.05$) activated in treated fish compared to controls (Fig. 4). These representative pathways found are associated with the haematological and immune systems response including acute-phase response signalling, liver and retinoid X receptors (LXR/RXR) activation, and intrinsic and extrinsic prothrombin activation pathways.

GO annotations were performed to obtain an overall biological and functional background of proteins in common carp serum; the results of this ontological categorization are presented in Fig. 5A, B, C. GO cellular localization annotations (Fig. 5A) showed that the proteins in carp serum mainly were extracellular proteins (57%) and other proteins are localized in plasma membrane, macromolecular complex, cytoplasm, nucleus, chromosome, cytoskeleton, and others. According to molecular function annotation, the largest fraction (46%) was binding to other molecules, 24% of proteins had catalytic activities, and a few proteins were assigned other molecular functions including molecular transduct activity (2%), structural molecular activity (1%), and antioxidant activity (1%) (Fig. 5B). Based on biological process annotations (Fig. 5C), proteins were mainly associated with regulation (24%) and metabolic processes (16%), cellular process (13%), localization (11%), immune system process (10%), interaction with cells and organisms (9%), developmental process (5%), response to stimulus (2%), and others (11%).

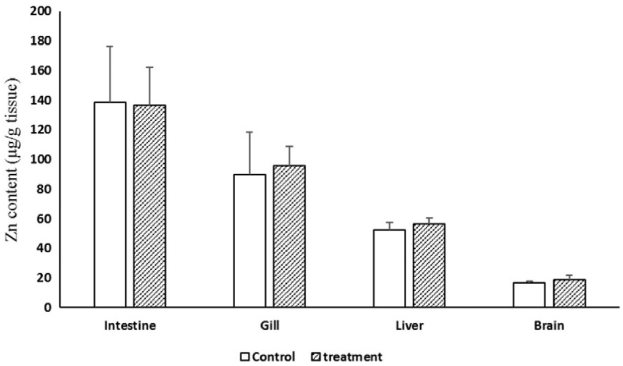

Fig. 2. Zinc content in common carp tissues after six weeks of dietary exposure to ZnO NPs.

4. Discussion

We considered the current study as sub-lethal regarding no mortality background during trial. The results from 500 mg kg^{-1} dietary exposure to Zno NPs in mice also showed a minimal toxicity (Wang et al. 2016). The obtained results were interpreted as toxic effects in term of presence of ZnO NPs in fish diets, the combined effect of ZnO chemistry and nano particle size. Our results did not show accumulation of zinc in carp tissues after six weeks of dietary exposure to ZnO NPs. The previous studies suggested that ZnO NPs were dissolved quickly in acidic condition of gastrointestinal tract and can be absorbed by intestine epithelium. If the nanomaterials dissolve to release free ions then they would show metal uptake models (Shaw and Handy 2011). Zinc is an essential micronutrient for freshwater fish and involves in a wide range of vital processes in body, hence its homeostasis is tightly regulated in body such that fish can maintain a constant state of body zinc over broad range concentrations by either regulating of absorption of zinc from diet or excretion of extra zinc (Bury et al. 2003).

The apparently lack of bioaccumulation of ZnO NPs and their low acute toxicity of dispersed does not mean that there are no toxicological concerns, since we report a range of important sub-lethal effects on serum proteome of fish treated with ZnO NPs.

We conducted the study to assess the sub-lethal effect of ZnO NPs on common carp serum proteome through dietary exposure. The proteins and canonical pathways which were differentially regulated may help exploring the molecular cascades underlying the toxicity mechanism of ZnO NPs. The results showed significant changes in the abundance of four proteins after treatment. We found that the level of soluble major histocompatibility complex (MHC) class I antigen was significantly reduced (fc 4.9) in ZnO NPs treaded fish versus controls. MHC class I molecules are expressed by all nucleated cells and proceed antigenic peptides in the cytoplasm from endogenously-derived peptides to CD8+ T lymphocytes (Neefjes et al. 2011). MHC class I has a fundamental role in initiating acquired immune response against intracellular pathogens through binding to peptides mainly derived from viral proteins (Kloetzel and Ossendorp 2004). Therefore, down-regulation of MHC class I could result in escaping of infected or affected cells from the specific immune response that may lead to health impairment and lower chance of survival. The knowledge on immunosuppression effects of nanomaterials is very limited to date. One study reported that subtoxic concentration of ZnO NPs affect the adaptive immune system by down regulation of CD16 expression in NK-cells in primary human peripheral blood mononuclear cells (Andersson-Willman et al. 2012). On the other hand, immunosuppressive properties of ZnO NPs could propose them as potential candidate for inhibiting allograft rejection, treating of autoimmune diseases, and an immunosuppressive drug delivery system. Clearly, more detailed research is required to confirm immunosuppression properties of ZnO NPs on different model animals.

We also observed an increased level of complement C4-2 in treated fish which is the ortholog for mammalian C4B (Boshra et al. 2006). Complement system is an essential part of the innate immune system and is involved in pathogens killing, phagocytosis, and inflammatory reactions (Holland and Lambris 2002). The complement system in teleost is not fully understood yet but it shares fundamental properties with the mammalian system and seems to possess activation pathways similar to those identified in mammals (Holland and Lambris 2002). The C4B protein in mammals is crucial for the activation of the complement pathway via propagation of the classical and the mannose-binding lectin complement pathways (Rupert et al. 2002). It seems that treatment of fish by ZnO NPs could induce activation of this complementary system. A number of studies have claimed that nanoparticles activate the complement system (Dykman et al. 2004; Hamad et al. 2008; Szebeni et al. 2007; Vauthier et al. 2011). Hamad et al. (2008) found that poly(ethylene glycol)-stabilized single-walled carbon nanotubes can activate the complement system in human serum through C4 cleavage. Activation of the complement system is tightly regulated in normal conditions because its hyperactivation potentially induces devastating effects on the healthy host tissues (Dunkelberger and Song 2010).

Table 1
List of proteins with significant fold changes in fish treated by ZnO NPs for six weeks in comparison with controls.

Accession number	Protein name	Gene ID	Source organism	*P* value	GO-molecular function	Fold change (treatments/control)
I7BBU6	MHC class I antigen	*cyca-UA*	*Cyprinus carpio*	0.021	Peptide antigen binding	Decreased (4.9)
F1R6G3	Uncharacterized protein	*lum*	*Danio rerio*	0.013	Collagen binding	Decreased (3.5)
Q9I932	Complement C4-2	*cycaC4-2*	*C. carpio*	0.031	Endopeptidase inhibitor activity	Increased (2.5)
Q1LYJ7	Uncharacterized protein	*kng1*	*D. rerio*	0.032	Cysteine-type endopeptidase inhibitor activity	Increased (5.8)

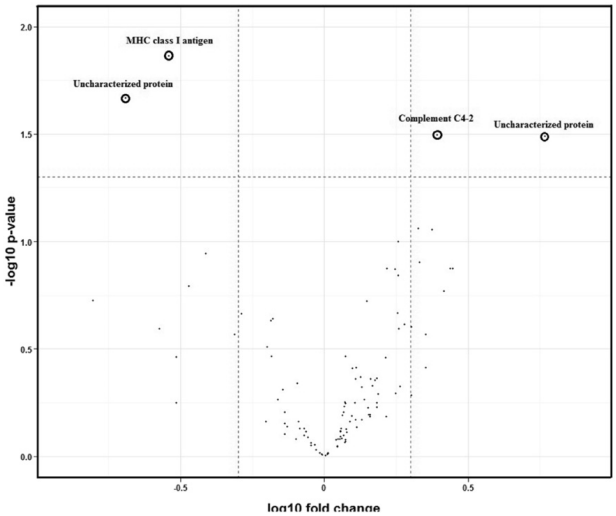

Fig. 3. Volcano plot of protein changes induced by ZnO NPs treatment. The protein expression ratio of ZnO NPs/control (log10 scale) in label-free protein quantitation was plotted against the −log10 of the probability calculated by *t*-test. The dashed lines represent the applied threshold values (p-value < 0.05, fc ≥ 2).

The results revealed an increased level of protein encoded by *kng1* gene (fc 5.75). The *kng1* gene was identified in fish (Tavares-Dias and Oliveira 2009), and is known to generate kininogens which are unique non-enzymatic cofactors of the plasmatic kallikrein–kinin system as well as participate in blood coagulation (Henkel et al. 2011). In addition to that, kininogen-1 is a mediator of inflammation and increases vascular permeability (Borlak et al. 2013) Kininogen-1 expression was reported in response to ZnO NPs (average diameter 20 nm) to human alveolar-basal epithelial A549 cells (Pan et al. 2014). Moreover, Kininogen-1 was reported as one of the serum proteins found in corona composition of various NPs such as carbon-based nanomaterials, silica and superparamagnetic iron oxide NPs after their entrance to biological systems (Fedeli et al. 2015; Sakulkhu et al. 2014; Sopotnik et al. 2015). However, several studies showed that ZnO NPs release zinc ions in biological media and their dissolution kinetic increases in the presence of acidic conditions like gastrointestinal tract (Benetti et al. 2014). Therefore, ZnO NPs seem to enter fish systemic circulation as zinc ions and we speculate that the elevated level of Kng1 protein could be a response to disturbance in coagulant and inflammatory pathways after ZnO NPs exposure.

Our results demonstrated that level of protein encoded by *lum* gene was reduced in the treated fish. This gene encodes lumican, a protein with leucine-rich repeats which is known as collagen fibrillogenesis and plays a role in maintenance and homeostasis of tissue and extracellular matrix (Yeh et al. 2010). Recently, it was reported that (Lum(−/−)) mice showed an impaired response to bacterial lipopolysaccharides and considering that, it is proposed that lumican modulates host response to bacterial infection and acts in innate immune response (Wu et al. 2007). The down-regulation of lum-protein in carp serum could weaken the elimination of invading pathogens via impairment of innate immune response.

The canonical pathways analysis showed significant association with the acute-phase response signalling, liver and retinoid X receptors

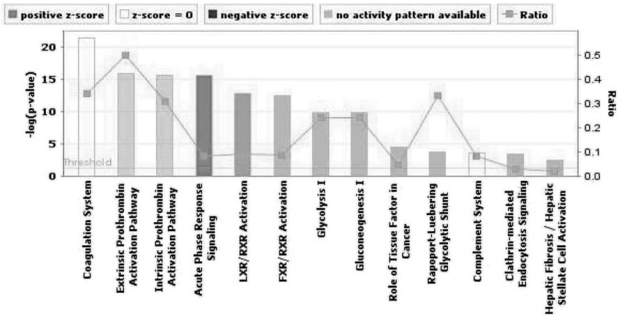

Fig. 4. Significantly enriched canonical pathways in ZnO NPs treated vs. control group identified by IPA. The bars demonstrate the p-value of the pathway; orange bars illustrate a positive z-score (up-regulation of the pathway). The orange boxes represent the ratio of the number of input molecules to total number of molecules in the pathway.

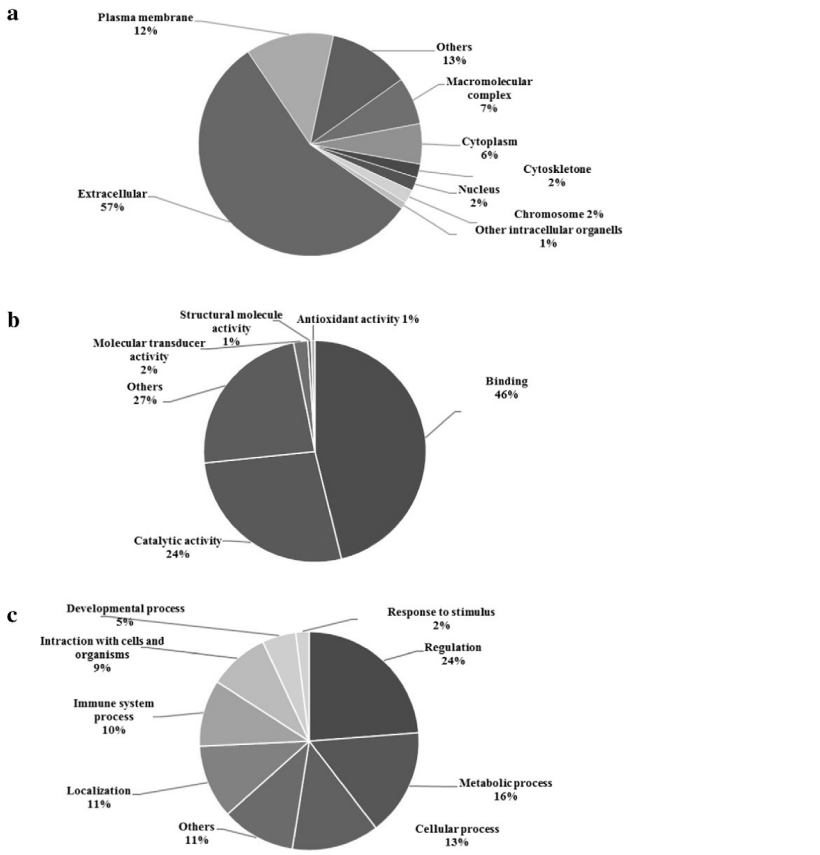

Fig. 5. Ontology analysis of identified carp blood serum proteins. The classification of the protein set was performed by STRAP according to the gene ontology terms: A. Cellular component, B. Molecular function and C. Biological process.

(LXR/RXR) activation, and intrinsic and extrinsic prothrombin activation pathways.

Host homeostasis could be disturbed by infection, tissue injury, trauma, neoplastic growth or immunological disorders. The organism combats these disturbances by a prominent systemic reaction known as acute phase response to restore tissue integrity and its function (Gruys et al. 2005). The acute phase proteins were elevated in plasma of mice after exposure to silica nanoparticles and proposed as a biomarker for risk assessment of exposure to nanomaterials as well as their likely toxicities (Higashisaka et al. 2011).

RXRs physically bind with LXRs and regulate the expression of LXR-responsive genes (Zhang-Gandhi and Drew 2007). Recently it has also been reported that LXRs modulate inflammatory response (Zelcer and Tontonoz 2006). The extrinsic (propagation pathway) and intrinsic (initiation pathway) prothrombin activation pathways are part of the coagulation (homeostasis). Coagulation is a critical physiological process evolving in vertebrate and contributes in formation of clot in damaged blood vessels (Ali et al. 2013). To our knowledge, this is the first study on the serum proteome after dietary exposure to ZnO NPs in fish. However, others have investigated the effects of other nanomaterials in vivo or in vitro using omics techniques and reported the modulation of inflammatory processes, the coagulation system and RXLs/RXRs activation (Ali et al. 2013; Maurer et al. 2016; Pisani et al. 2015; Poulsen 2015). Serum is the most commonly clinical samples for evaluation of health statues and recently has gained great attention for the screening of biomarkers for various diseases and chemical exposures. Label-free protein quantification allowed the identification of 326 proteins in serum of common carp. This is almost four times higher than the number of proteins (88) which were identified in carp blood plasma by 1D-SDS-PAGE prefractionation method combined with LC-MS/MS in the previous study (Dietrich et al. 2014). However, the number of identified protein is still lower in comparison with mammals due to the lack of available complete genome sequence for carp. Interestingly, in the study by Dietrich et al. (2014) parvalbumin was only identified in the seminal plasma and considered as the most discriminating protein between carp seminal and blood plasma. However, parvalbumin beta-1 was identified in our study. Therefore, the results from the current study provide a general view on the basic protein profile of carp serum, which could be valuable for future related studies about fish blood.

5. Conclusion

In summary, our results showed that dietary intake of ZnO NPs could affect the innate and adaptive immune systems in common carp. To some extent, it seems that homeostasis of fish could be affected after a six week exposure to ZnO NPs. The regulated proteins and pathways were related to immune and coagulation systems and could be a response to homeostasis disturbance in fish treated with ZnO NPs. In view of the fact that both mentioned systems are strongly interconnected and share proteins whose expressions are modulated during host defense response to homeostasis disturbing agents, in an attempt to minimize adverse effects and maintain homeostasis in balance. The current study provides basic hints on the pathways that might be affected by ZnO NPs, and may thus further increase our understanding of interaction of NPs in biological systems. This study is a proof of concept to go further to comprehensive studies in order to fulfil list of affected pathways in different biological systems.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.11.154.

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CHAPTER 4

INSIGHT INTO THE MODULATION OF INTESTINAL PROTEOME OF JUVENILE COMMON CARP (*CYPRINUS CARPIO* L.) AFTER DIETARY EXPOSURE TO ZnO NANOPARTICLES

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INSIGHT INTO THE MODULATION OF INTESTINAL PROTEOME OF JUVENILE COMMON CARP (*CYPRINUS CARPIO* L.) AFTER DIETARY EXPOSURE TO ZnO NANOPARTICLES

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Abstract

ZnO nanoparticles (NPs) are widely used in industrial and consumer products. Therefore understanding their interaction with biological systems is key to their safe application. Proteomics was applied to assess the sub-lethal effects of dietary ZnO NPs on two parts of carp intestine, the intestinal folds and the muscular parts. A carp feed containing 500 mg kg^{-1} of ZnO NPs was fed to fish for six weeks. The abundances of 32 proteins in the treated intestinal folds were significantly changed and in addition, 28 proteins were significantly changed in the muscular parts. Pathways analysis revealed downregulation of pathways attributed to protein synthesis in both parts of the treated intestine. Remodelling of actin cytoskeleton pathways were regulated positively and negatively in intestinal folds and muscular parts, respectively, albeit via different mechanisms. Apoptosis response was indicated in exposed intestinal folds, whereas elevated levels of protein associated with cancerous cell survival were observed in the muscular parts. Results showed that ZnO NPs affected the protein abundances associated with cell motility, immune system response, oxidative stress response, as well as cell metabolism. Data are available via ProteomeXchange with identifier PXD006867.

Keyword: *Apoptosis; common carp; intestine; proteomics; ZnO nanoparticles*

Introduction

Rapid advances in the interdisciplinary field of nanotechnology has led to the previously-unimaginably small dimensions of nanomaterials being commonly employed for different areas of application such as energy storage, sensing, data storage, optics, environmental protection, cosmetics, biology, and medicine. Among all nanomaterials which are being used, metal oxide nanoparticles are considered as one of the most versatile materials, due to their various properties and functionalities. Zinc oxide (ZnO) nanoparticles (NPs) are one of the metal oxide nanoparticles most widespread used because of their potential applications in diverse areas such as chemical sensor, bio-sensor, cosmetics, personal care products, solar cells, and drug-delivery (Vaseem et al., 2010).

ZnO NPs are of increasing interest in food sector as additives and in packaging due to their antimicrobial properties as well as the human body's ability for their degradation (Ben-Slama et al., 2015). They are also being tested for their potential application as fungicides in agriculture

(He et al., 2011), anticancer drugs and imaging in biomedical applications (Rasmussen et al., 2010). Currently, ZnO is listed as a generally recognized as safe (GRAS) material by the U.S. Food and Drug Administration (21CFR182.8991) and is permitted to be used as a food additive. Thus, even if it has been proved to be safe at larger sizes, it is necessary to ensure that they are safe in the nanoparticle form as well.

Along with extensive application of ZnO NPs in various areas, intentional or unintentional exposure of human body to NPs is conceivable via several possible routes such as oral ingestion, inhalation, and dermal penetration (Baek et al., 2012). Likewise, it is highly possible that these NPs could leach into aquatic ecosystems from NPs-containing products through widespread human application of such products and may thereafter be ingested by aquatic organisms. Among various exposure routes to NPs, gastrointestinal exposure is considered as one of the most important routes in the human body (Baek et al., 2012) as well as in aquatic organisms (Shaw and Handy, 2011). Several *in vitro* and in vivo studies regarding ZnO NPs ingestion exposure have shown that these NPs can induce toxicity. For example, ZnO NPs (30 nm) caused oxidative stress mediated by DNA damage and apoptosis in mice after 14 days oral administration of ZnO NPs (Sharma et al., 2012). A 90-day study showed that administration of ZnO NPs lead to inflammatory damage, adverse histopathological alterations and significant changes in hematological and blood biochemical parameters in Sprague Dawley rats (Kim et al., 2014). Similarly, dietary exposure of ZnO NPs in rainbow trout caused subtle oxidative stress in liver (Connolly et al., 2016). The cytotoxicity of ZnO NPs and microparticles have been investigated on RKO human colon cancer and revealed that NPs induced higher cytotoxic potency per unit mass. According to their findings, ZnO NPs but not Zn^{2+} induced cell death through the disruption of mitochondrial function (Moos et al., 2010). In contradiction, Liu et al, (2017) reported remarkably low toxicity of ZnO-NPs in mice after a 270-day consecutive dietary supplementation and have suggested that ZnO NPs are relatively biocompatible as the nutritional additive at the commonly used doses (Liu et al., 2017). Based on discrepancies in the literature, this is still a controversial issue and comprehensive studies are required to assess the biological effects of these NPs especially at cellular and molecular levels.

Omics methods have successfully been applied in experimental toxicology to identify molecular profiles which are indicative of exposure or effects of toxic substances (Marx-Stoelting et al., 2015). Application of Omics approaches for assessment of *in vitro* and *in vivo* effects of ZnO NPs on the gastrointestinal tract are limited to only a few studies mostly on gene expression. For instance, Bacchetta et al. (2012) investigated the mechanisms of the ZnO NPs-induced intestinal lesions in *Xenopus laevis*. According to their results, ZnO NPs induced a marked increase in antioxidant gene expression, high lipid peroxidation level in the enterocytes as well as disarrangement of the cytoskeleton and cell junction integrity (Bacchetta et al., 2012).

Although the mechanism of cellular responses in intestinal epithelium to several NPs has been investigated by proteomics (Lai et al., 2013), to our knowledge, this approach has not yet been utilized to explore biological effects of ZnO NPs on fish gastrointestinal tract *in vivo*. Previously, we have shown that ingestion of ZnO NPs induced changes in serum protein profile of juvenile common carp without apparent accumulation in internal organs (Chupani et al., 2017).

Our earlier study showed changes in the protein abundances in serum of fish treated with ZnO NPs, despite no accumulation of zinc in the internal organs (Chupani et al., 2017). It seems that fish could keep zinc homeostasis but the exposure induced some changes in the protein levels. Therefore, in the current study we aimed to evaluate the potential sub-lethal, chronic impacts in the protein levels of the intestine of common carp, *Cyprinus carpio*, after

dietary exposure to ZnO NPs. To assess the toxicity of ZnO NPs and their toxic mechanism to gastrointestinal tract, we applied label-free protein quantification on carp intestine to identify and quantify any change in the protein abundances and subsequent alterations in their related pathways. Even though carp and zebrafish do not have five intestinal segments like mammals (Brugman, 2016), previous studies have revealed that intestinal anatomy and architecture in cyprinid teleost fish is closely related to mammals with functional homology (Curry, 1939). Therefore, the findings can also provide beneficial information to understand the mechanisms underlying NPs toxicity in mammals.

Materials and methods

Animal handling, diet and sampling

Juvenile common carp, *Cyprinus carpio*, (13.1 ± 0.1 cm, 36.7 ± 8.7 g) were obtained from a local hatchery (Nove Hrady, Czech Republic). Fish were acclimated to the laboratory conditions for four weeks in experimental 300 L glass aquaria (40 fish/tank, identical water conditions) in semi-static set up with aged tap water and aerated with air stone. Fish were fed with commercial carp feed twice daily (1.5% body weight). Fish were randomly distributed in six trial aquaria (triplicate) which were filled with aged tap water and aerated via air–stone using air pumps. During trial, fish were fed with either control (no added ZnO NPs) or treated (500 mg per Kg dry feed) for six weeks, twice a day (1.5% of body weight) at fixed times (8 a.m. and 4 p.m.). Feed leftover was removed from experimental aquaria five min after feeding.

The feed and ZnO NPs in the present study were the same as our earlier study (Chupani et al., 2017). Briefly, The commercial ZnO-nanopowder (average particle size: ca. 25 nm; specific surface area: 19 ± 5 m^2 g^{-1}; purity: > 99%) was purchased from PlasmaChem GmbH (Berlin, Germany). The size and morphology of ZnO NPs dispersed in distilled water was verified by transmission electron microscopy using JEM 2010 (JEOL, Japan). NPs exhibited spherical and oval shape with the average particle size 28 (Chupani et al., 2017). ZnO NPs was prepared in ultrapure (Millipore) water followed by sonication in a bath type sonicator (35 kHz frequency, DT 255, Bandelin electronic, Sonorex digitec, Berlin, Germany) for six hours. In order to prepare experimental diet, the NPs solution was sprayed on commercial pellets while slowly mixing and then covered by 3.3% gelatine solution (Sigma-Aldrich, USA). The thin gelatine cover was air-dried and kept in airtight containers. The control diet was prepared in same procedure except the ZnO NPs solution was replaced by 200 mL of ultrapure water. The leaching trial with pellets showed no release of Zn from the pellets into water for at least the first 10 minutes. The concentration of zinc in pellets was determined by atomic absorption spectroscopy and showed the recovery of 85.16% of the nominal ZnO NPs concentration in the 500 mg kg^{-1} ZnO NPs diet.

After 6 weeks of exposure treatment, 14 fish (7 fish from the control and 7 fish from the treated group) were anesthetized and sacrificed. The anterior segment of intestine was collected. The first 4 cm was snap frozen in liquid nitrogen for proteomics. A part of anterior segment of intestine was also fixed in 10% neutral buffered formalin for assessment of apoptotic rate in intestinal epithelium.

Protein extraction for proteome analysis

Samples were thawed on ice and the muscular and the intestinal folds were separated using sterile scalpel under sterile ventilation hood and analyzed individually. A detailed protocol for protein sample preparation is provided (Supplementary File 1). In brief, protein extraction was

performed in lysis buffer (1% SDS, 50 mM Tris-HCL, pH 7.5, 0.1% PMSF). Protein pellets were dissolved in 30 µL SDS sample buffer (2% SDS, 2 mM beta-mercaptoethanol, 4% glycerol, 40 mM Tris-HCl pH 6.8, 0.01% bromophenolblue), heated to 90°C for 5 min and separated on a 1D SDS gel. Each gel lane was cut into five gel pieces, destained and subsequently proteolytically cleaved overnight at 37 °C using trypsin (Sigma, Munich, Germany). Peptide lysates were desalted using C18 purification (Shevchenko et al., 2006). Peptide concentration in each sample was determined using a NanoDrop at 280 nm (NanoDrop2000, Thermo Scientific, USA) prior mass spectrometric analysis.

Mass spectrometric analysis

Five µL of the peptide lysates were separated and measured on a Dionex Ultimate 3000 nano-LC system (Dionex/Thermo Fisher Scientific, Idstein, Germany) coupled to a Q Exactive HF (Thermo Fisher Scientific, Waltham, MA, USA) mass spectrometer. TriVersa NanoMate (Advion, Ltd., Harlow, UK) is used as ion source. A detailed protocol is provided (Supplementary File 1). The mass spectrometry proteomics data have been deposited to the ProteomeXchange (Vizcaino et al., 2016) Consortium via the PRIDE partner repository with the dataset identifier PXD006867.

Protein identification and quantification

LC-MS files were processed using Proteome Discoverer (v2.0, Thermo Fisher Scientific). MS spectra were searched against a database containing the Uniprot sequences of the all the Bacterial genera (2016-01-15) identified by 16S rRNA gene sequencing analysis in the same study, common carp (*Cyprinus carpio*), and zebrafish (*Danio rerio*) using the SEQUEST algorithm. Bacteria genera were searched in UniProt database. Protein-coding sequences were downloaded and DBToolkit (Martens et al., 2005) was used for processing of protein sequence database. Enzyme specificity was selected to trypsin with up to two missed cleavages allowed using 5 ppm MS tolerance and 0.1 Da MS/MS tolerances. Oxidation (methionine) and acetylation (lysine) were set as dynamic modifications, carbamidomethlyation (cysteine) was selected as fixed modification. Peptide spectrum matches (PSMs) were validated using percolator with a false discover rate (FDR) less than 1% and quality filtered for only rank 1 peptides with XCorr $\geq$ 2.25 [+2] and $\geq$ 2.5 [+3]. Protein quantification was carried out using the precursor ion area detector (2 ppm mass precision) of Proteome Discoverer. Identified bacteria proteins were excluded from final results. The significance threshold for protein quantification and threshold for the minimum required fold change were $p < 0.05$ and ≥ 2, respectively.

Gene ontology (GO) analysis and Ingenuity Pathway Analysis (IPA)

GO annotations were performed to compare overall biological and functional background of proteins which changed in common carp intestinal folds vs muscular parts using Software Tool for Rapid Annotation of Proteins (STRAP) v1.5.0.0 (Bhatia et al., 2009).

Canonical pathway analyses of ZnO NPs treated fish versus control was performed using the Ingenuity Pathways Analysis informatics platform (IPA, QIAGEN Redwood City, CA, USA). Proteome data were uploaded for canonical pathways analysis. No value cut-off was selected and all sources of data in the Ingenuity Knowledge Base were considered. The probability of each biological function was calculated using Fisher's Exact test and $p < 0.05$ was considered significant.

Apoptosis assay

Apoptosis was detected by TdT-Fragel™ DNA Fragmentation Detection Kit (Calbiochem) which is based on the TUNEL assay using terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling. Standard paraffin blocks were prepared and 5 μm sections were cut using rotatory microtome. Paraffin sections were dewaxed and rehydrated through ascending alcohols and xylene. They were treated with 20 μg/mL proteinase K for 14 min at room temperature before labeling of DNA breaks by terminal deoxynucleotidyl transferase with TDT-dNTP mix according to the protocol provided by the with slight modifications. One hundred cells in 5 randomly chosen area in each sample were counted under a light microscope at ×400 magnification. Apoptotic rate (AR) was calculated as follows: AR (%) = (number of positive staining cells/ total number of cells) × 100.

Statistical analyses

Statistical analysis was conducted using R software package (v3.2.2). Protein abundances were determined from peak area of precursor ions from the MS scans. The normalization of protein intensity values was performed by an in-house written R-script. Briefly, intensity values from each sample were log10 transformed, normalized by the median value of all protein abundance values from the same sample and multiplied by the median of these medians intensity values across all samples. Significant changes in the level of protein abundances between controls and ZnO NPs treated fish were determined using a two-sided independent Student's t test. The threshold for minimum required fold change (fc) was set at $\geq$ 2.

Since proportional data (percent of apoptotic cells) did not have normal distribution, proportional data were converted by angular transformation (arcsin$\sqrt{p}$) prior to analysis by t-test. Transformed data were checked for distribution characteristics and homogeneity of dispersion using Kolmogrov–Sminov and Levene's tests, respectively. Data are mean ± SD of the untransformed data, but statistical tests were performed using transformed data.

Results

Protein identification and quantification

In total, 3310 protein groups were identified in intestinal folds of common carp (Supplementary Table 1). Thirty-two proteins showed significant ($fc \geq 2$, $p < 0.05$) changes in their abundances after ZnO NPs treatment (Table 1 and Fig. 1a). Nine and 6 proteins were only identified in ZnO NPs treated group and control, respectively. In the opposite group, those proteins were below the limit of detection (Table 2). In muscular parts, 3526 protein groups were identified (Supplementary Table 2). The abundances of 28 proteins were significantly ($fc \geq 2$, $p < 0.05$) changed in muscular parts of ZnO NPs treated fish compared to the control (Table 3 and Fig. 1b). Five and 7 proteins were only identified in muscular parts of ZnO NPs treated group and controls, respectively. In the opposite group, those proteins were below the limit of detection (Table 4).

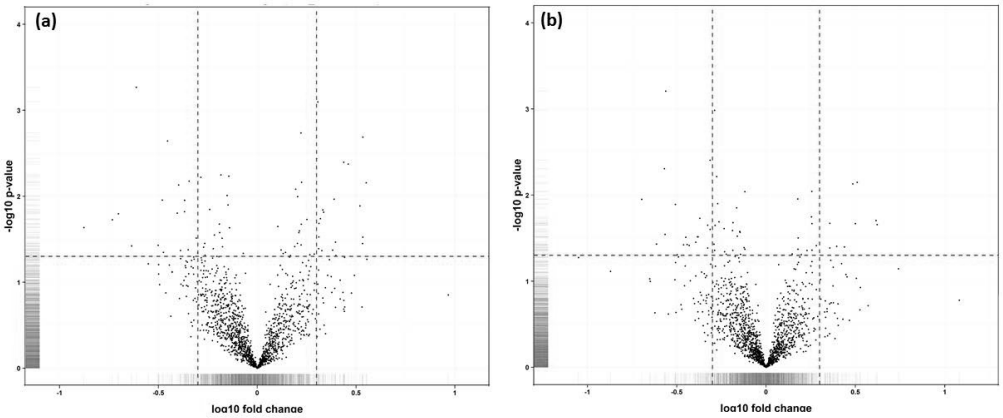

Figure 1. *Volcano plot of protein changes in intestinal folds (a) and muscular parts (b) induced by ZnO NPs treatment (control n = 7; treated n = 7). The protein expression ratio of ZnO NPs/Control (log10 scale) in label-free protein quantitation was plotted against the –log10 of the probability calculated by t-test. The dashed lines represent the applied threshold values (fc ≥ 2, p-value < 0.05).*

Table 1. *List of proteins with significant fold changes in intestinal folds of fish treated by ZnO NPs for six weeks in comparison with controls.*

Accession number	Protein name	Gene ID	Source organism	P value	GO-Biological process	Fold change (Treatments/Control)
Q6PCR7	Eukaryotic translation initiation factor 3 subunit A	eif3s10	*Danio rerio*	6.66×10^{-3}	Formation of cytoplasmic translation initiation complex	Decreased 2.21
Q7T2A5	Eukaryotic translation initiation factor 3 subunit L	eif3s6ip	*D. rerio*	4.54×10^{-2}	Translational initiation	Decreased 2.00
A3KQW5	40S ribosomal protein S18	rps18	*D. rerio*	4.18×10^{-2}	Translation	Decreased 2.23
O73870	S31 protein	S31	*Cyprinus carpio*	4.05×10^{-2}	Transport	Increased 2.04
M9MMD8	Immunoglobulin heavy variable 6-2	ighv6-2	*D. rerio*	1.53×10^{-2}	Response; metabolic cell organization and biogenesis; defense process; regulation of biological process; response to stimulus; transport	Increased 2.40
A0A0A6Z681	Cytochrome c oxidase subunit 2	COII	*Procypris mera*	4.01×10^{-3}	Metabolic process; transport	Increase 2.88
I6YYG2	Glyceraldehyde-3-phosphate dehydrogenase (Fragment)	N/A	*C. carpio*	4.27×10^{-2}	Metabolic process	Increased 2.12
E9QG66	ER membrane protein complex subunit 1	emc1	*D. rerio*	1.81×10^{-2}	Metabolic process	Increased 2.08
Q6P963	Hydroxyacylglutathione hydrolase, mitochondrial	hagh	*D. rerio*	6.94×10^{-3}	Metabolic process	Increased 3.55
Q1LYG5	Eukaryotic translation elongation factor 2a, tandem duplicate 1	eef2a.1	*D. rerio*	1.59×10^{-2}	Metabolic process	Decreased 2.33
R4GEX4	Eukaryotic translation initiation factor 3, subunit F	eif3f	*D. rerio*	1.51×10^{-2}	Metabolic process	Decreased 5.04
Q8AW07	Proteasome (Prosome, macropain) 26S subunit, non-ATPase, 12	psmd12	*D. rerio*	4.48×10^{-2}	Metabolic process	Decreased 2.98
Q6DRE5	NOP56	nop56	*D. rerio*	1.10×10^{-2}	Metabolic process	Decreased 3.02
Q7SXA1	Ribosomal protein L26	rpl26	*D. rerio*	3.40×10^{-2}	Metabolic process	Increased 2.49
Q6PBI0	Succinyl-CoA ligase subunit beta	sucla2	*D. rerio*	4.26×10^{-2}	Metabolic process	Decreased 2.45

Accession number	Protein name	Gene ID	Source organism	P value	GO-Biological process	Fold change (Treatments/Control)
Q561S3	O-sialoglycoprotein endopeptidase	osgep	*D. rerio*	2.26 x 10^{-3}	Metabolic process	Decreased 2.84
A8WGC0	Carboxylic ester hydrolase	si:ch211-93f2.1	*D. rerio*	1.11 x 10^{-2}	Metabolic process	Decreased 2.33
F1Q8Q0	Uncharacterized protein	N/A	*D. rerio*	1.87 x 10^{-2}	Metabolic process	Decreased 5.41
F1RBF9	Golgi reassembly-stacking protein 2		*D. rerio*	4.22 x 10^{-2}	Golgi organization	Increased 2.44
Q803X7	Nap1l1 protein	nap1l1	*D. rerio*	5.4 x 10^{-4}	Cell organization and biogenesis; response to stimulus	Decreased 4.09
Accession number	Protein name	Gene ID	*Source organism*	P value	GO-Biological process	Fold change (Treatments/Control)
A5D6R8	Wu:fb63a08 protein (Fragment)	wu:fb63a08	*D. rerio*	2.96 x 10^{-2}	Cell organization and biogenesis; response to stimulus	Increased 2.04
D6R707	Fibrinogen gamma polypeptide	N/A	*Hypophthalmichthys molitrix*	2.98 x 10^{-2}	Cell organization and biogenesis; regulation of biological process; response to stimulus	Increased 3.42
A9JRP8	Zgc:86896	zgc:86896	*D. rerio*	3.77 x 10^{-2}	Cell organization and biogenesis; regulation of biological process	Decreased 4.32
D6R713	Tubulin beta chain	N/A	*H. molitrix*	1.08 x 10^{-2}	Cell organization and biogenesis; metabolic process	Increased 2.48
E7FGA0	Myosin VIb	myo6b	*D. rerio*	3.05 x 10^{-2}	Cell differentiation; metabolic process; response to stimulus	Decreased 2.03
A5WWI6	Profilin	pfn2l	*D. rerio*	4.22 x 10^{-3}	Actin cytoskeleton organization	Increased 3.30
F1Q5R8	Aldehyde oxidase 6	aox6	*D. rerio*	2.07 x 10^{-2}	–	Increased 2.05
F8W2I6	Galectin	lgals2b	*D. rerio*	7.9 x 10^{-4}	–	Increased 2.02
A7MBU8	Zgc:171682 protein	zgc:171682; nagk	*D. rerio*	7.40 x 10^{-3}	–	Decreased 2.50
A0A0M4UG63	Microsomal glutathione S-transferase 1	N/A	*C. carpio*	1.29 x 10^{-2}	–	Increased 3.40
Q6PBW6	Chaperonin containing TCP1, subunit 2 (Beta)	cct2	*D. rerio*	1.57 x 10^{-2}	Protein folding	Decreased 2.53
A2CEW3	Fibronectin 1b	fn1b	*D. rerio*	2.04 x 10^{-3}	Heart field specification; somitogenesis	Increased 3.42

Table 2. *List of proteins identified in intestinal folds of only one of the two experimental groups (controls or exposed to dietary ZnO NPs for six weeks). In the opposite group, those proteins were below the limit of detection.*

Accession number	Protein name	Gen ID	Source organism	Identified in	GO-Biological process
I7AHI7	MHC class I antigen	cyca-UA	*Cyprinus carpio*	ZnO NPs	Regulation of biological process; response to stimulus
E7FCV8	Collagen, type VI, alpha 2	col6a2	*D. rerio*	ZnO NPs	--
Q6NYE1	Fibrinogen, B beta polypeptide	fgb	*D. rerio*	ZnO NPs	Cell organization and biogenesis; metabolic process; regulation of biological process; response to stimulus
E7FCY6	Zgc:64065	zgc:64065	*D. rerio*	ZnO NPs	Metabolic process; regulation of biological process; response to stimulus
F1R5J9	Protein-S-isoprenylcysteine O-methyltransferase	icmt	*D. rerio*	ZnO NPs	Metabolic process
Q6DBW5	LSM1, U6 small nuclear RNA-associated	lsm1	*D. rerio*	ZnO NPs	Metabolic process
Q6PGY7	CDGSH iron sulfur domain 1	cisd1	*D. rerio*	ZnO NPs	–
F1R8P8	Protein tyrosine phosphatase, receptor type, C	ptprc	*D. rerio*	ZnO NPs	Metabolic process
Q7SXW4	ER membrane protein complex subunit 3	emc3	*D. rerio*	ZnO NPs	Metabolic process
Q8UVE7	Serotransferrin	N/A	*C. carpio*	Control	Cellular homeostasis; transport
A0A097C2M3	TNF receptor-associated protein 1	Trap1	*Ctenopharyngodon idella*	Control	Metabolic process; response to stimulus
E7F6D1	Immunity-related GTPase family, f3	irgf3	*D. rerio*	Control	Metabolic process
E7F7E3	DEAH (Asp-Glu-Ala-His) box polypeptide 8	dhx8	*D. rerio*	Control	Cell division; metabolic process
A3KPX2	ATP-binding cassette, sub-family F (GCN20), member 1	abcf1	*D. rerio*	Control	Metabolic process
F1REP0	TBC1 domain family, member 22a	tbc1d22a	*D. rerio*	Control	Activation of gtpase activity; intracellular protein transport; regulation of vesicle fusion

Table 3. *List of proteins with significant fold changes in intestine muscular parts of fish treated by ZnO NPs for six weeks in comparison with controls.*

Accession number	Protein name	Gene ID	Source organism	P value	GO-Biological process	Fold change (Treatments/Control)
Q9YIA8	Complement C3-H2	N/A	*Cyprinus carpio*	1.11×10^{-2}	Regulation of biological process	Decreased 4.97
A5PL98	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial	sdhb	*Danio rerio*	2.23×10^{-2}	Metabolic process; transport	Decreased 2.12
L7US09	Catalase	N/A	*Carassius auratus*	1.28×10^{-2}	Metabolic process; response to stimulus	Decreased 3.22
B0S789	Signal transducer and activator of transcription	stat1b	*D. rerio*	3.04×10^{-2}	Metabolic process; regulation of biological process; response to stimulus	Decreased 2.40
Q8JHJ1	60S ribosomal protein L35	rpl35	*D. rerio*	3.92×10^{-3}	Metabolic process; cell differentiation; development; regulation of biological process	Decreased 2.05
Q6P7E3	Lancl1 protein (Fragment)	lancl1	*D. rerio*	3.69×10^{-2}	Metabolic process; regulation of biological process; response to stimulus	Decreased 4.11
Q503D3	Polypyrimidine tract binding protein 1a	ptbp1a	*D. rerio*	2.85×10^{-2}	Metabolic process; cellular homeostasis; regulation of biological process	Decreased 3.67
F1QR78	Proteasome subunit beta type	zgc:92791	*D. rerio*	3.52×10^{-2}	Metabolic process	Decreased 2.47
A2BIM7	Threonyl-tRNA synthetase	tars	*D. rerio*	1.85×10^{-2}	Metabolic process	Decreased 2.35
Q7SZR0	Ddc protein	ddc	*D. rerio*	3.04×10^{-2}	Metabolic process	Decreased 2.42
A9C3Q7	Aminomethyltransferase	amt	*D. rerio*	3.77×10^{-2}	Metabolic process	Decreased 2.89
Q0GZP7	Glutathione S-transferase	N/A	*C. carpio*	2.50×10^{-2}	Metabolic process	Decreased 2.05
B3DIX3	Glutamine-fructose-6-phosphate transaminase 2	gfpt2	*D. rerio*	6.2×10^{-4}	Metabolic process	Decreased 3.64
Q6NX09	Hexokinase	hk1	*D. rerio*	4.09×10^{-2}	Metabolic process; cellular homeostasis;	Increased 2.27

Accession number	Protein name	Gene ID	Source organism	P value	GO-Biological process	Fold change (Treatments/Control)
F1QTF9	Inter-alpha-trypsin inhibitor heavy chain 3a	itih3a	*D. rerio*	2.14 x 10^{-2}	Metabolic process; regulation of biological process	Increased 3.15
Q502Q7	Prph protein	prph	*D. rerio*	7.40 x 10^{-3}	Metabolic process; cell organization and biogenesis;	Increased 3.05
A5WWI1	UMP-CMP kinase (Fragment)	cmpk	*D. rerio*	1.97 x 10^{-2}	Metabolic process	Increased 4.12
E7EYF3	Ubiquitin-like modifier-activating enzyme 7	uba7	*D. rerio*	3.92 x 10^{-2}	Metabolic process	Increased 2.48
C5H5I1	Plexin B2	plxnb2a	*D. rerio*	4.12 x 10^{-2}	Development; regulation of biological process; response to stimulus	Decreased 0.47
Accession number	Protein name	Gene ID	*Source organism*	P value	GO-Biological process	Fold change (Treatments/Control)
E7FAZ5	Heterochromatin protein 1,-binding protein 3	hp1bp3	*D. rerio*	7.09 x 10^{-3}	Cell organization and biogenesis	Increased 3.23
Q803W9	Transgelin	tagln2	*D. rerio*	4.93 x 10^{-3}	Cell organization and biogenesis	Decreased 3.71
A4IG64	Zgc:162287 protein	exoc8	*D. rerio*	4.92 x 10^{-2}	Cell organization and biogenesis	Decreased 3.14
B2X124	Tapasin	tpsn	*Ctenopharyngodon idella*	2.19 x 10^{-2}	–	Increased 4.18
Q92155	Vimentin	vim	*C. carpio*	3.99 x 10^{-2}	–	Increased 2.74
F1QEL0	Transmembrane protein 214	tmem214	*D. rerio*	3.08 x 10^{-2}	–	Decreased 2.81
F1QCQ7	Stomatin	stom	*D. rerio*	2.12 x 10^{-2}	–	Increased 2.27
Q1PSH4	Disabled1 transcript variant 2	dab1a	*D. rerio*	3.74 x 10^{-2}	–	Decreased 2.76
E9QED5	Si:dkey-30j10.5	si:dkey-30j10.5	*D. rerio*	3.84 x 10^{-2}	–	Decreased 2.69

Table 4. *List of proteins identified in intestine muscular parts of only one of the two experimental groups (controls or exposed to dietary ZnO NPs after six weeks). In the opposite group, those proteins were below the limit of detection.*

Accession number	Protein name	Gen ID	Source organism	Identified in	GO-Biological process
I7AHI7	MHC class I antigen (Fragment)	Cyca-UA	*Cyprinus carpio*	ZnO NPs	Regulation of biological process; response to stimulus
E9JM89	Beta-actin (Fragment)	N/A	*Mylopharyngodon piceus*	ZnO NPs	–
F1QNV4	Nucleoporin 133	nup133	*Danio rerio*	ZnO NPs	–
Q6NUY0	Adenylate kinase 4, mitochondrial	ak4	*D. rerio*	ZnO NPs	Metabolic process
F1R4P0	Formin-like protein 3	fmnl3	*D. rerio*	ZnO NPs	–
E7FBD0	Neurofibromin 1a	nf1a	*D. rerio*	Control	Cell proliferation; regulation of biological process; response to stimulus
Q0P3U4	Cytochrome c oxidase subunit Vb 2	cox5b2	*D. rerio*	Control	Metabolic process; transport
Q8UVE7	Serotransferrin	N/A	*C. carpio*	Control	Cellular homeostasis; transport
E7EXW1	Integrator complex subunit 11	cpsf3l	*D. rerio*	Control	Metabolic process; regulation of biological process
B0V1I3	Ureidopropionase, beta	upb1	*D. rerio*	Control	Metabolic process
Q7SXC0	Carboxylic ester hydrolase	cel.1	*D. rerio*	Control	Metabolic process
Q7T1G9	Serotransferrin	TF	*Carassius cuvieri*	Control	Cellular homeostasis; transport

GO analysis and pathway analysis

The ontological categorizations are presented in figure 2. Based on GO biological process (GO-BP), the largest fraction was cellular process and GO-BP terms unique for intestinal folds included immune system process and interaction with cells and organisms (Fig. 2a). Even though proteins subjected to GO analysis were different, except for serotransferrin and MHC class I antigen which were identified in both, the same GO terms such as response to stimulus, regulation, metabolic process, localization, developmental process, and cellular process, were enriched in both muscular parts and intestinal folds. This phenomenon was also observed in GO molecular function (GO-MF) analysis. GO-MF terms such as binding, catalytic activity, and structural molecular activity were enriched in both studied groups. GO-MF terms including antioxidant activity and molecular transducer activity were unique for muscular parts (Fig. 2b).

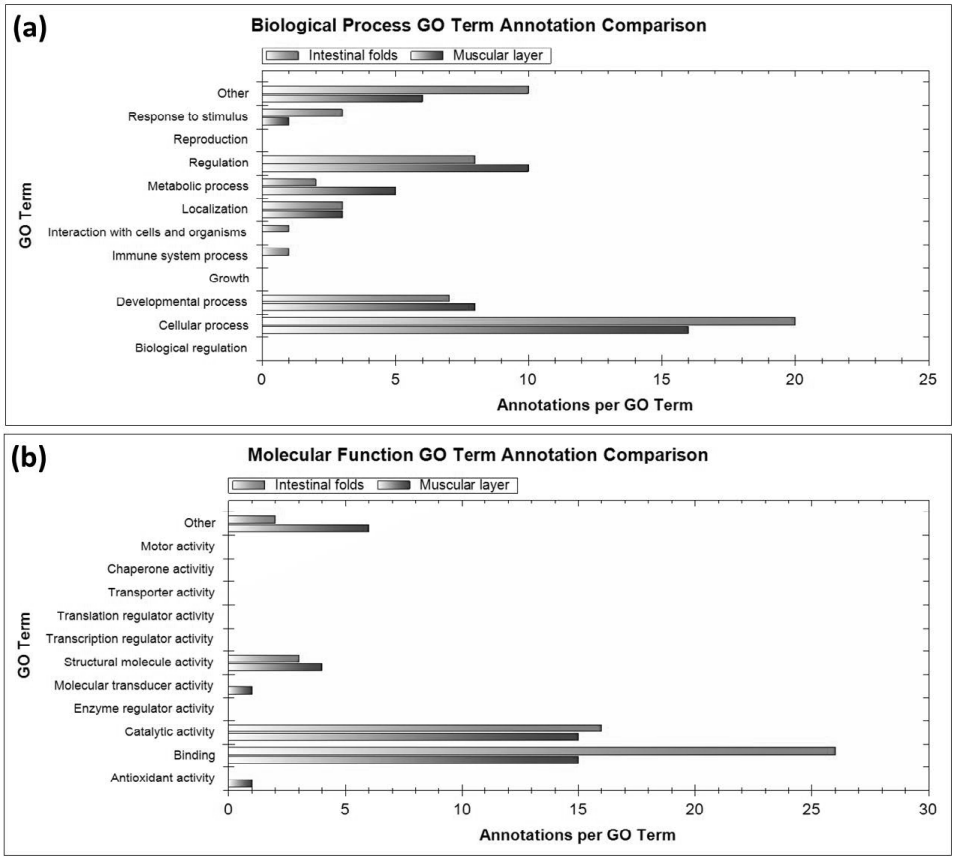

Figure 2. *Comparison of GO distribution of changed proteins (fc ≥ 2, p < 0.05) in intestinal folds vs muscular parts in the categories of biological process (a) and molecular function (b). (control n = 7; treated n = 7).*

Pathway analysis of identified proteins in intestinal folds and muscular parts is shown in figures 3a and 3b, respectively. The results for intestinal folds revealed that pathways which are mainly associated with translation including EIF2 signaling and regulation of eIf4 and p70s6k signaling, were negatively regulated, while pathways being related to remodeling of actin cytoskeleton and cell adhesion such as remodeling of epithelial adhesions junctions, integrin signaling, actin cytoskeleton signaling, RhoA signaling, and regulation of actin based motility by Rho, were positively regulated (Fig. 3a). According IPA results from muscular parts, EIF2 signaling and regulation of eIf4 and p70s6k signaling were negatively regulated similar to intestinal folds whereas pathways which are associated to remodeling of actin cytoskeleton and cell adhesion were negatively regulated (Fig. 3b).

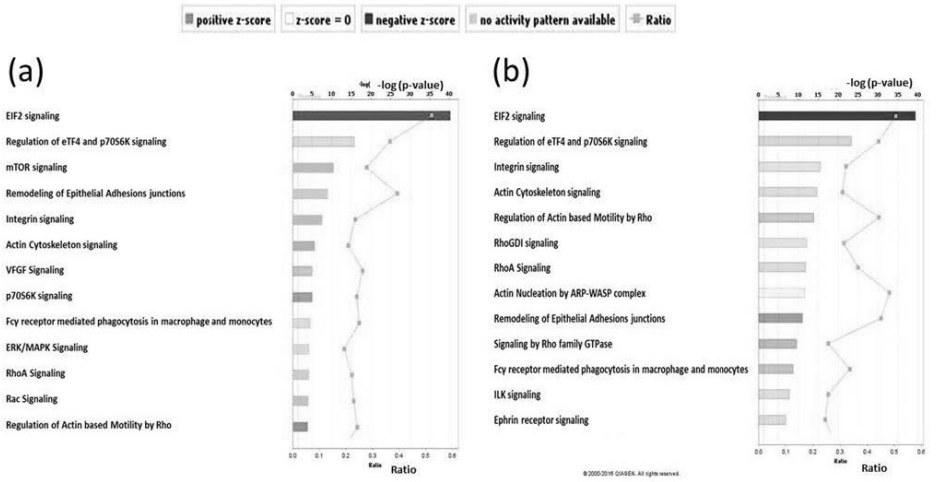

Figure 3. *Significantly enriched canonical pathways in ZnO NPs treated vs. control groups identified by IPA (a) intestinal folds and (b) muscular parts (control n = 7; treated n = 7). The bars demonstrate the p-value of the pathway; orange bars illustrate a positive z-score (up-regulation of the pathway). The orange boxes represent the ratio of the number of input molecules to total number of molecules in the pathway.*

Apoptosis assay

The presence of ZnO NPs slightly induced apoptosis in treated fish. The rate of apoptosis in intestinal epithelium cells of ZnO treated fish was significantly higher ($p < 0.05$) in comparison with control fish (Fig. 4).

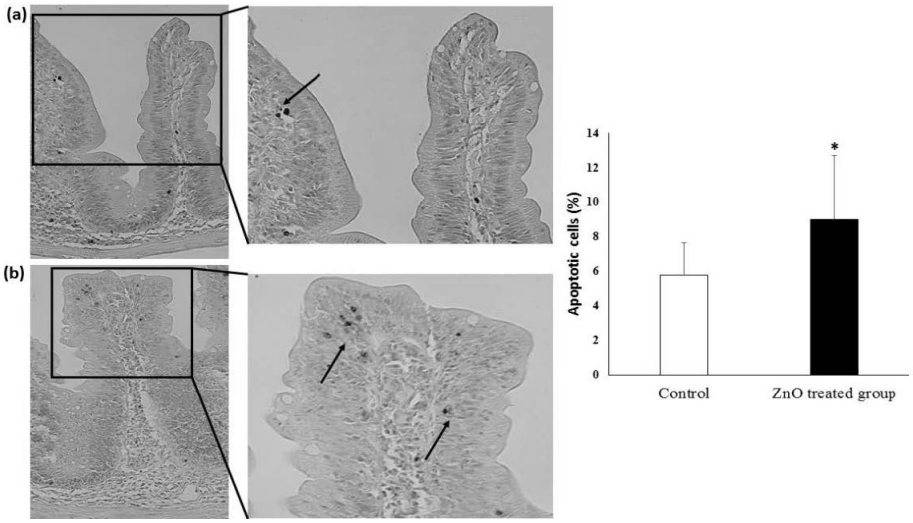

Figure 4. *TUNEL-positive cells (brown stained) were slightly increased in intestinal epithelial cells of ZnO NPs treated fish (b) compared to control (a) (magnification ×200). (c) Graphic representation of apoptotic rate (mean ± SD). * $p < 0.05$ vs. control group.*

Discussion

Results of the present study revealed that the presence of ZnO NPs in fish feed could induce substantial changes in the abundance of proteins and perhaps subsequently in several important biochemical pathways in both intestinal folds and muscular parts of carp intestine. Our observation demonstrated slightly induction of apoptosis in epithelium cells and changes in the abundances of proteins associated with cytoskeleton remodelling, cell metabolism and in both intestinal folds and muscular parts of treated fish. Moreover, the proteomic analysis of the muscular parts revealed an increase in the levels of proteins involved in cell proliferation and known to be oncogenes.

Effects on intestinal folds after dietary exposure to ZnO NPs

Our results revealed some decreases in the abundance of proteins which are involved in the initiation of translation and protein synthesis and other metabolic process such as eukaryotic translation initiation factor 3 subunit L and A, proteasome 26S subunit, O-sialoglycoprotein endopeptidase, carboxylic ester hydrolase. The pathway analysis also indicated that eIF2 signaling was negatively and eIF4 and p70S6K was positively affected. This may suggest inhibition of the cellular protein synthesis by ZnO NPs exposure, probably at the initial step of translation. This inhibition could be an adaptive response indicating the molecular mechanism behind the DNA damage which may be induced in cells exposed to ZnO NPs. This inhibition of the protein synthesis could be a double-edge sword in regard to cell fate decisions. While its initial inhibition could be cytoprotective as a result of reduced ER stress, prolonged or unresolved inhibition could drive cells to undergo apoptosis. Our observation showed increased level of proteins which might be associated with DNA damages and early apoptotic process including cytochrome c oxidase subunit 2, glyceraldehyde-3-phosphate dehydrogenase, and ribosomal protein L26 that seems to be a response to DNA damage induced by ZnO NPs. Furthermore, in the present study, TUNEL assay also confirmed apoptosis in ZnO NPs treated fish compared to control. Taken all together, it seems that presence of ZnO NPs in pellets have the capability to slightly induce apoptosis in intestinal cells. Induction of apoptosis by ZnO NPs has been previously reported in intestinal cell lines (Setyawati et al., 2015).

The level of fibrinogen gamma and beta were higher in ZnO NPs treated fish. These increases could be a response to cell injury which might be induced in treated fish. Because it has been suggested that extrahepatic fibrinogen biosynthesis is invoked only during inflammation and plays a role during localized injury and repair to restore tissue homeostasis (Lawrence et al., 2004). This hypothesis may be further supported by reduction in the level of serotransferrin in treated fish in which its reduction has been reported during inflammation (Rosa et al., 2014). Moreover, increased levels of complement C4-2, Kininogen 1 and positively regulation of intrinsic and extrinsic prothrombin activation pathways in serum proteome were observed in our earlier report (Chupani et al., 2017). Inflammation and coagulation are two main host-defence systems that tightly linked and interact with each other. Under normal physiological conditions, the molecules within the microcirculation of tissues hinder coagulation and inflammation processes. However, when inflammation initiates, it activates coagulation cascades which in return participates in the spreading of inflammation (Vetrano et al., 2011). An inflammatory response was reported after ZnO NPs exposure in intestinal cells *in vitro* (Setyawati et al., 2015). Considering all, it seems that ZnO NPs induced an inflammatory response in the treated fish.

Our results displayed changes in the abundances of several cytoskeleton related proteins. We observed an elevation in the levels of profilin, protein-S-isoprenylcysteine O-methyltransferase

(Icmt), beta tubulin, and galectin in intestinal epithelium of treated fish. The pathway analysis showed positive regulation of pathways associated with cytoskeleton remodelling including mToR signalling, remodeling of epithelial adherens junctions, integrin signaling, actin cytoskeletal signalling, regulation of actin based motility by Rho, and RhoA signalling. ZnO NPs were shown to induce actin polymerization which is indicated by cytoskeleton re-organization (Goncalves and Girard, 2014). One possible scenario for induction of polymerization of actin filaments in ZnO NPs treated fish could be contribution of Icmt in activation of Rho GTPase family. It could subsequently mediate actin polymerization through a profilin-dependent mechanism.

We showed an increased level of β-tubulin and decreased level of chaperonin containing TCP-1 (CCT), subunit 2 (beta). Excess β-tubulin is known to induce microtubule depolimerization via several ways including by interacting with components which are necessary for microtubule assembly, direct interaction with assembled microtubules or microtubule organization centre to impede their assembly (Kappas, 1990). CCT functions as a protein folding machine for de novo unfolded polypeptides such as actin and tubulin without impeding their polypeptide synthesis (Lin et al., 2012). Therefore, depletion of CCT complex seems to perturb both microfilament- and microtubule-based cytoskeleton activities. Inhibition of β-tubulin folding could also disrupt tubulin dimer formation and microtubule assembly. Thus, the presence of misfolded tubulin may be linked to decreased stability of microtubule network. Microtubule disruption could affect intracellular tensile and may hamper directional cell motility by preventing the cells from establishing the forward-reverse polarity (Elbaum et al., 1998). Reduction of microtubule stabilization activates Rho which induce stress fiber, cellular contractility, and focal adhesions (Small et al., 2002). These results were also supported by our pathway analysis showing Rho activation and cytoskeleton remodelling. Furthermore, increased levels of galectin could be linked to a stronger substrate adhesion as is found to play an important role in formation of the adhesion complex and cell-cell adhesion (Elola et al., 2007). Remodelling of cell junctions by TiO2-NPs has already been described in the regular ileum epithelium and it has been suggested that NPs are transported through the cell via the paracellular route (Brun et al., 2014). Clearly, further investigation needs to be carried out to fully elucidate the effects of ZnO NPs on cell migration and motility.

The ability of ZnO to induce cytoskeleton re-organization in the current study is in agreement with previous studies where different NPs were able to induce cell shape changes (Goncalves and Girard, 2014) as well as limited cell migration through microtubule destabilization and increased contractility (Tay et al., 2013).

Effects on muscular parts after dietary exposure to Zno NPs

We observed high level of tapasin and MHC class I in muscular parts of treated fish. Tapasin is a type I transmembrane glycoprotein MHC which has critical role in assembly of MHC class I in endoplasmic reticulum (Rizvi and Raghavan, 2010). MHC class I was not found to be expressed in smooth muscular parts of intestine under normal conditions in rainbow trout (Dijkstra et al., 2003). This protein was also under limit of detection in common carp intestine in controls of the present study. The exact mechanism and reason of its induction in intestinal muscular by ZnO NPs exposure is difficult to explain. Its induction might be in response of inflammation caused by ZnO NPs treatment as overexpression of MHC class I was demonstrated in mammals idiopathic inflammatory muscular diseases (Li et al., 2009).

A decline in antioxidant protection regarding reduced levels of glutathione S-transferase (GST) alpha, Lancl1 protein, and catalase in treated fish was observed. GST alpha down-regulation was observed in response to pro-inflammatory cytokinesin in the cultured human

intestinal cells (Romero et al., 2002). Lancl1 protein is a part of the glutathione defense pathway and it appears to contribute in protection of neurons against oxidative stress (Huang et al., 2014). Therefore, we hypothesize that a decline in Lancl1 protein may be an indicative of antioxidant dysfunction in enteric nervous system after ZnO NPs. Down-regulation of catalase may be a response to increased level of ROS induction due to NPs exposure. It has been demonstrated that zinc can reduce expression of catalase through ROS-dependent processes (Patrushev et al., 2012).

The abundances of vimentin increased in the treated fish which may suggest adaptive regeneration response to oxidative damage in muscular parts. Chablais et al. (2011) observed accumulation of vimentin-positive fibroblasts during myocardium regeneration in zebrafish (Chablais et al., 2011). On the other hand, induction of vimentin as a mesenchymal marker was demonstrated in human bronchial epithelial cells after chronic exposure to silver NPs suggesting a malignant transformation (Choo et al., 2016). Increased levels of hexokinase I, Heterochromatin protein 1,-binding protein 3, formin-like protein 3, adenylate kinase-4 (AK4), actin, and decreased levels of transgelin, succinate dehydrogenase (SDH) may support carcinogenic potential of ZnO NPs regarding their higher expression levels in cancer cells. Hexokinase promotes tumor cell survival through either augmentation of cellular energy supply or protection against apoptosis (Abu-Hamad et al., 2008). Heterochromatin protein 1,-binding protein 3 has been associated with tumorigenesis and functional studies demonstrated that this protein mediates chromatin condensation during hypoxia enhanced growth and viability in cancer cells (Hadac et al., 2016). SDH down-regulation may result in accumulation of succinate, a TCA cycle metabolite, and convey an "oncogenic" signal from mitochondria to the cytosol (Selak et al., 2005). AK4 is suggested as a progression-associated gene in human lung cancer contributed to metastasis (Jan et al., 2012). Actin-associated proteins have been found to be down-regulated; whereas, actin was detected as up-regulated in pancreatic neoplasia (Hamacher et al., 2006). Down-regulation of transgelin, an actin-binding protein, is proposed as critical early event in tumor progression in colon and breast cancers (Shields et al., 2002) as well as tend to promote cell survival under stress conditions (Thompson et al., 2012). In addition, reduction of transgelin abundance appears to protect muscular cells against stressful stimuli that results in ROS accumulation (Thompson et al., 2012).

We could demonstrate that several proteins associated with protein biosynthesis and metabolism including 60S ribosomal protein L35, aminomethyltransferase, glutamine-fructose-6-phosphate amidotransferase 2, and polypyrimidine tract binding protein 1a were down-regulated. In keeping with this, the pathway analysis indicated negative regulation of eIF2 signaling which could be a sign of pause in protein synthesis pathways in muscular parts as it is demonstrated in intestinal folds. As discussed before, prolonged protein synthesis inhibition can induce apoptotic cell death. However, apoptosis could be hampered in the absence of *de novo* protein synthesis by inhibition of the proteasome degradative pathway (Bannerman et al., 2001). Our observation indicated a significant reduction in the level of proteasome subunit beta type, one of subunits of 20S catalytic core complex of the proteasome 26S which may result in inhibition of proteasome activity and could also cause a failure in apoptosis induction thereby supporting hypothesis of carcinogenic potential of ZnO NPs on intestine muscular parts.

The pathway analysis revealed that the signaling pathways associated with the regulation of the actin cytoskeleton were negatively affected. Positive regulation of RhoGDI signalling and negative regulation of Rho A signalling, regulation of actin-based motility by Rho, and actin cytoskeleton signalling may suggest affecting of motility of intestinal muscular after dietary exposure to ZnO NPs. RhoGDIs have been identified as negative regulators of Rho GTPases and microinjection of RhoGDI into fibroblasts resulted in inhibition of motility (Takaishi et

al., 1993). RhoA belongs to a family of small GTPases which has a critical role in the smooth muscular motility and contraction through cytoskeletal reorganization (Wang and Bitar, 1998).

Our observation demonstrated inhibition of Rho and actin polarization in muscular parts which is opposite to what is seen in the intestinal folds. Furthermore, increased levels of proteins related to cell survival were demonstrated in muscular parts, while an apoptosis response was observed in the intestinal epithelium. These opposing results might be associated with differences among target cells or may attribute to difference in exposure route between intestinal folds and muscular parts. Even if the presence of ZnO NPs seems to have potential carcinogenic effect on muscular parts of exposed fish, we cannot exclude the possibility of Zn^{2+} being released inside the cells, which could be another potential mechanism of their toxicity.

Our first study on the effects of dietary exposure to ZnO NPs showed alterations in the blood serum proteins and pathways (Chupani et al., 2017). We observed decreased level of MHC class I in serum whereas its abundance increased in the treated intestine. The presence of any link between the intestine and serum MHC class I level must be addressed in the future studies. Serum is a circulating body fluid of which many of its proteins are secreted or shed by cells of organs during different physiological and pathological conditions (Chandramouli et al., 2009). Therefore, its composition could be referred to as an indicator of the overall physiological situation of an organism (Anderson et al., 2002). Because serum circulates and is in contact with all tissues, any changes in its protein levels might also be an indicator of responses by other tissues to exposure of ZnO NPs. But, the intestine is directly in contact with the food containing NPs and is considered as the important organ for zinc homeostasis.

The observed changes could be considered as compensatory mechanisms to maintain homeostasis in fish treated with ZnO NPS. In the current study, we hypothesise that the changes in the protein abundances may participate in the maintenance of the normal physiological functions via compensatory mechanisms. On the other hand, these compensatory mechanisms may compromise the overall fitness of the organism. Whether those particular changes are reversible, and if so what would be the cost for maintenance of normal physiological function is a challenging field requiring further studies.

Conclusion

We showed that presence of ZnO NPs in pellets could induce changes in the abundance of proteins in proteome of intestine of common carp. Those proteins are mostly associated with immune response, cell metabolism, oxidative stress response, and cell motility. The slightly induced apoptotic death in exposed epithelium folds versus elevated levels of proteins associated with cancerous cell survival may further underline the biological importance of exposed cells in nanotoxicological studies. Our findings provide insight on the possible mechanisms involved in ZnO NPs toxicity in fish intestine as well as has highlighted a need for further investigations for their safe application particularly in food industry.

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CHAPTER 5

EVALUATION OF THE TOXIC EFFECT OF PERACETIC ACID ON GRASS CARP (*CTENOPHARYNGODON IDELLA*) JUVENILES

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Evaluation of the toxic effect of peracetic acid on grass carp (*Ctenopharyngodon idella*) juveniles

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Abstract

OBJECTIVES: The aim of present study was to evaluate the effect of peracetic acid (PAA) on haematotological and biochemical indices, antioxidant status, micronucleus induction and histopathological alterations of liver and gill in grass carp.
METHODS: Grass carp (*Ctenopharyngodon idella*) juveniles were exposed to therapeutic concentrations (1, and 3 mg.l^{-1}) of PAA for a period of 10 days. Selected haematotological indices – the erythrocyte count (RBC), haematocrit (PCV), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and leukocyte count (WBC), and biochemical indices – glucose (Glu), total protein (TP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), and lactate dehydrogenase (LDH) were evaluated in plasma. Activity of superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR), as well as levels of thiobarbituric acid reactive substances (TBARS) were assessed in gill and liver. Micronucleus frequency in peripheral erythrocytes was counted in control and experimental fish. Histological examinations of gill and liver were performed.
RESULTS: No significant differences were found in haematological parameters measured. Statistically significant ($p<0.05$) alterations in the activities of AST, CK and LDH were found in treated fish compared to control groups. Fish exposed to 1 mg.l^{-1} of PAA showed significantly lower ($p<0.05$) SOD activity in liver and gill while catalase activity indicated a significant decrease ($p<0.05$) only in gill tissue. Other significant changes were observed in GR activity in gill in both PAA exposed groups, while GR activity in liver remained unchanged. There was no significant difference in the count of micronuclei between control and exposed fish. Haemorrhage, fusion of primary lamellae, degeneration of secondary lamellae, some clubbing on primary and secondary lamellae tips, and lifting of epithelial cells were found in gill tissues in both control and treated fish.
CONCLUSION: The results show that PAA could induce alterations in biochemical parameters in blood plasma, antioxidant enzymes response and histopathological changes in gill; however, it seems that these changes are reversible. Subsequently, lower concentration (1 mg.l^{-1}) is useable as a treatment concentration for grass carp.

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Abbreviations:

ALT	- alanine aminotransferase
AST	- aspartate aminotransferase
BCA	- bicinchoninic acid
CAT	- catalase
CK	- creatine kinase
Glu	- glucose
GPx	- glutathione peroxidase
GR	- glutathione reductase
GSH	- glutathione
GSSG	- oxidized glutathione
LDH	- lactate dehydrogenase
MN	- micronucleus
NBT	- nitro blue tetrazolium
PAA	- peracetic acid
PMS	- phenazine methosulfate
SOD	- superoxide dismutase
TBA	- thiobarbituric acid
TBARSs	- thiobarbituric acid reactive substances
TP	- total protein

INTRODUCTION

Peracetic acid (PAA) is a strong oxidant agent which has been known for its germicidal properties for a long time (Duong 2005). It has been widely used as a disinfectant in laboratories, the food, beverage, medical and pharmaceutical industries as well as for the treatment of municipal waste water to inactivate many pathogenic and indicator microbes (Kitis 2004). PAA have been recently approved for the use in aquaculture as a sanitizer (Straus *et al.* 2012a; Kouba *et al.* 2012) which has drawn a high attention as an alternative biocide for malachite green and formaldehyde in aquaculture. The use of malachite green for food fish has been forbidden in Europe since 2000 by the reason of its accumulation in exposed fish (Sudova *et al.* 2007). The application of formaldehyde has been recommended to be limited as regards workers' safety and possible harmful effects on water body (Pedersen *et al.* 2013). The most desirable attributes for PAA include its wide spectrum of antimicrobial activity, relative stability in environments containing low organic matter, harmless by-products, neutral residuals, easy-to-use in water bodies (Pedersen *et al.* 2009). PAA products are commercially available in the form a quaternary equilibrium aqueous solution containing PAA, hydrogen peroxide (HP), acetic acid, and water (Falsanisi *et al.* 2006). Although there is no comprehensive data about the distinction activity of PAA, two feasible mechanisms are hypothesized. First one is the production of active oxygen (Lefevre *et al.* 1992; Liberti *et al.* 1999) and hydroxyl radicals (-OH) (Lubello *et al.* 2002) which would lead to disruption of sulphydril (-SH) and sulphur (-S-S) bonds in the cell membrane proteins (Reichert & Young 1997) and the second one is the disruption of the chemiosmotic function of lipoprotein cytoplasmic membrane, denaturation of microbial macromolecules and metabolites (Gómez-López 2012).

In recent years, some data has been published about PAA treatment against fish ectoparasite *Ichthyophthirius multifiliis* (Straus & Meinelt 2009; Meinelt *et al.* 2009; Sudová *et al.* 2010; Meinelt *et al.* 2007), its use in the control of fungal infections of channel fish (Straus *et al.* 2012a) and of crayfish plague (Jussila *et al.* 2011). The results of these studies have shown promising perspectives of PAA as a disinfectant useful in fish cultures. However, there is a paucity of information on the toxic impacts potential in terms of PAA application on fish (Straus *et al.* 2012b). It identifies the necessity of toxicity data on different fish species for safe use of PAA in aquaculture.

Haematological and blood biochemical parameters have been considered as valuable indicators for the assessment of physiological and pathological alterations in fish health statue through toxicological research (Saravanan *et al.* 2011). Furthermore, fish antioxidant system factors, either enzymatic or non-enzymatic component with histopathological alteration have been approved as sensitive indices for the evaluation of fish health condition (Li *et al.* 2010). Erythrocyte micronucleus frequency, which was originally adapted for mammalian species, has been proposed as a useful tool for estimating of cytogenetic damages in fish under laboratory and field conditions (Udroiu 2006).

Given the narrow available information on consequences of PAA treatment in fish, we designed the recent study to assess possible changes in health status of grass carp juveniles (*Ctenopharyngodon idella)* exposed to PAA through the analysis of the alterations in haematological and biochemical parameters as well as the alterations in antioxidant system, micronuclei induction in peripheral erythrocytes and histopathological changes.

MATERIAL & METHODS

Fish and water parameters

Grass carp (mean weight ± SD, 72.3±14.0 g) were obtained from a local fish hatchery and maintained for 2 weeks in aquaria with dechlorinated tap water. Fish were fed a commercial diet (BioMar, 47%protein, 26% fat) every day, at 2.5% of total body weight. Water quality parameters were: temperature 16.3–18.2 °C; dissolved oxygen >69%; pH 7.2–7.9; ANC4.5 (acid neutralisation capacity) 1.2 mmol.l^{-1}; COD_{Mn} (chemical oxygen demand) 1.5 mg.l^{-1}; total ammonia 0.04 mg.l^{-1}; sum of Ca^{2+}+Mg^{2+} 14 mg.l^{-1}; Cl^{-} 11 mg.l^{-1}; PO_4^{3-} 0.01 mg.l^{-1}.

Experimental protocol

The 10-day test was carried out using duplicate groups of 7 fish held in six 150-l tanks. Grass carp juveniles were subjected to 0 (C, control group), 1 mg.l^{-1} (E1, experimental group) and 3 mg.l^{-1} (E2, experimental group) of PAA for ten days. PAA concentrations (1 and 3 mg.l^{-1}) were obtained by addition of Persteril 36 (Eurosarm) two-times a day (at 8:30 a.m. and 4:30

p.m.) to the waterThe desired concentration of PAA was first carefully diluted with enough water from the target aquarium and then was applied into the rest of water volume with fish to ensure even distribution of the active ingredient. The test baths were constantly aerated and renewed daily before the first application of PAA. The range of examination consisted of haematological, biochemical and, histopathological analyses, the evaluation of antioxidant status and counting of micronuclei in erythrocytes.

Fish sampling and haematological examination

The blood samples were taken by puncturing the caudal vessel with heparinised syringes and the blood was stabilised by 40 IU of sodium heparin (Heparin inj., Leciva, Czech Republic) per 1 ml blood. Immediately after sampling, erythrocyte count (RBC), haematocrit (PCV), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and leukocyte count (WBC) were determined in the blood samples according to *Svobodova et al. (1991)*.

Biochemical examination

Blood plasma was separated by centrifugation (10 min at 12 000 × *g*) at 4 °C and then glucose (Glu), total proteins (TP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and creatine kinase (CK) were determined using VETTEST 8008 Analyser (IDEXX Laboratories, Westbrook, ME, USA). The analyser works on the basis of dry chemistry and colorimetric analysis. The analyses were carried out using testing slides (Multi-layer film slides, Kodak).

Biomarkers of oxidative stress and antioxidant parameters

Livers and gill samples were taken, quickly frozen and stored in –80 °C until analysis. For the determination of catalase (CAT) and superoxide dismutase (SOD) activities, frozen samples were homogenized in 50 mM potassium phosphate buffer (KH_2PO_4 with 1 mM EDTA, pH 7.4), centrifuged at 30 000 × *g* for 30 minutes, and supernatants were taken. Total SOD activity was assessed according to the method based on the inhibition of nitro blue tetrazolium (NBT) and production of superoxide anions by NADH and phenazine methosulfate (PMS). SOD activity was measured spectrophotometrically at 560 nm and reported as the nmol of NBT per min per mg protein (Ewing & Janero 1995). CAT activity was determined regarding to the decomposition rate of hydrogen peroxide by spectrophotometric method at 240 nm (Abei 1984). Glutathione reductase (GR) activity was evaluated spectrophotometrically by the rate of NADPH oxidation at 340 nm (Carlberg & Mannervik 1975). In order to determine GR and lipid peroxidation rate, the tissues were homogenized in phosphate saline buffer at pH 7.2. The level of TBARS as an index of lipid peroxidation was calculated spectrophotometrically at 532 nm according to the method of TBA (thiobarbituric acid) assay with some slight modifications (Uchiyama & Mihara 1978).

Total protein concentration in various samples was calculated by bicinchoninic acid (BCA) method using bovine serum albumin as a standard (Bradford 1976).

Micronucleus test

Peripheral blood samples were taken from caudal vein by heparinized syringe and smeared on microscope glasses. The blood smears were fixed in absolute ethanol for 20 minutes, air-dried and stained by 10% Giemsa solution for 25 minutes. Two slides were prepared for each fish and 1000 erythrocyte cells per slides were analyzed under light microscope (1 000×). Micronuclei were identified according to Al-Sabti and Metcalfe (1995) as non-reflective, small ovoid or circular chromatin bodies, displaying the same staining properties and with a diameter 1/3–1/20 of the nucleus.

Histopathology

Liver and gill from each fish were fixed in 10% neutral buffered formalin. The fixed tissue samples were dehydrated through a series of graded ethanol, cleared in xylene, and embedded in paraffin. Sections were cut using a rotary microtome at 3–4 µm, stained with haematoxylin and eosin (H&E) and observed under light microscope.

Statistical analysis

The statistical analysis was performed using the STATISTICA (version 8.1 for Windows, StatSoft). Data was expressed as means ± SD and *p*-value less than 0.05 was considered as statistically significant. Kolmogorov-Smirnov and Bartlett's tests were conducted to evaluate normality and homoscedasticity of variance respectively. One-way ANOVA was applied in the case of data normal distribution. If the data showed a "non-normal" distribution, a non-parametric test (Kruskal-Wallis) was used.

RESULTS

Mortality

No mortality occurred in control and E1 groups. In contrast, high mortality rate (71.5%) was observed in fish exposed to 3 mg.l^{-1} PAA (E2).

Haematological and biochemical indices

Haematological and biochemical values are shown in Table 1 and 2. There are no significant differences in haematological parameters between PAA exposed groups (E1 and E2) and control. On the other hand, the results of biochemical values show a significant increase in AST, CK and LDH activities in E1 group compared with control.

Biomarkers of oxidative stress and antioxidant parameters

No significant differences were observed in the levels of TBARS in liver and gill tissues between control and exposed groups. SOD activity in liver and gill tissue was significantly lower ($p<0.05$) in treated fish (E1, E2).

Tab. 1. Haematological parameters.

Parameters	C n=14; mean±SD	E1 n=14; mean±SD	E2 n=4; mean±SD
RBC ($T.l^{-1}$)	2.20±0.13	2.32±0.51	1.95±0.08
WBC ($G.l^{-1}$)	42.56±7.45	37.94±7.15	29.25±4.75
PCV ($l.l^{-1}$)	0.31±0.03	0.32±0.02	0.30±0.01
Hb ($g.l^{-1}$)	66.99±9.18	65.56±7.22	63.02±8.24
MCV (fl)	142.02±13.41	142.10±30.32	155.34±5.09
MCH (pg)	29.63±4.34	28.84±7.55	32.20±2.90
MCHC ($l.l^{-1}$)	0.22±0.03	0.21±0.02	0.21±0.03

Tab. 2. Biochemical parameters.

Parameters	C n=14; mean±SD	E1 n=14; mean±SD	E2*
GLU ($mmol.l^{-1}$)	3.21±0.83[a]	2.87±0.42[a]	–
TP ($g.l^{-1}$)	29.00±4.50[a]	28.30±2.70[a]	–
AST ($U.l^{-1}$)	93.00±24.00[a]	158.30±30.27[b]	–
ALT ($U.l^{-1}$)	18.80±6.80[a]	23.50±8.00[a]	–
CK ($U.l^{-1}$)	576.30±101.70[a]	1 160.00±232.70[b]	–
LDH ($U.l^{-1}$)	1 780.00±821.00[a]	2 837.50±366.80[b]	–

* all samples missed for this group
Values with the different alphabets within each row are significantly different ($p<0.05$)

There was a significant decrease in CAT activity of gill in both groups of E1 and E2 compared with control, whereas in liver significant decrease was just observed in E group. On the other side, GR activity significantly increased in gill tissues of E1 and E2 groups compared with control (Tables 3 and 4).

Micronucleus frequency

Micronucleus assay revealed no significant differences in E1 and E2 groups compared with control.

Histopathology

The liver of exposed fish (E1 and E2) showed normal physiological structure, while gill tissue revealed some pathological alterations in secondary and primary lamellae including haemorrhagiae, fusions, focal degeneration of secondary lamellae, clubbing of primary lamellae tips and lifting of epithelial cells (Figure 1). Furthermore, as side findings, there were observed undifferenced protozoan parasites in histological section of gill of control group.

DISCUSSION

Peracetic acid is a disinfectant, which was relatively recently introduced into aquaculture for fish treatment (Elia *et al.* 2006). Regarding the growing interest in the application of PAA as therapeutic agent, toxicity data on different fish species is needed for the safe application security.

Haematological and biochemical indices in peripheral blood provide a reliable index of health status in various organisms including fish (De Pedro *et al.* 2005). The activity of enzymes can be affected as a result of environmental stress, diseases and tissue damage (Kori-Siakpere *et al.* 2011). The present study indicated higher

Tab. 3. Lipid peroxidation and antioxidant enzymes activities in liver of grass carp.

Group	SOD (nmol NBT/min/mg protein) (mean±SD)	CAT (H_2O_2/min/mg protein) (mean±SD)	GR (nmol NADH/min/mgprotein) (mean±SD)	TBARS (nmol/mg protein) (mean±SD)
C (n=14)	0.1017±0.0332[a]	2.0956±0.0683[a]	0.2274±0.0927[a]	0.1808±0.0249[a]
E1 (n=14)	0.0706±0.0267[b]	2.1505±0.2668[a]	0.2292±0.0833[a]	0.2034±0.0333[a]
E2 (n=4)	0.0683±0.0054[b]	1.3102±0.1425[b]	0.2708±0.1177[a]	0.1670±0.0018[a]

Values with the different alphabets within each column are significantly different ($p<0.05$).

Tab. 4. Lipid peroxidation and antioxidant enzymes activities in gill of grass carp

Group	SOD (nmol NBT/min/mg protein) (mean±SD)	CAT (H_2O_2/min/mg protein) (mean±SD)	GR (nmol NADH/min/mgprotein) (mean±SD)	TBARS (nmol/mg protein) (mean±SD)
C (n=14)	0.1809±0.0896[a]	0.0585±0.0119[a]	0.2399±0.0683[a]	0.1140±0.0093[a]
E1 (n=14)	0.0080±0.0038[b]	0.0287±0.0072[b]	0.3535±0.0435[b]	0.1510±0.0218[a]
E2 (n=4)	0.0110±0.0060[b]	0.0392±0.0124[b]	0.2889±0.0292[b]	0.1145±0.0048[a]

Values with the different alphabets within each column are significantly different ($p<0.05$).

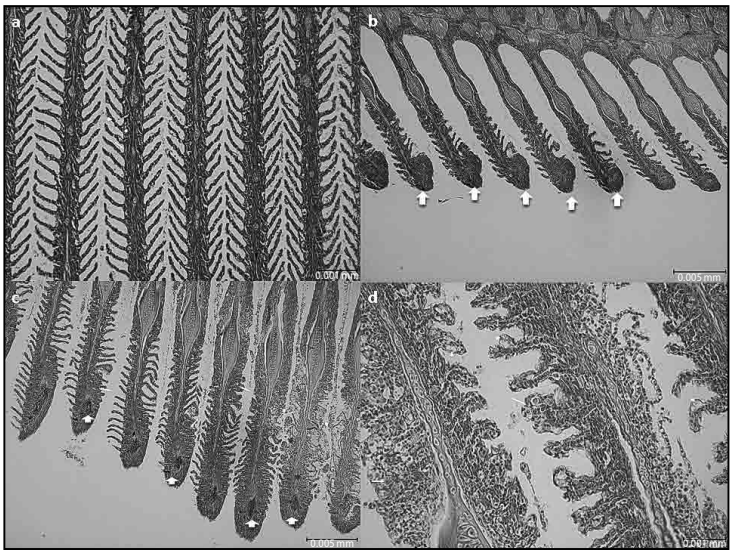

Fig. 1. Histological sections of gill of grass carp (H&E). a) Normal structure of gill; b–d) gill with histopathological alterations; b) club deformation of primary lamellae tips c) aneurysm (head arrows); fusion of some secondary lamellae (arrows); sever degeneration of the secondary lamellae (asterisks); d) hyperplasia in primary lamellae (arrows); lifting of epithelial cells (head arrows).

AST, LDH, and CK activities in fish exposed to PAA. The increase in the activities of the relevant enzymes is well-known as an indicator of physiological disorder due to stress (Velisek *et al.* 2006) reported under different contamination exposure and stress condition in fish (Banaee *et al.* 2014a; Agrahari *et al.* 2007; Almeida *et al.* 2002). Furthermore, AST is one of important enzymes involved in nitrogen metabolism, amino acid oxidation and liver gluconeogenesis. Increased levels of the aminotransferases can have a vital role in supplying energy the under the stress condition (Banaee *et al.* 2014b). LDH activity is generally associated with the reduction of pyruvate, the final product of glycolytic pathway, which allows keeping glycogenesis. This could be vital in the conditions that additional amount of energy required such as stress situation related to chemical exposure (Diamantino *et al.* 2001). The elevation of LDH may attribute to a shift from aerobic metabolism to anaerobic pathway in treated fish. CK, engaged in the regeneration of ATP by reversible transfer of phosphate from the phosphoryl group of phosphocreatine to ADP, play an essential role in providing required energy in the process of environmental adaptation (Kori-Siakpere *et al.* 2011). The reduction in SOD and CAT activities was observed in gill tissues of treated fish, whereas, GR activity was increased. PAA can produce free radicals (Booth & Lester 1995). GR is one of important enzymes engaged in the detoxification of reactive oxygen species through the reduction of oxidized glutathione (GSSG) to reduced form (GSH) (Chang *et al.* 1978). In general, high activity of GR is proposed as an indices of oxidative stress in living cells (Stageman *et al.* 1992). Increase in the activity of GR in treated fish could indicate an increment in the consumption of glutathione used by glutathione peroxidase (GPx), one of crucial enzymes involved in antioxidant system, to protect cells against oxidative stress. Weakening SOD and CAT activities observed in our study might be attributed to enhanced ROS production. The ROS overproduction could overwhelm the oxidative detoxification, resulting in decrease of antioxidant enzymes activities or inactivation of enzymes (Kono & Fridovich 1982). Furthermore, PAA causes the inactivation of catalase (Block 1983).

The data obtained for MN assay indicated that micronuclei frequency was not elevated by fish exposure to PAA. Our results are in line with the finding of exposure of common carp with PAA (Sapone *et al.* 2007) and could be a confirmation for non genotoxic effect of PAA as reported in other studies with other organisms (Maffei *et al.* 2005; Bolognesi *et al.* 2004).

The histopathological analysis of liver tissue in both control and treated fish did not show any alterations. Our data from histopathology corresponded to the constant level of ALT activity in plasma as already confirmed that ALT activity is more specific enzyme for the evaluation of liver damage than AST (Singh *et al.* 2011). Normal structure with slight variations in antioxidant enzyme activities may point out that liver is not a target organ for PAA exposure in fish.

As fish gills have an extensive surface area exposed to the external environment, they could be affected by

multiple factors in water, such as physical and chemical changes (Flores-Lopes & Thomaz 2011). There is rare published data on gill histopathological alterations due to PAA exposure of fish. The histopathological alterations including fusion, club-shape of primary lamellae tips, and lifting of epithelial cells observed in gill tissues during present study have been reported in several studies which have investigated the effects of hydrogen peroxide on fish (Tort *et al.* 2002; Rach *et al.* 1997). It is reported that the recovery of minor damage gill can begin relatively fast, but intensive damage caused by hydrogen peroxide can be recovered within weeks (Henriksen *et al.* 2014). The mechanism of action of PAA is thought to be similar to the mechanism of hydrogen peroxide (Finnegan *et al.* 2010) and since the main structure of gill revealed unchanged in most cases in our study, histological changes reported in our study may be repairable. The lesions observed in gill tissues in control group could be caused by protozoan parasites. Protozoan parasites constitute the most common parasites group encountered in fish (Omeji *et al.* 2011). Gill filament fusion, aneurism, filament hyperplasia, epithelial lifting were reported in fish infected by protozoan parasites (Khorramshahr 2012; Baticados *et al.* 1984; Singh & Kaur 2013). No parasites were found in gill of treated fish which might be due to biocide impact of PAA on protozoan parasites.

Although PAA sub-acute exposure (1 mg.l^{-1}) of grass carp can affect some enzymes of antioxidant system and induces histopathological changes in gill, it seems that these alterations are slight and can be recovered. The present results may propose that the concentration of 1 mg.l^{-1} of PAA can be applied as a therapeutic concentration in grass carp.

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CHAPTER 6

HISTOLOGICAL CHANGES AND ANTIOXIDANT ENZYME ACTIVITY IN SIGNAL CRAYFISH (*PACIFASTACUS LENIUSCULUS*) ASSOCIATED WITH SUB-ACUTE PERACETIC ACID EXPOSURE

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Histological changes and antioxidant enzyme activity in signal crayfish (*Pacifastacus leniusculus*) associated with sub-acute peracetic acid exposure

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ABSTRACT

Peracetic acid (PAA) is a powerful disinfectant recently adopted as a therapeutic agent in aquaculture. A concentration of 10 mg L^{-1} PAA effectively suppresses zoospores of *Aphanomyces astaci*, the agent of crayfish plague. To aid in establishing safe therapeutic guideline, the effects of PAA on treated crayfish were investigated through assessment of histological changes and oxidative damage. Adult female signal crayfish *Pacifastacus leniusculus* (n = 135) were exposed to 2 mg L^{-1} and 10 mg L^{-1} of PAA for 7 days followed by a 7 day recovery period in clean water. Superoxide dismutase activity was significantly lower in gill and hepatopancreas after three days exposure to 10 mg L^{1} PAA than in the group treated with 2 mg L^{-1} PAA and a control in only clean water. Catalase activity in gill and hepatopancreas remained unaffected by both exposures. Glutathione reductase was significantly decreased in gill of 10 mg L^{-1} PAA treated crayfish and increased in group exposed to 2 mg L^{-1} compared to control after 7 days exposure. Antioxidant enzyme activity in exposed groups returned to control values after recovery period. Gill, hepatopancreas, and antennal gland showed slight damage in crayfish treated with 2 mg L^{-1} of PAA compared to the control group. The extent and frequency of histological alterations were more pronounced in animals exposed to 10 mg L^{-1}. The gill was the most affected organ, infiltrated by granular hemocytes and displaying malformations of lamella tips and disorganization of epithelial cells. After a 7 day recovery period, the infiltrating cells in affected tissues of the exposed crayfish began to return to normal levels. Results suggested that the given concentrations could be applied to signal crayfish against crayfish plague agent in aquaculture; however, further studies are required for safe use.

1. Introduction

Crayfish plague, caused by the oomycete *Aphanomyces astaci*, is the most severe crayfish disease known to date and is lethal to all native European species [22]. The disease was introduced in Italy in the 1860's [15] and has been responsible for catastrophic mortality in European indigenous crayfish populations [2]. Three North American species, the spiny-cheek crayfish *Orconectes limosus*, signal crayfish *Pacifastacus leniusculus*, and red swamp crayfish, *Procambarus clarkii*, known as carriers of *A. astaci* playing a major role in its spread and transmission to native crayfish species [45]. These invaders are widely distributed while the number of newly confirmed North American crayfish plague carries is increasing [45]. Moreover, the range of *A. astaci* hosts is wider than previously expected, covering also freshwater crabs [37,41,43] and shrimps [44].

In Europe, numerous chemicals and disinfectants have been employed in commercial crayfish culture and in attempts to prevent the spread of the disease to native crayfish populations [28,36]. Crayfish suffer variety of further diseases, but suitable therapies are often missing [18].

Peracetic acid (PAA) is a strong oxidizing agent that is widely used as sanitizer and disinfectant agent in laboratory facilities, medical settings, food and beverage processing, and pulp and textile industries [34]. It is widely used in aquaculture to combat a wide spectrum of fish pathogens [35]. Recently it has been reported that a concentration of 10 mg L^{-1} of PAA can effectively suppress the pathogenicity of *A. astaci* zoospores [27] and further

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applications have been suggested for crayfish culture [31].

Signal crayfish have been introduced into European freshwater bodies as suitable replacement of lost populations of European noble crayfish, *Astacus astacus* considering occupation of similar ecological niches and increased plague resistant [47]. Since signal crayfish vulnerability to crayfish plague agent [3], the proposed concentration can potentially be used for disinfection of the water containing the *A. astaci* spores as well as treatment of signal crayfish against *Aphanomyces astasi* in crayfish culture. For establishing safe therapeutic guidelines, it is necessary to understand the effects of the given concentration of PAA on the health status of treated animals. However, the concentration is lower than the reported LC50 > 70 mg L^{-1} for signal crayfish adults [31]. Furthermore, PAA use in aquaculture is recent, limited toxicity data exist on aquatic species [14,19,40]. Further data on toxicological effects to different species is still required to confirm its safe use in aquaculture.

Increased levels of oxidative stress in an organism can lead to reduced survival and reproductive capacity. Thus, oxidative damage can be considered a relevant marker of general health status [6]. Proposed oxidative indicators include antioxidant enzyme activity, lipid peroxidation products, and glutathione redox status [33]. Histopathological lesions are also considered sentinel indicators of a stressed condition, as they can provide visible biological endpoints of chemical exposure and stressors [39].

The present study evaluated oxidative damage to gill and hepatopancreas and histological alterations in gill, hepatopancreas, and antennal gland of signal crayfish exposed to sub-acute levels of PAA.

2. Materials and methods

2.1. Crayfish and experimental conditions

Adult intermoult female signal crayfish *P. leniusculus* (n = 135) were caught from the wild populations in the Vysočina Region. They were transferred into nine 350 L glass aquaria (98 × 64.5 × 100 cm; used water volume 100 L) (15/tank) acclimated for a period of 5 days prior to exposure. To reduce aggressive behaviors, polypropylene shelters (2.6 pcs per animal) were provided. The aged tap water was used with basic parameters as follows: temperature 21 ± 1 °C; total ammonia 0.02 mg L^{-1}; NO_3^- 1.55 mg L^{-1}; NO_2^- 0.006 mg L^{-1}; chemical oxygen demand 0.6 mg L^{-1}, PO_4^{3-}, 1 ± 0.0 mg L^{-1}, Ca^{2+} 32.9 ± 0.8 mg L^{-1}, and Mg^{2+} 2.9 ± 0.0 mg L^{-1}. Crayfish were fed once a day during the adaptation phase until 24 h before initiation of the trial. The animals were not fed during the trial. The mean weight and carapace length of the crayfish were 28.5 ± 9.7 g and 44.3 ± 4.7 mm, respectively.

Crayfish were exposed to 2 mg L^{-1} and 10 mg L^{-1} of peracetic acid, for 7 days with trials conducted in triplicate. Required amounts of PAA were applied twice a day to maintain the appropriate concentration of PAA. Prior to applying of PAA water in each aquarium was renewed. The concentration of PAA was measured immediately after application of PAA in each aquarium using a RQflex®plus reflectometer and Reflectoquant® Peracetic Acid (Merck KGaA, Darmstadt, Germany) test strips with a metering capacity of 1–22.5 mg L^{-1} PAA. The measured concentrations were >80% of nominal concentration (data not shown). The experiment was followed by a 7 day recovery period in clean water. The control group was kept in clean water with the same water exchanging as trial groups.

2.2. Antioxidant enzyme activity

Hepatopancreas and gill samples were obtained at three and 7 days exposure to PAA and after 7 days recovery period, immediately frozen, and stored at −80 °C. The frozen samples were homogenized in 50 mM potassium phosphate buffer (KH_2PO_4 with 1 mM EDTA, pH 7.4) and centrifuged at 30,000 × rpm for 30 min. The supernatants were used for quantification of catalase (CAT) and superoxide dismutase (SOD) activity. Total SOD activity was determined by the inhibition of reduction of nitro-blue tetrazolium (NBT) to phenazine methosulfate (PMS) with nicotinamide adenine dinucleotide (NADH). Activity was assessed spectrophotometrically at 560 nm and expressed as nmol of NBT per min per mg protein [20]. CAT activity was determined using the method described by Ref. [1] based on the reduction of absorbance at 240 nm due to the decomposition of hydrogen peroxide by CAT. To determine glutathione reductase (GR) activity tissues were homogenized in phosphate saline buffer at pH 7.2 and centrifuged at 10,000 × g for 15 min, and the supernatant was used for assay of GR activity, measured according to the rate of NADPH oxidation at 340 nm [12]. Total protein concentration in each sample was calculated according to the Bradford method using bovine serum albumin as a standard [10].

2.3. Histological analysis

Samples of gill, hepatopancreas, and antennal gland were collected from five crayfish after three and 7 days of exposure and at the end of the recovery phase. Tissue samples were fixed in Davidson's fixative overnight and then transferred to 70% ethanol. The tissues were processed using routine histological techniques, embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin and eosin for examination by light microscopy [5].

2.4. Statistical analysis

Data was expressed as mean ± SD. STATISTICA version 12.0 for Windows (StatSoft, Inc.) was used to perform the statistical analysis. Kolmogorov–Smirnov and Bartlett's tests were applied to assess data for normal distribution and homoscedasticity of variance respectively. For data with normal distribution, one-way ANOVA was used. In case of non-normal distribution, a non-parametric Kruskal–Wallis test was performed. The significance level for all tests was $P < 0.05$.

3. Results

3.1. Antioxidant enzyme activity

After three days exposure, SOD activity in hepatopancreas of both PAA treated groups was significantly lower than control ($P < 0.05$; Table 1). In gill of crayfish treated with 10 mg L^{-1} PAA, SOD showed lower activity compared to controls after three days treatment. There was no significant difference in CAT activity in tissues of exposed crayfish compared to controls (Table 2). GR activity was significantly higher ($P < 0.05$) in hepatopancreas of crayfish exposed to 2 mg L^{-1} PAA after 7 days treatment. After 7 days exposure, GR activity in gill was significantly lower ($P < 0.05$) in crayfish treated with 10 mg L^{-1}, while it was higher in gill of crayfish exposed to 2 mg L^{-1} compared to controls (Table 3). After the recovery period, antioxidant enzyme activity in PAA-treated crayfish did not show significant differences from controls in any tissue.

3.2. Histology

3.2.1. Gill pathology

Generally, no abnormalities or lesions were observed in gill of untreated crayfish. A thin cuticle covered the lamella which is lined

Table 1
SOD activity (nmol NBT/min/mg protein) in gill and hepatopancreas, after 3 days, 7 days and during the recovery phase.

	Gill			Hepatopancreas		
	3 days Exposure	7 days Exposure	Recovery period	3 days Exposure	7 days Exposure	Recovery period
Control	0.08 ± 0.02[a]	0.02 ± 0.01[a]	0.07 ± 0.02[a]	0.16 ± 0.02[a]	0.03 ± 0.01[a]	0.03 ± 0.01[a]
2 mg L^{-1}	0.07 ± 0.02[a]	0.01 ± 0.00[a]	0.05 ± 0.03[a]	0.10 ± 0.02[b]	0.02 ± 0.01[a]	0.02 ± 0.01[a]
10 mg L^{-1}	0.04 ± 0.01[b]	0.01 ± 0.01[a]	0.06 ± 0.02[a]	0.03 ± .008[b]	0.03 ± 0.01[a]	0.01 ± 0.01[a]

*Different letters in columns show significant difference ($P < 0.05$).

Table 2
CAT activity (μmol H_2O_2/min/mg protein) in gill and hepatopancreas after 3 days, 7 days, and after the recovery phase.

	Gill			Hepatopancreas		
	3 days Exposure	7 days Exposure	Recovery period	3 days Exposure	7 days Exposure	Recovery period
Control	0.16 ± 0.10[a]	0.12 ± 0.07[a]	0.29 ± 0.17[a]	0.4 ± 0.16[a]	0.65 ± 0.13[a]	0.5 ± 0.2[a]
2 mg L^{-1}	0.19 ± 0.10[a]	0.16 ± 0.15[a]	0.22 ± 0.06[a]	0.61 ± 0.18[a]	0.49 ± 0.33[a]	0.4 ± 0.2[a]
10 mg L^{-1}	0.26 ± 0.10[a]	0.21 ± 0.07[a]	0.24 ± 0.08[a]	0.79 ± 0.38[a]	0.32 ± 0.16[a]	0.25 ± 0.19[a]

*Different letters in columns show significant difference ($P < 0.05$).

Table 3
GR activity (nmol NADPH/min/mg protein) in gill and hepatopancreas after 3 days, 7 days, and after the recovery phase.

	Gill			Hepatopancreas		
	3 days Exposure	7 days Exposure	Recovery period	3 days Exposure	7 days Exposure	Recovery period
Control	0.04 ± 0.01[a]	0.07 ± 0.05[b]	0.06 ± 0.01[a]	0.23 ± 0.17[a]	0.057 ± 0.03[a]	0.06 ± 0.03[a]
2 mg L^{-1}	0.088 ± 0.06[a]	0.11 ± 0.04[a]	0.03 ± 0.03[a]	0.11 ± 0.06[a]	0.24 ± 0.10[b]	0.11 ± 0.04[a]
10 mg L^{-1}	0.04 ± 0.01[a]	0.02 ± 0.01[c]	0.03 ± 0.01[a]	0.11 ± 0.06[a]	0.17 ± 0.11[a]	0.10 ± .08[a]

*Different letters in columns show significant difference ($P < 0.05$).

by a single layer of epithelial cells. Each filament contains an afferent and efferent vessels separated by a fine septum (Fig. 1A). Gill of crayfish treated with 2 mg L^{-1} exhibited normal structure after three days exposure, while disorganization of epithelial cell was observed in gill tissue after 7 days. Gill of crayfish exposed to 10 mg L^{-1} displayed hemocyte aggregation in vessels, infiltration of granular hemocytes, malformations of lamella tips, and disorganization of epithelial cells (Fig. 1B–D). The extent and frequency of histological alterations were more pronounced after 7 days exposure in comparison with those treated for three days. After the 7 days recovery period, gills of treated crayfish showed some histological changes such as disorganization of epithelial layer and hemocyte aggregation, whereas some parts exhibited the normal structure (Fig. 1E and F). However, histological alterations were not as extensive as in crayfish exposed to 10 mg L^{-1} of PAA.

3.2.2. Antennal gland pathology

The structure of the antennal gland in control group consists of the coelomosac, labyrinth, and two nephridial parts including proximal and distal tubules (Fig. 2A). The gland tissues showed normal structure after three days exposure at both tested concentrations. After the 7-day exposure 10 mg L^{-1} PAA, antennal gland tissue displayed infiltration of hemocytes (Fig. 2B).

3.2.3. Hepatopancreas pathology

The hepatopancreas is composed of numerous blind-end tubules and connective tissue. The lumen of each tubule is surrounded by four types of epithelial cells: secretory (B), absorptive (R), fibrillar (F), and embryonic (E) (Fig. 3A). B-cells, located mainly in distal part of tubules, are the largest cells and contain a large vacuole with a basal nucleus. R-cells, distributed throughout the tubule, are distinguished by the presence of numerous small lipid vacuoles. F-cells show a fibrillar appearance and usually a central oval nucleus. E-cells are cuboidal and occur primarily in the distal part of hepatic tubules.

No differences were observed between hepatopancreas tissue of controls and the group exposed to 2 mg L^{-1} PAA. Hepatopancreas from crayfish exposed to 10 mg L^{-1} PAA exhibited enlargement of tubular lumen and increased hepatocyte vacuolation after 7 days exposure (Fig. 3B).

4. Discussion

We found the gill to be the main organ affected by sub-acute PAA exposure, with significant reduction in SOD activity after three days treatment at 10 mg L^{-1}. The degradation of PAA to hydrogen peroxide could be the source of the decreased SOD activity in gill, as high concentrations of hydrogen peroxide can strongly inhibit SOD activity [30]. SOD catalyses dismutation of superoxide ions to hydrogen peroxide, and CAT is responsible for neutralization of hydrogen peroxide [42]; therefore an intrinsic link between SOD and CAT activity is expected. However, we did not observe such correlation. The enzymes of the antioxidant defense system can interact for mutual modulation of activity [16]. Similar divergent SOD and CAT levels have been reported in other studies. For example, increased SOD level, but unaffected CAT activity, was observed in digestive gland of the freshwater mussel *Dreissena polymorpha* exposed to mercury chloride, methyl mercury chloride, cadmium, and Aroclor1260 for five days [21]. We observed lower GR activity in gill of crayfish in the higher PAA concentration group. The reduction could be due to oxidative damage. Research has shown GR to be highly susceptibility to oxidative damage resulting from un-neutralized free radicals generated *in vitro* and *in vivo* [4,25,46]. The low activity of SOD and GR may induce oxidative damage. It is generally accepted that hydrogen peroxide can penetrate membranes by free diffusion and is scavenged by CAT and glutathione peroxidase under normal conditions [9]. Excess hydrogen peroxide can react with free Cu and Fe in the cytosol and

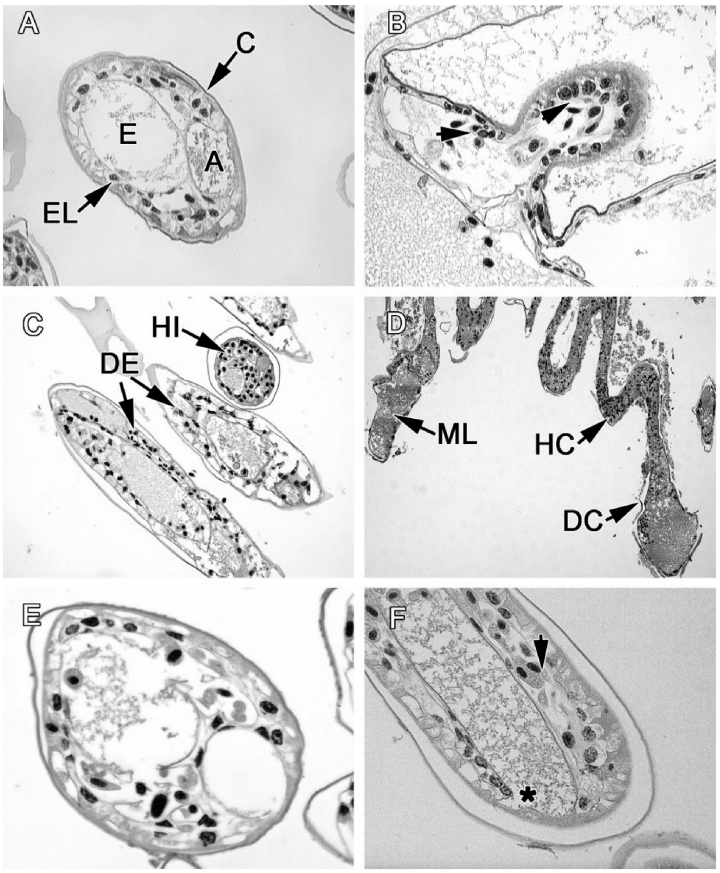

Fig. 1. Representative photomicrographs of H&E-stained gill sections. (A) Gill lamella in controls showing normal structure with uniform arrangement. Efferent vessel (E), afferent vessel (A), epithelium layer (EL), cuticle (C) (magnification ×40). Histological changes in gill lamellae of crayfish exposed to 10 mg L^{-1} of PAA for 7 days: (B) infiltration of granular hemocytes (arrows) (magnification ×40), (C) hemocyte infiltration (HI) in hemocoel and disorganization of epithelium layer (DE) (magnification ×20), (D) exhibiting malformations in tips of lamellae (ML), hemocytic aggregate in vessels (HC) and disruption of cuticle (DC) (magnification ×10). Gill lamella of crayfish after recovery period: (E) normal structure of lamellae (magnification ×40) and (F) hemocytic aggregate (arrow), disorganization of epithelium layer in some part (asterisk) (magnification ×40).

form hydroxyl radicals leading to damage in macromolecules [13].

Based on the histopathological results, gill of the experimental group exposed to 10 mg L^{-1} PAA displayed some alterations, including hemocytic infiltrations, abnormal lamella tips, and disorganization of epithelial cells. Previous studies have reported histological changes in gill of crustacean exposed to toxic chemicals and stress conditions [7,8,17,26,32]. Hemocyte infiltration may occur under stress to provide an alternative source of energy for cells by release of sugar from their granules or glycogen sources [11]. Disorganization of epithelial cells and abnormalities in lamella structure are known physiological adaptation responses to stress [38]. The changes observed in gill structure in this research could serve as a compensatory response to exposure to PAA. In crustaceans, the gill is a multifunctional organ that plays a vital role in physiological processes such as ion transport, acid-base balance, and ammonium excretion [23]. Therefore, the alterations observed in gill morphology and histology in crayfish exposed 7 days may lead to disruption of the physiological function of the gill, consequently, threaten their overall health [29]. However, our results showed reduction in histopathological alterations of gill after 7 days in clean water, suggesting that damages could be reversible with sufficient recovery period.

The histological examination showed the alterations in hepatopancreas of crayfish exposed to PAA after 7 days. These changes could alter the functional cytology of the hepatopancreas and may influence the metabolic activity. Since hepatopancreas serves as the main energy reserve for growth and moulting [49], its impairment can affect health status. The lesions described in hepatopancreas and antennal gland could be considered a secondary response to PAA treatment related to impaired health status. The PAA is

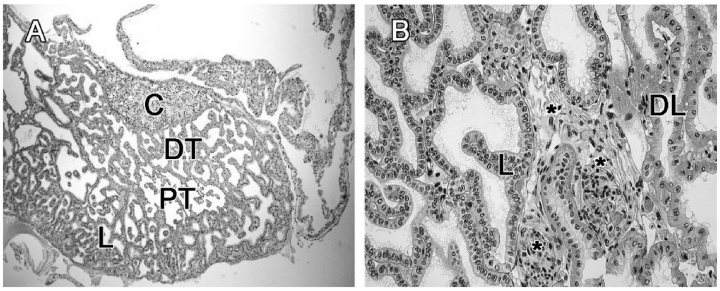

Fig. 2. Representative photomicrographs of H&E-stained antennal gland tissue. (A) Intact structure illustrating coelomosac (C), distal tubules (DT), proximal tubules (PT), labyrinth (L) (magnification ×4). (B) Hemocytic infiltration (asterisks) between distal tubules (DT) and labyrinth (L) after 7 days exposure to 10 mg L^{-1} of PAA (magnification ×40).

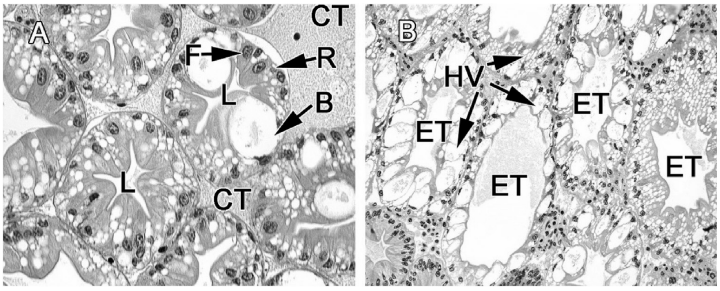

Fig. 3. Transverse H&E-stained sections of hepatopancreas tissue. (A) Hepatopancreas from control group with normal structure of tubules and connective tissue (CT), lumen (L), F-cell (F), B-cell (B), R-cell (R) (magnification ×40). (B) Extensive hepatocyte vacuolation (HV) and enlargement of tubules (ET) after 7 days exposed to 10 mg L^{-1} PAA (magnification ×40).

degraded into harmless residuals acetic acid and H_2O_2 and eventually to water in a dose dependent half-life. The approximate half-life of PAA for low (2 mg L^{-1}) and high (20 mg L^{-1}) concentrations are 24 min and 6–7 h in freshwater, respectively [24,35]. In addition, a hard exoskeleton protects internal organs as well as crayfish does not drink water. Thus, the internal organs, antennal gland and hepatopancreas, would not be directly exposed to PAA.

The results of this study indicated that the concentration of 10 mg L^{-1} of PAA could be proposed as a safe therapeutic for treatment of signal crayfish against *A. astaci*, with a sufficient recovery period to allow reversal of damage to gills and hepatopancreas. Moreover, considering PAA concentrations proposed for fish aquaculture and suggested applications in crayfish culture [31], even concentration of 2 mg L^{-1} PAA could be effective against some diseases. However, species-specific differences should be considered while treating more sensitive crayfish such as the European astacids. Toward establishing a safe application guideline, more studies will be required to investigate the given issues.

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CHAPTER 7

GENERAL DISCUSSION

Toxicity testing of chemical pollutant and therapeutic compounds have evolved into a battery of standardized tests conducted in laboratory organisms to make sure those chemicals are safe. The pollutants in the environmental media and therapeutic chemicals may have a negative impact on biological systems which can be monitored through endpoints based on blood chemistry, biochemical biomarkers, histopathological criteria, and body and organ weights. Thanks to advances in scientific knowledge as well as analytical techniques, an increasing importance is directed to characterise the mode of action to identify the biochemical pathways that link exposure to a chemical with predictive consequences. The comprehensive, validated and up-to-date databases are the essential foundation for the development of the strategies in environmental risk assessment as well as new therapeutic chemicals with higher benefit/risk ratio.

Assessment of cellular and physiological response of common carp (*Cyprinus carpio* L.) to dietary ZnO NPs toxicity on

Investigation of tissue accumulation and physiological responses induced by dietary administration of ZnO NPs in carp

Our results did not show any accumulation of zinc in tissues of treated fish. Fish may be able to adapt with the higher amounts of zinc in thier diet and reduce its concentration to normal levels. The maintenance of zinc homeostasis in teleost fish is more complicated than in mammals considering that there are more variabilities in aquatic environment (Jiang et al., 2014). Fish can maintain a constant state of total body zinc content over a wide range of concentrations by either regulating zinc absorption from diet or excretion of extra zinc from gills or kidneys (Bury et al., 2003).

We did not show that a diet-borne metal accumulates, in contrast to the consensus view. Based on this view, metals are absorbed via intestinal mucus and then transported to the liver, and subsequently to other internal organs (Ramsden et al., 2009). Our observation found no accumulation of zinc in the liver. The first sampling time point in the present study was 2 weeks after exposure, thus, we hypothetize that a homeostasis process in the liver was regulated and could eliminate the extra amount of zinc in less than 2 weeks. The results of ZnO NPs dietary exposure in rainbow trout could support this idea. Connolly et al. reported significant bioaccumulation of zinc in gills and intestine but no accumulation in liver after 10 days dietary exposure to ZnO NPs (Connolly et al., 2016).

The histological analysis showed some alterations in kidneys in higher treatment group during experiment. Tubular regeneration observed after the recovery period may be a compensatory response of organism to nephrotoxicity induced during exposure period. The finding may support the idea that kidney function was affected during exposure to ZnO NPs. Studies related to histopathological changes in the kidneys of fish from nanoparticle exposure are limited. Similar to our observations, histological alterations, including deformations of the renal tubule epithelium and necrosis, have been observed in the kidneys of tilapia (*Oreochromis niloticus*) following exposure to ZnO NPs (Kaya et al., 2016). The alterations could be due to the role of kidney in zinc excretion and/or elimination. Our results may indicate that the kidney has a major role in zinc homeostasis in diet-borne zinc exposure in carp; however, a more detailed study is needed.

Our results did not show significant disturbances in haematological, blood biochemical parameters, and lipid peroxidation in carp during 6-week exposure to ZnO NPs. The results suggest that dietary ZnO NPs are not sufficiently potent in the studied doses to affect blood in haematological, biochemical parameters and the level of lipid peroxidation.. This agrees with the findings for dietary exposure to TiO2 for rainbow trout (*Oncorhynchus mykiss*) (Ramsden et al., 2009).

After a 2-week recovery period, in both treatments (50 and 500 $mg.kg^{-1}$), a significant reduction in the level of TBARS was obsereved. It is difficult to explain reduction in the levels of lipid peroxidation in liver after recovery period because we did not measure other antioxidant parameters. However, we hypothesize that those reductions might be related to an adaptive process in this organ which enhanced antioxidant defense.

The level of aspartate aminotransferase: alanine aminotransferase (AST:ALT) ratio increased in higher concentration group and the level of AST rise in lower concentration group after recovery period. This, together with a lack of histopathological changes in liver of treated fish after recovery period, may indicate that the modification in zinc homeostasis could affect liver biochemical function which led to imbalance in AST and ALT level.

ZnO NPs dietary exposure and susequent changes in serum protein profile

Serum is the most commonly used clinical sample for evaluation of health status and recently has gained great attention for the screening of biomarkers for various diseases and chemical exposures. We conducted the current experiment to assess weather ZnO NPs can induce some modification in protein level in common carp serum through dietary exposure despite no observed effect in blood biochemical parameters. The proteins and canonical pathways which may differentially regulate, provide information for exploring the molecular cascades underlying the toxicity mechanism of ZnO NPs.

The results showed significant changes in the abundance of four proteins in serum after a 6-week exposure to 500 $mg.kg^{-1}$ ZnO NPs. Our findings showed a decreased level of MHC class-I which has a fundamental role in initiating adaptive immune response against intracellular pathogens through binding to peptides mainly derived from viral proteins (Kloetzel and Ossendorp, 2004). Sub-toxic concentration of ZnO NPs have been shown to affect the adaptive immune system by down regulation of CD16 expression in NK-cells in primary human peripheral blood mononuclear cells (Andersson-Willman et al., 2012). Impaired response of adaptive immune could result in escape of infected or affected cells from the specific immune response that may lead to health impairment and lower chance of survival. On the other hand, we also observed an increased level of complement c4-2 in treated fish which is the ortholog for mammalian C4B (Boshra et al., 2006). The C4B protein in mammals is crucial for the activation of the complement pathway via propagation of the classical and the mannose-binding lectin complement pathways (Rupert et al., 2002). Thus, its increased level could induce activation of the complementary system in fish treated by ZnO NPs. Activation of complement system in human serum through C4 cleavage was induced by poly(ethylene glycol)-stabilized single-walled carbon nanotubes (Hamad et al., 2008). Activation of the complement system is tightly regulated in normal conditions because its hyperactivation potentially induces devastating effects on the healthy host tissues (Dunkelberger and Song, 2010).

Ingenuity pathway analysis revealed four canonical pathways including acute-phase response signaling, liver and retinoid X receptors activation, and intrinsic and extrinsic prothrombin activation pathways as significantly regulated in the treated fish. The all mentioned pathways and modified proteins are strongly interconnected and share proteins whose expressions are

modulated during host defense response to homeostasis disturbing agents, in an attempt to minimize adverse effects and maintain homeostasis in balance. Therefore, ZnO NPs exposure to common carp probably disturbs the fish homeostasis by affecting proteins of the haematological and the immune systems.

Insight into modulation of intestinal proteome of juvenile common carp after exposure to dietary ZnO NPs

Our results did not show a zinc accumulation and changes in histological level in intestine during 6 weeks of exposure; whereas, we observed some changes in protein level in serum. Based on this, we aimed to investigate whether the presence of ZnO NPs in feed can induce modification in the intestines at molecular level. A label-free protein quantification approach was applied to assess the sub-lethal effects of dietary exposure to ZnO NPs on proteome profiles of two parts of juvenile carp intestine including intestinal folds and muscular layer.

In both intestinal folds and muscular layer, our results revealed some down-regulated proteins, albeit different in each part, as well as negatively regulated pathways which were involved in the initiation of translation and protein synthesis and other metabolic process. This inhibition could be an adaptive response indicating the molecular mechanism behind the DNA damage which may be induced in cells exposed to ZnO NPs. It could act as a double-edge sword in cell fate decisions. While its initial inhibition could be cytoprotective as a result of reduced ER stress, prolonged or unresolved inhibition could drive cells to undergo apoptosis. Our observation showed increased level of proteins associated with DNA damages and early apoptotic process in intestinal folds. The TUNEL assay also confirmed apoptosis in ZnO NPs treated fish compared to control. It seems that presence of ZnO NPs in pellets have the capability to induce apoptosis in intestinal cells.

However, apoptosis could be hampered in the absence of *de novo* protein synthesis by inhibition of the proteasome degradative pathway (Bannerman et al., 2001). In muscular layers, our results indicated a significant reduction in the level of proteasome subunit beta type, one of subunits of 20S catalytic core complex of the proteasome 26S which may result in inhibition of proteasome activity and could also cause a failure in apoptosis induction, and thereby supporting hypothesis of carcinogenic potential of ZnO NPs on intestine muscular layer. In keeping with this, we observed the elevated level of proteins involved in cell proliferation and known to be oncogenes. The enhanced in vitro malignant cell transformation in non-tumorigenic BEAS-2B cells was reported after long-term exposures to low doses of silver nanoparticles (Choo et al., 2016). This findings could provide useful information for required assessment of the carcinogenic potential of ZnO NPs.

Our finding showed that the presence of ZnO NPs in fish pellets seems to have affected mitochondria function in intestinal folds of treated fish considering down-regulation of succinate-CoA ligase, subunit beta and tumor necrosis factor receptor-associated protein 1 (TRAP1). Succinate-CoA ligase is a mitochondrial enzyme operating within the Krebs cycle and TRAP1 is a molecular chaperon involved in protection of mitochondria against damaging stimuli by reducing ROS production (Tian et al., 2014). Thus, their suppression may result in mitochondrial dysfunction which could contribute to prompt the demise of the cell.

Our results displayed changes in the abundances of several cytoskeleton related proteins in both studied parts. In treated intestinal folds, our result showed increased level of β-tubulin and down-regulation of chaperonin containing TCP-1 (CCT), subunit 2 (beta). Excess β-tubulin is known to induce microtubule depolimerization in several ways (Kappas, 1990). Depletion of CCT complex also seems to perturb both microfilament and microtubule-based cytoskeleton activities. Because CCT functions as a protein folding machine for *de novo*

unfolded polypeptides such as actin and tubulin (Lin et al., 2012). Inhibition of β-tubulin folding could also disrupt tubulin dimer formation and microtubule assembly. Thus, the presence of misfolded tubulin may be linked to decreased stability of the microtubule network. Microtubule disruption could affect intracellular tensile and may hamper directional cell motility by preventing the cells from establishing the forward-reverse polarity (Elbaum et al., 1998). Reduction of microtubule stabilization activates Rho which induce stress fiber (Small et al., 2002). Regarding modified proteins in treated fish, the possible polymerization of actin filaments scenario could be a contribution of *Icmt* in activation of Rho GTPase family. It subsequently mediates actin polymerization through a profilin-dependent mechanism. The ability of ZnO NPs to induce cytoskeleton re-organization in the current study fits well with previous studies where different NPs were capable of inducing cell shape changes (Goncalves and Girard, 2014) as well as limited cell migration through microtubule destabilization and increased contractility (Tay et al., 2013).

In muscular layer, the pathway analysis revealed the signalling pathways associated with the regulation of the actin cytoskeleton such as positive regulation of RhoGDI signalling and negative regulation of Rho A signalling, which may suggest affecting of motility of intestinal muscular after dietary exposure to ZnO NPs. RhoGDIs have been identified as negative regulators of Rho GTPases and microinjection of RhoGDI into fibroblasts resulted in inhibition of motility (Takaishi et al., 1993). Rho A belongs to a family of small GTPases which has a critical role in the smooth muscular motility and contraction through cytoskeletal reorganization (Wang and Bitar, 1998). Therefore, it seems cell motility in muscular layer affected as intestinal folds, albeit through different mechanisms.

Our observation demonstrated inhibition of Rho and actin polarization in muscular layer which is opposite to intestinal folds. Furthermore, increase levels of proteins related to cell survival were demonstrated in muscular rather than induced apoptosis in intestinal epithelium. These opposing results might be associated with differences among target cells or may attribute to difference in exposure route between intestinal folds and muscular layer. Even if ZnO NPs seems to have potential carcinogenic effect on muscular layer of exposed fish, we cannot exclude the possibility of Zn^{2+} release inside cells, which could be another potential mechanism of their toxicity.

Peracetic acid safety evaluation

Biochemical and histological biomarkers to evaluate effects of sub-acute PAA exposure on grass carp (*Ctenopharyngodon idella*)

No mortality was observed in fish exposed to 1 $mg.L^{-1}$ of PAA. Our results indicated higher aspartate aminotransferase, lactate dehydrogenase, and creatine kinase activities in both treated groups compared to controls. The increase in the activities of the relevant enzymes is well-known as an indicator of physiological response for supplying energy in response to stress and have been reported under different contamination exposures and stress condition in fish (Agrahari et al., 2007; Almeida et al., 2002; Banaee et al., 2014). The reduction in superoxide dismutase (SOD) and catalase (CAT) activities were observed in gills of treated fish; whereas, glutathione reductase (GR) activity was increased. In general, high activity of GR is proposed as an index of oxidative stress in living cells (stegemenj et al., 1992). Increase in the activity of GR in treated fish could be compensatory response to increased consumption of reduced glutathione by antioxidant enzymes to protect cells against oxidative stress. Decreased SOD and CAT activities might be attributed to enhanced ROS production. ROS overproduction

could overwhelm the oxidative detoxification resulting in decrease of antioxidant enzymes activities or inactivation of enzymes (Kono and Fridovich, 1982).

The histological analysis did not show changes in the liver, while fused, club-shaped primary lamellae tips, and lifting of epithelial cells were observed in treated gills. The alterations have been reported in several studies which investigated the effects of hydrogen peroxide on fish (Rach et al., 1997; Tort et al., 2002). Hydrogen peroxide is one of PAA degradation products in water, thus exposure of gills to that compound could induce the histological alteration. It is reported that the recovery of minor damage in gills can begin relatively fast, but intensive damage caused by hydrogen peroxide can only be recovered within weeks (Henriksen et al., 2015). The mechanism of action of PAA is thought to be similar to the mechanism of hydrogen peroxide (Finnegan et al., 2010). Accordingly, the intact main structure of the gills in most cases in our study, suggests that histological changes are reversible. Although PAA sub-acute exposure (1 $mg.L^{-1}$) in grass carp can affect some enzymes of antioxidant system and induces histopathological changes in gills, it seems that these alterations are not severe and can recover. The present results may propose that the concentration of 1 $mg.L^{-1}$ of PAA can be applied as a therapeutic concentration in grass carp.

Biochemical and histological biomarkers to evaluate effects of sub-acute PAA exposure on crayfish

Ten $mg\ L^{-1}$ of PAA proposed as effective dose for suppression of crayfish plague, *Aphanomyces astaci* spore germination, is a higher concentration than generally used for wastewater disinfection (0.2–1.3 $mg.L^{-1}$) and is considerably higher than has been applied in aquaculture (0.2–1.3 $mg\ L^{-1}$) (Jussila et al., 2011). Although the concentration is lower than the reported LC50 > 70 $mg.L^{-1}$ for signal crayfish (Kouba et al., 2012). For establishing safe therapeutic guidelines, it is necessary to understand the effects of a given concentration of PAA on the health status of treated crayfish. Therefore, we assess the health status of healthy signal crayfish (*Pacifastacus leniusculus*) through oxidative stress and histological biomarkers. We found the gills to be the main organ affected by sub-acute PAA exposure, with significant reduction in SOD and GR activities after three and a seven days treatment at 10 $mg.L^{-1}$. The degradation of PAA to hydrogen peroxide could be the source of the decreased SOD activity in gills, as high concentrations of hydrogen peroxide can strongly inhibit SOD activity (Kosenko et al., 1997). The lower GR activity could be due to oxidative stress. Research has shown GR to be highly susceptibility to oxidative damage resulting from un-neutralized free radicals generated *in vitro* and *in vivo* (Barker et al., 1996; Huang and Philbert, 1996; White et al., 1999). The low activity of SOD and GR may result in excess free radicals which can react with macromolecules and induce oxidative damages.

Gills of the experimental group exposed to 10 $mg.L^{-1}$ PAA displayed some alterations, including haemocytic infiltrations, abnormal lamella tips, and disorganization of epithelial cells. Similar alterations have been reported in crustaceans exposed to toxic chemicals and stress conditions (Desouky et al., 2013; Ikerd et al., 2014). Haemocyte infiltration may occur under stress to provide an alternative source of energy for cells by release of sugar from their granules or glycogen sources (Bubel, 1976). Disorganization of epithelial cells and abnormalities in lamellar structure are known physiological adaptation responses to stress (Stalin et al., 2013). Our results showed reduction in histopathological alterations of gills after 7 days in clean water. This may suggest that damage is reversible with sufficient recovery period. However, the alterations observed in gill morphology and histology in crayfish exposed 7 days may lead to impairment of vital gill function such as osmoregulation and ion transfer. According our observation, the concentration of 10 $mg.L^{-1}$ of PAA could be proposed as

a therapeutic treatment of signal crayfish against A. astaci, with a sufficient recovery period to allow reversal of histological damage.

Conclusion

Although response of aquatic organisms at biochemical and histological levels are known as reliable biomarkers of aquatic pollution. But molecular response have the potential to identify the earliest events in the natural history of exposure and can open a window to potential mechanisms related to the pollutant exposure which may lead to modification of risk prediction. As our finding showed that the apparent lack of bioaccumulation of ZnO NPs and their low acute toxicity do not mean that there are no toxicological concerns. We reported a range of important sub-lethal effects on serum and intestine proteome of fish treated with ZnO NPs. Our results revealed that dietary exposure to ZnO NPs could induce substantial changes in the regulation of proteins and may subsequently in several important biochemical pathways. The ZnO-NP-induced biological responses include affecting immune response, disruption in cell metabolism, relatively weak oxidative stress response, and limited cell motility in intestine in treated fish. Our findings provide insight into the possible mechanisms involved in ZnO NPs toxicity in fish intestine as well as has highlighted a need for further investigations for their safe application particularly in food industry.

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ENGLISH SUMMARY

Physiological and molecular responses of aquatic organisms to chemicals exposure

The biochemical and physiological responses of organisms to xenobiotics have been investigated for many years as general indicators of health status of an organism. These markers have been used in the development of the synthetic pharmaceuticals and screening effects of environmental pollutants on biological systems, and in clarifying their modes of action. Recently, use of "OMICS" approaches has received great attentions in exploring the effects of chemical contaminants at the molecular level and is one of the rapidly developing areas in the field of toxicology. Use of molecular response within aquatic organisms has been reviewed and are considered as early changes occurring in response to chemical exposure.

ZnO nanoparticles (NPs) are widely used in various fields of industry and possess great potentials in food industry and agriculture. Their subsequent release into environment has raised concerns about potential effects on aquatic organisms. Although, the dietary exposure perhaps is the main route of exposure of aquatic animals to nanomaterials, the majority of studies have focused on assessment of waterborne exposure. The molecular mechanisms inducing their toxicity on biological system even less investigated. The present study was conducted to explore whether ZnO NPs can be accumulated in internal organs of carp, used as a model fish species, as well as to assess fish responses to the presence of ZnO NPs in the feed at protein, biochemical, and histological levels. Our results showed neither apparent accumulation of ZnO NPs nor major changes in haematological parameters, lipid peroxidation level, and histology of internal tissues. Our observations showed that 500 mg ZnO NPs per kg of feed caused changes in the level of proteins associated with cell motility, immune system response, protein synthesis, cell metabolism, and cell survival in intestine. Moreover, it affected the proteins related to immune system in serum. These changes can be considered as compensatory mechanisms to maintain homeostasis after exposure. If energetic demands costs for coping with stressor exceed the capacity of limit, then health of the exposed organism will be negatively affected.

In the second part of study, peracetic acid in was tested therapeutic doses (1 $mg.L^{-1}$ in grass carp and 10 $mg.L^{-1}$ in signal crayfish) and caused some histological alterations in gills as well as changes in the activities of antioxidant enzyme in treated animals. It seems that the observed changes were not large enough to induce mortality in treated animals. According to our observation, it seems that those changes are reversible if the exposure is followed by a sufficient recovery period allowing animals to restore their disturbed homeostasis. However, species-specific differences should be considered when treating more sensitive species. Toward establishing a safe application guideline, more studies will be required to investigate the given issues.

CZECH SUMMARY

Fyziologické a molekulární odezvy vodních organizmů na účinky chemických látek

Biochemické a fyziologické odezvy organizmů na působení cizorodých látek jsou zkoumány již řadu let a obecně odrážejí zdravotní stav sledovaných organizmů. Hlavní ukazatele těchto reakcí jsou využívány při vývoji syntetických léčiv a prověřování účinků a mechanizmu působení znečišťujících látek na biologické systémy. V poslední době je věnována velká pozornost využívání „OMICS" přístupů, jakožto jedné z nejrychleji se rozvíjejících oblastí v oboru toxikologie. Účinky cizorodých znečišťujících látek na vodní organizmy jsou zde zkoumány na molekulární úrovni, na které se změny promítnou nejdříve.

ZnO nanočástice jsou hojně využívány v potravinářském průmyslu a zemědělství. Jejich následné uvolnění do životního prostředí vyvolává obavy z možných negativních účinků na organizmy. Ačkoli jsou vodní organizmy v nejvyšší míře vystavovány nanočásticím pravděpodobně perorální cestou, většina publikovaných studií je zaměřena na hodnocení expozice ve vodním prostředí. Velmi málo jsou rovněž popsány molekulární mechanizmy odpovídající za jejich toxicitu v biologickém systému. Předkládaná práce se zabývá schopností akumulace ZnO nanočástic podávaných v krmivu ve vnitřních orgánech kapra jako modelové ryby. Odezvy organizmu na ZnO – medikované krmivo byly posuzovány na proteinové, biochemické a histologické úrovni. Výsledky neprokázaly významnou akumulaci ZnO nanočástic, ani změnu hematologických parametrů, peroxidace lipidů a histologické struktury tkání. Významné změny po aplikaci 500 mg ZnO nanočástic na kg krmiva byly prokázány v hladině proteinů spojovaných s buněčnou motilitou, v ukazatelích odezvy imunitního systému, v syntéze proteinů a buněčném metabolizmu. Rovněž bylo ovlivněno přežívání buněk ve střevě a skladba sérových proteinů souvisejících s činností imunitního systému. Aplikované nanočástice ZnO na ryby pravděpodobně působí jako vnější stresor a popsané změny fyziologických a molekulárních pochodů se projevují jako kompenzační mechanizmus nutný k udržení vnitřní homeostázy a energetické rovnováhy. Pokud kompenzační energetické nároky na zvládnutí stresu překročí adaptační kapacitu, je následně negativně ovlivněno zdraví organizmu.

Ve druhé části studie byla testována kyselina peroctová v terapeutických dávkách 1 a 3 $mg.L^{-1}$ u amura bílého a 10 $mg.L^{-1}$ u raka signálního. Testované koncentrace způsobily histologické změny žaber a změny v aktivitě antioxidačních enzymů. Sledované terapeutické koncentrace však nezpůsobily mortalitu ošetřovaných ryb a po přesazení do čisté vody se změněné hodnoty navrátily do původního stavu. Z práce vyplývá, že popsané změny jsou reverzibilní, jestliže následuje dostatečná doba zotavení, která dovoluje živočichům obnovit jejich mírně narušenou homeostázu. Při léčbě citlivějších druhů je však třeba vzít v úvahu druhově specifické rozdíly. K vytvoření bezpečného aplikačního manuálu pro jednotlivé vodní organizmy by bylo žádoucí provést další rozšiřující studie.

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LIST OF PUBLICATIONS

Peer-reviewed journals with IF

Chupani, L., Niksirat, H., Lunsmannb, V., Haangeb, S., Bergen M., Jehmlich, N. Zuskova, L., 2017. Insight into the modulation of intestinal proteome of juvenile common carp (Cyprinus carpio L.) after dietary exposure to ZnO nanoparticles . Sci. Total Environ. Accepted. (IF 2016 = 4.900)

Chupani, L., Niksirat, H., Velišek, J., Stara, A., Hradilova, Š., Kolařik, J., Panaček, A., Zuskova, E., 2017. Chronic dietary toxicity of Zinc Oxide nanoparticles in common carp (Cyprinus carpio L.): tissue accumulation and physiological responses. Ecotox. Environ. Safe. Accepted. (IF 2017 = 3.743)

Chupani, L., Zusková, E., Niksirat, H., Panáček, A., Lünsmann, V., Haange, S.-B., von Bergen, M., Jehmlich, N., 2017. Effects of chronic dietary exposure of zinc oxide nanoparticles on the serum protein profile of juvenile common carp (*Cyprinus carpio* L.). Sci. Total Environ. 579, 1504–1511. (IF 2016 = 4.900)

Aminzadeh, Z., Jamalan, M., **Chupani, L.**, Lenjannezhadian, H., Ghaffari, M., Aberomand, M., Zeinali, M., 2016. *In vitro* reprotoxicity of carboxyl-functionalised single-and multi-walled carbon nanotubes on human spermatozoa. Andrologia. (IF 2015 = 1.441)

Chupani, L., Savari, A., Zolgharnein, H., Rezaie, A., Zeinali, M., 2016. Enzymatic and histopathologic biomarkers in the flatfish *Euryglossa orientalis* from the northwestern Persian Gulf. Toxicol. Ind. Health 32, 866–876. (IF 2015 = 1.688)

Chupani, L., Zuskova, E., Stara, A., Velisek, J., Kouba A., 2016. Histological changes and antioxidant enzyme activity in signal crayfish (*Pacifastacus leniusculus*) associated with sub-acute peracetic acid exposure. Fish Shellfish Immunol. 48, 190–195. (IF 2015 = 3.025)

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Abstracts and conference proceedings

Zusková, E., **Chupani, L.**, Stará, A., Velíšek, J., 2014. The effect of peracetic acid on grass carp (*Ctenopharyngodon idella*). In: Abstracts book of the 19th Interdisciplinary Toxicological Conference TOXCON. 24–26 September, 2014, Stara Lesná, Slovensko, Interdisciplinary Toxicology 7 (Suppl. 1): 88.

Zuskova, E., **Chupani, L.**, Velisek, J., 2014. Evaluation of Peracetic acid application in therapeutic dose to grass carp (*Ctenopharyngodon idella*). In: Abstracts book of the Konferencja szkoleniowo-naukowa Polskiego Towarzystwa Toksykologicznego. 17–19 September, 2014, Olsztyn, Poland, p. 132.

Chupani, L., Zusková, E., Kouba, A., Stará, A., Velíšek, J., 2015. Investigation on histopathological alterations of sub.acute Peracetic acid exposure to signal crayfish (*Pacifastacus leniusculus*). In: Book of abstracts. 20th Interdisciplinary Toxicology Conference TOXCON 2015. 27–29 May, 2015, Brno, Czech Republic, p. 60.

Zusková, E., Piačková, V., Kolářová, J., Máchová, J., **Chupani, L.**, Steinbach, Ch., Velíšek, J., 2015. Experimental treatment of *Pomphynchus laevis* (Acanthocephala infection in barbel (*Barbus barbus*). In: Book of abstracts. 20th Interdisciplinary Toxicology Conference TOXCON 2015. 27–29 May, 2015, Brno, Czech Republic, p. 132.

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Helmholtz Centre for Environmental Research, Department of Molecular Systems Biology, Leipzig, Germany under supervision of Dr. Nico Jehmlich/ Proteomics and metabolomics study of fish serum and gut epithelium in common carp (4 months).	2015
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Responsible leader of project
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October 2014, University of Murcia, Faculty of Biology, Department of Cell Biology & Histology, Spain

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