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Effects of xenobiotics on oxidative stress, lipid metabolism, DNA integrity and cell viability in human cells and fish spermatozoa *in vitro*

Účinky xenobiotik na oxidační stres, metabolizmus lipidů, integritu DNA a životaschopnost lidských buněk a rybích spermií *in vitro*

Pavla Linhartová

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

1.1. WATER POLLUTION AND THEIR EFFECTS ON HUMAN HEALTH

Pollution is defined as an undesirable change in the physical, chemical or biological characteristics of air, water and land brought by man's activities that may harmfully affect living organisms and other resources. Man, in his quest for a better life, is getting more and more dependent on technology and industries. Industry together with technological inventions and the modern lifestyle are making life easier but at the same time it contaminates water, releases unwanted toxins into the air and reduces the quality of soil all over the world. Pollution of the aquatic environment by inorganic and organic chemicals is a major factor posing serious threat to the survival of aquatic organisms including fish. This is one of the main reasons why risks verus benefits of fish consumption are an intensively discussed public health topic (Sioen et al., 2007a, b, 2009). The health benefits of eating fish are well-known. Fish consumption has been linked to a decrease in several diseases of our modern lifestyle like rheumatoid arthritis, psychiatric disorders (Mcmanus et al., 2011; Cherubini et al., 2007) as well as cardiovascular health or cancer (Kinsella et al., 1990; Hooper et al., 2006). On the other hand, the consumption of contaminated fish might cause health problems (Gochfeld and Burger, 2005) to humans (Domingo et al., 2007) and especially to pregnant women, where dietary intake of essencial n-3 fatty acids (FA) is important (Ruyle et al., 1990).

Water pollutants, are often released into domestic wastewater treatment facilities through washing off cremes, sunblockers, or as waste products of excreted metabolites from ingested pharmaceuticals or in the manufacture of industrial products. The increase of these substances have been documented in the last decade (Ferhi et al., 2016; https://www.epa.gov). Pollutants can be generaly divided in the following groups: heavy metals, persistent organic pollutants (POP), environmental persistent pharmaceutical pollutants (EPPP), polycyclic aromatic hydrocarbons, volatile organic compounds and other environmental xenobiotics (Banjoko, 2014). All pollutants are defined as xenobiotics, presenting extraneous artificial substances created mostly by humans and not found in nature (Mansuy, 2013).

Heavy metals can be defined as metallic elements that have a relatively high density compared to water. They also include metalloids, such as arsenic which is able to induce toxicity at low levels of exposure (Ferdosi et al., 2016). Not all heavy metals have been classified as toxic compounds (Duffus, 2002; Fergusson, 1999). The term heavy metal has particular application to cadmium, mercury, lead and arsenic (Srivastava et al., 2010). These metals are listed in the World Health Organisation's list of 10 chemicals of major public concern (http://www.who.int) and have the capacity to accumulate in the soil, water sediments and in the food chain (Celechovska et al., 2008). The contamination chain of xenobiotics via the aquactic system to human occurs usually in the following cycle: industry, atmosphere, soil, water, phytoplankton, zooplankton, fish and human (Kadar et al., 2000). Huge quantities of inorganic and organic compounds, found in agricultural drainage water contain pesticides and fertilizers, as well as effluents from manufacturing these materials, in addition to sewage effluents (ECDG, 2002). Another group of xenobitics important to consider are phenols and their derivatives, and oxidative and toxic metabolic products (quinones and bisphenols). In the present thesis, the metal ions (e.g. cadmium chloride) and three xenobiotics (e.g. duroquinone, bisphenol or tetrabrombisphenol A) were chosen for study. These have been shown to accumulate in fish organs to a level that affects their physiological state as well as a contaminated source in the human diet (Tirkey et al., 2012). The bioaccumulation of dangerous pollutants in humans can cause malfunctions of the liver, kidneys, circulatory system, and the transmission of nerve signals (Tchounwou et al., 2012). By neurological depositions, xenobiotics containing redox active metals ions have been recognized to increase oxidative damage, a key component of

chronic inflammatory disease (Umanzor et al., 2006) and have been suggested as an initiator of cancer (Elst et al., 2007). As inflammation is a characteristic feature of a wide range of diseases, further potential pathological effects for metal ions and xenobiotics are emerging, as exemplified by premature ageing (González-Cortijo et al., 2008).

In the present study, the following water pollution chemicals were then studied using *in vitro* cellular models and reported in Chapters 2 & 3 – cadmium chloride (Cd^{2+}), Chapter 4 – duroquinone (DQ), Chapter 5 – tetrabrombisphenol A (TBBPA) and bisphenol A (BPA) in Chapter 6.

1.1.1. Water pollutants and their effects on human and fish health

The xenobiotic cadmium (Chapters 2 and 3) is a heavy metal environmental pollutant. This dangerous heavy metal is taken up with drinking water and food, both by humans and fish. The TDI (tolerable daily intake) for Cd^{2+} in the human diet according to the guidelines of the Institute of Standard and Industrial Research of Iran (ISIRI) was calculated. Based on ISIRI, the TDI for Cd^{2+} is 1 $\mu g.day^{-1}$ kg of bw (body weight) (Chamannejadian et al., 2013). Another world organistion the Joint FAO/WHO Expert Committee on Food Additives recommended a tolerable monthly intake (TMI) for Cd^{2+} at 25 $\mu g.kg^{-1}$ (Chapters 2 and 3; JECFA, 2010). In addition to the effect on humans, Cd^{2+} can affect fish and other aquatic organisms. Valova et al. (2010), found increased Cd^{2+} concentrations of 0.001–0.254 $mg.kg^{-1}$ in chub (*Leuciscus cephalus* L.) and brown trout (*Salmo trutta m. fario* L.) in the Czech river Morava (Danube basin). Considering the highest found values, a person weighing 100 kg and consuming 10 kg of fish would reach the limit in less than a month. The highest average metal concentration found in fish muscle was: Hg > Pb > Cd (Valova et al., 2010), suggesting that Cd^{2+} has the lowest concentration in fish muscle compared to the other metals. However, the exposures to low levels of Cd^{2+} causes DNA damage and stress in common carp (*Cyprinus carpio*) (Jia et al., 2011). Furthermore, in Japan, Belgium, Sweden and China, Cd^{2+} has caused bone and reproductive cancer in in human (Nordberg, 2006), suggesting that consuming fish or other water animals that have accumulated Cd^{2+} may pose a threat to human health. Consequently, the IARC (International Agency for Research on Cancer) decided to classify cadmium as a human carcinogen, group I (IARC, 1993). In Chapters 2 and 3, we focused on the effect of cadmium in combination with fatty acids on lipid metabolism and oxidative stress in human liver (Hep G2) cells. The Hep G2 cell line was chosen, since liver is a target organ for cadmium and the cell line is a suitable model for studying lipid metabolism in humans.

The xenobiotic DQ (Chapter 4) is a compound receiving considerable attention nowadays. DQ is produced through nitration of durene (1, 2, 4, 5-tetramethylbenzene) followed by reduction to the diamine and then oxidation. Quinones are widespread in the environment and form an important class of toxic metabolites generated by the metabolism of phenols and related compounds (diethyl stilbesterol, phenol, 1-naphthol); these occur naturally and as water pollutants at concentrations up to 480 $\mu g.L^{-1}$ (Usenko, 2012; Smith., 1985). The EFSA committee did not assess the hazard from oxidation products of phenol, e.g. quinones/hydroquinones. Therefore, the TDI only covers phenol (not DQ) and has established a limit of 0.5 $mg.kg^{-1}$ bw per day (EFSA, 2013). DQ is a derivate of 1, 4- benzoquinone, which is very soluble in water, and at low concentrate ions (50–150 μM) induces cytotoxic effects in fish through rective oxygen species (ROS) production (Lemaire et al., 1994; Linhartova , 2013; Linhartova et al., 2013a, Chapter 4). Zhou et al. (2006) used this substance as a model of ROS inducer in sperm of common carp and they showed that reproduction of carp can be impaired through oxidative damage of their spermatozoa.

The xenobiotic TBBPA (Chapter 5) was the subject of an eight-year evaluation under the EU risk assessment procedure that reviewed over 460 studies. TBBPA was already regulated under the chemical policy of the European Union: registration, evaluation, and authorization of chemicals (REACH) in October 2010 and was classified in the EU as an R50-53 substance, indicating toxicity to aquatic organisms. TBBPA is widely used as brominated flame retardant (BFR) and has also additive applications in several types of polymers. Lethal TBBPA concentrations found in different fish species, where LC_{50}s reported by WHO/IPCS (1995) include 0.51 $mg.L^{-1}$ (0.9 μM) for bluegill sunfish (*Lepomis macrochirus*), 0.4 $mg.L^{-1}$ (0.7 μM) for rainbow trout (*Oncorhyncus mykiss*) (WHO/IPCS, 1995), and a relatively high 3 $mg.L^{-1}$ (5.5 μM) for zebrafish (*Brachydanio rerio*) (Lee et al., 1993; Linhartova, 2013; EFSA, 2011a). The concentrations of TBBPA found in herring (*Clupea harengus*) from the Baltic waters were reported by EFSA (2011a) in the range of 0.5–5 $ng.g^{-1}$ fresh weight, Northern pike (*Esox lucius*) from Swedish inland waters 2–4 $ng.g^{-1}$ fresh weight and moose (*Alces alces*) 1–3 $ng.g^{-1}$ fresh weight. The most serious cases of TBBPA pollution were found in China in Chaohu Lake, Anhui (industry concentration site) with concentrations of TBBPA reaching 850–4870 $ng.L^{-1}$ in water (Liu et al., 2016). However, regarding human consumption, European Food Safety Authority (EFSA, 2011) reported TBBPA exposure and its derivatives in food based on: more than 344 food samples from the fish and other seafood products. They concluded that the dietary exposure to TBBPA within the European Union does not raise a health concern for humans, and the committee recommended then the TDI of 1 $mg.kg^{-1}$ bw per day (EFSA, 2011a). However, this pollutant might have an important effect on fish health.

The xenobiotic BPA (Chapter 6) is classified as one of the most common endocrine disruptor compounds worldwide. EFSA reviewed new scientific information on BPA in 2008-2015 and concluded that there was no evidence to revise the present secure levels of BPA exposure. However, EFSA recognizes some uncertainties and will continue to investigate them (EFSA, 2015). For example in February 2016, French reasearchers proposed BPA as a REACH Regulation candidate of very high concern (SVHC). This chemical is mostly used in combination with others to manufacture plastics, resins or in polycarbonates. Polycarbonates are used for the production of food containers, such as returnable beverage bottles, infant feeding bottles for babies, tableware (plates and mugs) and storage containers (EFSA, 2015). Small amounts of BPA can potentially leach from food containers into foodstuffs and therefore can be ingested. The permissable level of BPA in food-contact plastics in the European Union were settled with a specific migration limit of 0.6 $mg.kg^{-1}$ food (EFSA, 2011b). BPA is capable of interfering with vertebrate reproduction and may induce deregulation of epigenetic mechanisms (Santangeli et al., 2016). The TDI for BPA is 0.05 $mg.kg^{-1}$ bw per day EFSA (2010). Singh et al. (2016) tested the reproductive toxicity of BPA in adult Kadaknath chicken using two oral dosages (1 or 5 $mg.kg^{-1}$ body weight) for seven weeks. Sperm concentration was significantly decreased, but not fertilization rate. Additionally Mita et al. (2011) found that fish livers are about 2.5 times more polluted with BPA than muscle and fish caught in the Gulf of Naples cointained higher levels than those which naturally inhabitat the Italian Latium coast. Moreover, this endocrine disrupting chemical was shown to act as a xenoestrogen modulating the endocrine pathways through a receptor-mediated process in sperm of goldfish (*Carassius auratus*) (Hatef et al., 2012).

Xenobiotics studied in this thesis are known to enhance production of ROS (Livingstone, 2001) that attack a variety of biological macromolecules such as DNA, proteins, and lipids, leading to oxidative damage in mammals and fish (Kelly et al., 1998). Cd^{2+} also plays a role in the development of various cancers (Mates et al., 2010). The chosen compounds bioaccumulate in fish muscles and fat; fish exposed to excessive concentrations of these toxic substances are not suitable for human consumption. Besides the effects on human nutrition, they also

can have direct effects on fish, especially during reproduction and fish feeding. During the natural reproduction of most fishes, spermatozoa are released to the environment where there are a number of substances which affect spermatozoan functions. It has been reported that osmotic stress and various xenobiotics can provoke ROS generation in fish spermatozoa (Zhou et al., 2006; McCarthy et al., 2010). Moreover, ROS can modulate cell signaling events and physiological functions (O'Flaherty et al., 2006, Baumber et al., 2003). ROS production has been reported in the sperm and cells of fish and mammals (Aitken et al., 1993 and 2006; Li et al., 2010a). Furthermore, it has been postulated that the large quantities of ROS could be toxic for fish sperm (Chapters 4–6) or Hep G2 cells (Chapter 3).

1.2. HEP G2 CELLS AS A MODEL TO STUDY THE EFFECTS OF XENOBIOTIC ON HUMAN LIVER

Human hepatocellular cells (Hep G2) (Fig. 1), a perpetual cancerogenic cell line, was derived from the liver tissue of a 15-year-old Caucasian American male with a well-differentiated hepatocellular carcinoma. Liver is one of the most affected organs by Cd^{2+} as well as playing a key role in lipid metabolism (Javitt, 1990). Primary human hepatocytes (HH) can be considered as a gold standard model to study the metabolic pathways and cytotoxic effects of xenobiotics (Guillouzo et al., 2007). However, fresh human liver samples are scarce and there are limits in use; for example, isolation is complicated, individual samples are variable, thus, life span limitations and their price, seriously restricts *in vitro* use of such systems in screening (Madan et al., 2003). To overcome these limitations, immortalized liver-derived cell lines were suggested as an impeccable alternative for the studies of drug metabolism. Their unlimited availability and phenotypic stability are advantageous. As a good model organism the widely used human hepatocellular carcinoma cell line (Hep G2) can be considered; it is highly differentiated and displays many of the genotypic features of normal liver cells (Sassa et al., 1987). Moreover, the present line can be used as a proposal screening material for new cytotoxic chemical entities at the lead generation phase (Gerets et al., 2009). Hep G2 cells have the ability to activate and detoxify xenobiotics and repel their metabolic pathways in the human body better than other cells line used (Mersch-Sundermann et al., 2004). Hepatic cancer has been classified as the sixth most common cancer and the second disease causing death in the world (Jemal et al., 2011). The statistic from 2012 indicate that liver cancer occurred in 782,000 cases and caused death to 746,000 people (9.1% of total cancer death) (Stewart and Wild, 2014). Nowadays studies focusing on hepatic cancer have received increasing attention (Neergheen et al., 2010). Cancer of liver cells (hepatoma) is now being classified as one of the most detected types of cancer diseases all over the word. Various worldwide laboratories are trying to find new biologic markers leading to easier prognoses for hepatocellular cancer disease (HCC) and in this way help diagnose and maybe cure cancer in its first stages. Therefore, cultures of primary hepatocytes and hepatoma cell line Hep G2 are frequently used *in vitro* as a tool for studying genotoxicity, oxidative stress, mitochondrial dysfunction, changes in gene expressions and apoptosis related to HCC (Knasmuller et al., 2004; Constantini et al., 2013).

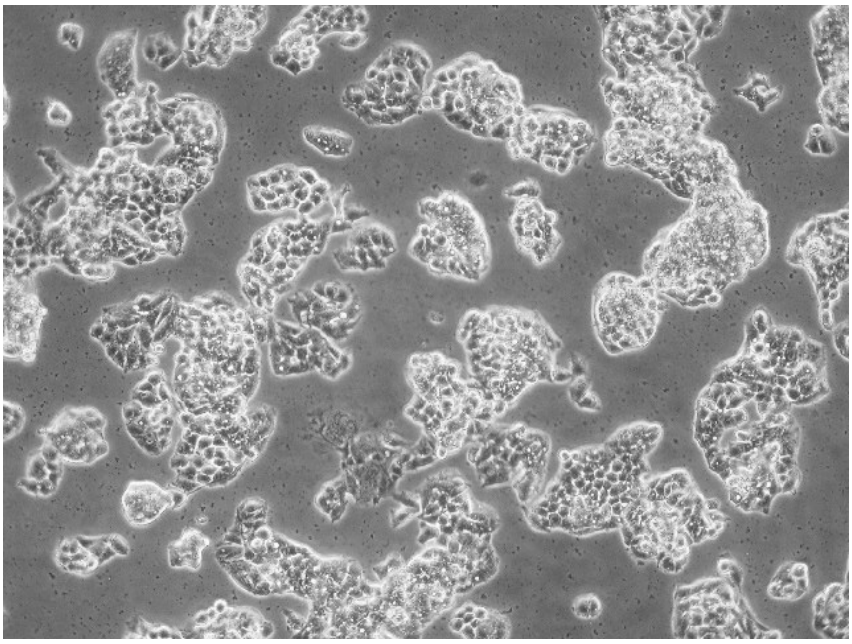

Fig. 1. *Liver-Hep G2 cells (http://www.medchem.upol.cz).*

1.3. IMPORTANCE OF N-3 FATTY ACIDS IN HEALTH AND DISEASE

Fatty acids (FA), both free and as part of complex lipids like triacylglycerosl and phospholipids, play key roles in metabolism: as major metabolic fuel (storage and transport of energy), as essential components of many membranes, and as gene regulators (Tvrzicka et al., 2011). Long-chain FA have number of important biological functions, as energy substrates (Distel et al., 1992), as precursors for glyco- and phospholipid components of cell membranes (Amri et al., 1994), as biological mediators like eicosanoids (Noy and Zakim, 1993), and as mediators of cellular processes (e.g., gene expression and growth regulation) (Distel et al., 1992; Amri et al., 1994). FA are the building blocks of lipids and generally comprise 90% of the fat in foods. They are important sources of fuel because, when metabolized, they yield large quantities of ATP. Most cell types can use either glucose or FA for this purpose. In particular, heart and skeletal muscle prefer FA. Despite long-standing assertions to the contrary, the brain can use FA as a source of fuel in addition to glucose and ketone bodies (Domingo, 2007).

Mammals cannot synthesize the long chain polyunsaturated FA (PUFA): alpha-linolenic acid (ALA) 18:3 (n-3) and linolenic acid (LA) 18:2 (n-6), but have the desaturase enzymes to elongate them in small but not sufficient amount towards the longer chain metabolites. ALA and LA are naturally found in plants. Fish and especially herbivorous and ominvorous species are capable of biosynthetizing eicosapentaenoic acid (EPA) 20:5 (n-3) and docosahexaenoic acid (DHA) 22:6 (n-3) through desaturation and elongation of ALA which are present in plants in greater amounts. For this reason the FA elongase and desaturase metabolism capabilities of farmed fish are nowadays attracting significant research attention (Stubhaug et al., 2005). Additionally freshwater fish (both herbivorous and omnivorous) can synthetisize long chain FA (not having them naturally in their diet) in better way than seawater fish species. Seafish have a natural intake of EPA and DHA in their diet from algae (herbivorous) or seafish (predators) and have been considered as a rich source of both FA for human nutrition (Zajic et al., 2013).

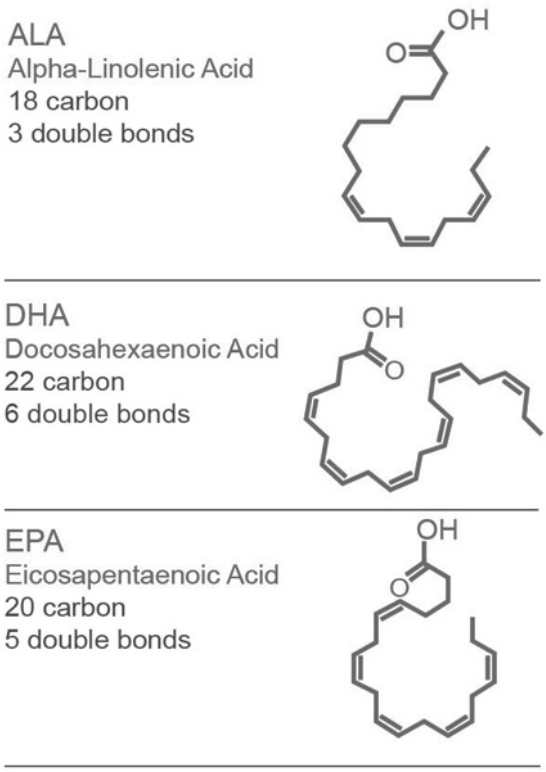

Fig. 2. *ALA, EPA and DHA (http://www.cargillfoods.com/).*

Although it is possible for the human body to convert ALA to EPA and DHA by enlongase and desaturase enzymes, but research suggests that only a small amounts can be synthesized with this process (Simopoulos, 2008). Therefore EPA and DHA (having a longer structural chain) are classified as essential FA for humans (Fig. 2).

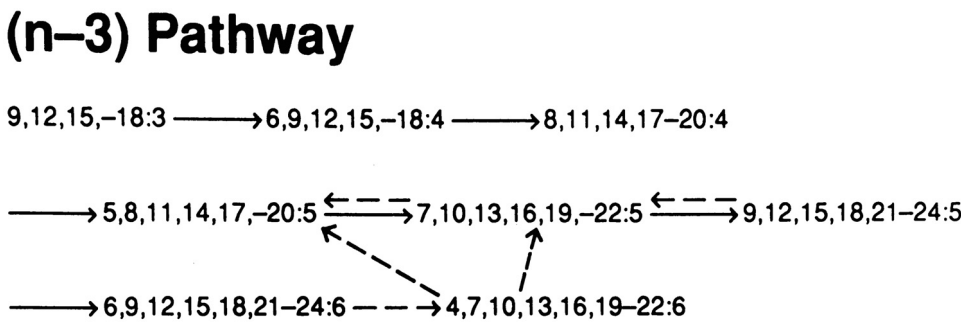

Fig. 3. *Biosynthesis of n-3 PUFA (Sprecher, 2000).*

Essential EPA and DHA may obtained in the diet through oily fish; these are excellent sources as they contain approximately 2 g of EPA + DHA per portion of fish fillet (150 g), (Simopoulos et al., 1991). These FA have a number of pleiotropic effects on lipid and energy metabolism.

After FA are absorbed by the organism, they are transported as lipoprotein particles. The role of lipoproteins is to transport triacylglycerols and cholesterol in the blood to various tissues, the most common being the liver and the adipocytes (Hegele, 2009). Two main factors control the degree of FA oxidation by the liver. The supply of FA to the liver is delivered via lipolysis and by separation between oxidation and microsomal esterification (Nguyen et al., 2008). The n-3 PUFA affect hepatic lipid metabolism by regulation and expression of key enzymes in lipid synthesis and catabolism. They are able to activate ligands of peroxisome proliferator-activated receptor (PPAR-α) and decrease lipogenesis and secretion of very low density lipoprotein (VLDL) by suppressing sterol response element binding protein (SREBP-1) (Tvrzicka et al., 2011; Burdge et al., 2009). Also, EPA has the capability to decrease the production of proinflammatory cytokines (e.g. IL-1β, TNF-α) through its mono- and trihydroxy-derivatives, thus, considering fish oil (containing both EPA and DHA) as an important antiinflammatory component in human nutrition (Simopoulos, 2002; Calder et al., 2009). Increased dietary intake of n-3 resulted in enhanced transcription of antioxidant enzymes and in suppressed transcription of enzymes taking part in the production of ROS (Kremmyda et al., 2011).

1.4. FISH AND ITS ROLE IN HUMAN NUTRITION

Fish are consumed by many animals; they have been an important source of protein and other nutrients for humans throughout recorded history. Additionally, fish are an important and integral part in the human diet, playing a critical role as supplier of essential FA. Research over the past few decades has shown that the nutrients and minerals and particularly the n-3 FA found in fish and seafood, have several benefits for heart functions (good prevention against heart diseases) and have capability to improve brain development and human reproduction system (Domingo, 2007; Simopoulos, 2008; Liu and Li, 2000). For example, long chain n-3 FA have these following effects: decrease the risk of arrythmias, decrease triglyceride (blood fat) levels, decrease growth rate of atherosclerotic (arterial) plaque and lower the blood pressure (Serhan et al., 2008). There is little doubt that n-3 FA have a decisive importance in our diet, providing significant structural components of the phospholipid membrane tissues throughout the body. These components are especially important in the retina, brain, and spermatozoa where DHA constitutes up to 36.4% of total FA (Neuringer et al., 1986; Lin et al., 1993). It is widely known for the n-3 FA family, that they are essential FA, necessary from conception, throughout pregnancy, in infancy and undoubtedly throughout our whole life (Connor et al., 1996). This has highlighted the role for fish in the functionality of the human body. Above all, not only its nutritional value of fish as human food, but also it is important in fish breeding (including feeding) which is vital to the suitable production of food (availability, cost and nutrition value) for the world (Calder, 2012).

The composition of FA in fish tissue is mostly determined by their diet (food intake) and lipid metabolism. Fish have the ability to synthesize the saturated FA (SFA) and monounsaturated FA (MUFA) and, moreover to absorb and metabolise dietary FA, including PUFA, to obtain an optimal fatty acid composition (Bell et al., 1997). Two important n-3 FA, EPA and DHA, where chosen for the present study (Chapters 2 and 3). These beneficial FA are classified as PUFA, because they contain 5 and 6 double bonds on their long structural chains. As already mentioned, humans cannot synthetize these long chain n-3 FA in sufficient amounts, so we must ingest them via the diet. N-3 FA are found in every kind of fish, but are especially high in fatty fish (for example common carp or salmon, butter fish, eel, mackerel etc.). The absolute amount of EPA + DHA in common carp, silver carp or tench is 234 mg/100 g, 471 mg/100 g 319 mg/100 g flesh, respectively; while lean fish from semi-intensive freshwater production showed 195 mg/100 g and 169 mg/100 g for pike perch and northern pike (Linhartova Z., et

al., unpublished data). Therefore, fish and seafood are recommended for today's population as a regular item in their diet (twice a week, portion of 150–200 g of fish). More recently it has been discussed that fish protein (Pilon et al., 2011) and other fish nutrients (Rudkowska et al., 2010) are beneficial for human health. However, an adverse health effect of fish from environmental contaminants must be considered along with the beneficial aspects (Gochfeld and Burger, 2005; Mahaffey et al., 2011).

Nowadays, a key factor for the supply of fish to feed a growing world population is reproductive success in fish breeding. Also from an ecological viewpoint, successful reproduction is important in determining species survival. Water pollution can decrease the quality of gametes of aquatic organisms, which in turn affects fertilization success, hatching of embryos, and subsequent survival of offspring. Additionally, the gametes of most fishes are released into the water, where they can be directly exposed to man-made compounds that can interrupt cellular metabolism on various levels (Linhartova, 2013). It is important to mention that fish spermatozoa differ from mammals'. For example, fish sperm are immotile on ejaculation, but are activated when they contact the surrounding water, and they have an extremely short period of motility, typically ranging from several seconds to minutes (Li et al., 2010b; Cosson, 2010) depending on fish species. Many studies have shown that different factors (of the external medium) can induce a significant decrease of spermatozoan motility and thus a reduction of fertilization rate (Zhou et al., 2006; Dietrich et al., 2012). It can be hypothesized that the fertilization ability strongly depends on the capacity of spermatozoa to move rapidly during a short time so as to contact the egg (Kaspar et al., 2007, 2008). In the present study, spermatozoa from sterlet (*Acipenser ruthenus*) were used; this species is considered by many international organizations as endangered (Chapters 4–6). Populations have drastically decreased, due to over-fishing, destruction of their natural habitat, as well as water pollution (Birstein and DeSalle, 1998). Figure 4 demonstrates the structure of sterlet spermatozoa with the acrosome, which is present only in cartilaginous fishes (Linhartova, 2013).

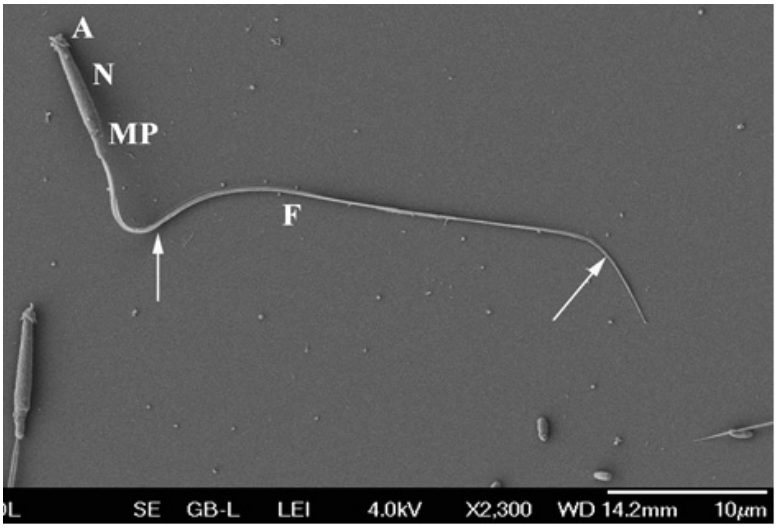

Fig. 4. *Structure of sperm of sterlet (A – acrosom, N – nukleus, MP – midpiece, F – flagellum), (Linhartova et al., 2013b).*

1.5. INCREASED OXIDATIVE STRESS AND ROS INDUCED DAMAGE IN DIFFERENT CELL TYPES

Oxidative stress can be defined as an imbalance between the production of free radicals and the ability of the body to counteract their harmful effects or to detoxify the radicals through neutralization by antioxidants. Disturbances from normal redox conditions in cells can cause toxic effects through the production of free radicals or peroxidases which are responsible for damage of all components inside of the cells (proteins, lipids, or DNA). Detoxification of ROS is paramount to the survival of all aerobic life forms. There exist numbers of defense mechanisms that have evolved to meet ROS and need to and should provide a balance between production and removal of ROS. While an imbalance toward the pro-oxidative state appears, this situation is often referred to as "oxidative stress" (Sies 1995, 2007).

ROS can be classified as highly reactive chemical compounds containing oxygen ions (peroxides, superoxide, hydroxyl radical, and singlet oxygen). The high reactivity of ROS is caused by free shell electrons, having the capacity to damage cellular structures, even resulting in apoptosis and hence playing an important role in cell death (Evan and Vousden, 2001). In fish and human cells, ROS can be generated endogenously (Ray et al., 2012), through the process of normal cell respiration or from the interactions of exogenous sources (Aitken et al., 1992; Gazo, 2015). When ROS reach pathological levels, reactive oxygens can exert significant damage to different biomolecules (proteins, lipids and nucleic acids).

ROS, at appropriate concentrations, also play an important physiologic role in cellular signaling, regulation of cell growth, cell differentiation, vascular tone, inflammation, and immune responses (Lushchak, 2011, 2014). However, excessive levels of ROS from both exogenous and endogenous sources have been shown to be harmful to different cell types, including fish spermatozoa (Chapters 4–6) and hepatocytes (Hep G2) (Chapter 3). ROS can also cause inflammation, which in turn can induce or increase oxidative stress, and both inflammation and oxidative stress can result in injury to cells (Touyz, 2005; Ishibashi, 2013).

It is known that Cd^{2+} can act as a catalyst in forming ROS; Cd^{2+} increases lipid peroxidation and it depletes cellular antioxidants, as for example glutathione and protein-bound sulfhydryl groups. Additionally, ROS is often involved in Cd^{2+} toxicology, as found in different cell culture systems by Hart et al. (1999) and He et al. (2008) and the acute toxicity of Cd^{2+} may result in advanced production of oxidative stress such as superoxide ion, hydrogen peroxide, and hydroxyl radicals (Bagchi et al. 1997; Liu and Jan 2000). Many studies have demonstrated that Cd^{2+} can induce cell death (apoptosis), which is connected with increased p53 protein and p53 mRNA levels in different cell lines after Cd^{2+} exposure (Achanzar et al., 2000; Lag et al., 2002). Also different contaminants are known to enhance production of ROS (Livingstone, 2001) by attacking different biological macromolecules (DNA, proteins, and lipids) leading to oxidative damage (Kelly et al., 1998, Moore et al., 1987). Related to this, DQ is of interest because it induces mitochondrial Ca^{2+} release through H_2O_2 (hydroperoxide) generation by redox cycling, followed by NADPH oxidation and gluthatione oxidation (Moore et al., 1987; Linhartova et al., 2013a, Chapter 4) and in case of TBBPA and BPA, these xenobiotics can produce ROS in cell cultures (Reistad et al., 2005; Li et al., 2009).

Reproductive success is a key factor in determining species survival. Since spermatozoa movement is a major requisite for successful fertilization, assessment of motility and velocity may be a sensitive and accurate as bio-indicator of aquatic pollution (Li et al., 2010a). Sperm cells of externally fertilizing fishes are particularly vulnerable to oxidative stress when they are released into the environment (water). Gametes of most fishes are prone to oxidative injury because of their lack of protective cytoplasmic enzymes as well as the PUFA that are present in plasma membranes of spermatozoa (Drevet, 2006). Therefore, different pollutants in the

aquatic environment can lead to ROS production and impair normal reproduction processes of fish (Zhou et al., 2006).

Another part of this thesis points out the role of n-3 FA and their metabolites, which exert multiple effects in our bodies, including the antiinflammatory actions by diminishing the oxidative stress (Fukui et al. 2013). These reduce the cardiovascular disease in patients with hypertension (Maksimenko, 2005, Houston, 2014, Ghiadoni et al., 2012). However, long chain PUFA are prone to oxidation, which may lead to an increase of lipid peroxidation (LPO) in the human body (Mori 2004). Many studies have shown that the accumulation of certain PUFA would result in higher LPO and formation of lipid hydroperoxides or other lipid degradation manners (Cognault et al., 2000; Gago-Dominguez et al., 2005). Also, Kang et al. (2010) showed that EPA and DHA can cause ROS accumulation and can consequently cause caspase-8-dependent apoptosis in human breast cancer cells.

1.6. AIMS OF THE THESIS

The main aims of this thesis were:

1) To evaluate the effects of Cd^{2+},with or without the co-incubation with n-3 FA, on the viability and lysosomal integrity of human hepatocytes (Hep G2) as model cells.
2) To identify changes in n-3 FA composition and phospholipid classes in the cells caused by repeated incubation with FA and Cd^{2+}.
3) To assess viability parameters (motility and velocity), DNA integrity and ATP content of fish spermatozoa as a model cell after exposure to different xenobiotics (DQ, TBBPA and BPA).
4) To measure the effects of various xenobiotics on lipid and protein oxidation parameters (ROS) and antioxidant response in the different model cell types.

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CHAPTER 2

COMBINED INCUBATION OF CADMIUM, DOCOSAHEXAENOIC AND EICOSAPENTAENOIC ACID RESULTS IN INCREASED UPTAKE OF CADMIUM AND ELEVATED DOCOSAPENTAENOIC ACID CONTENT IN HEPATOCYTES *IN VITRO*

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Combined incubation of Cadmium, docosahexaenoic and eicosapentaenoic acid results in increased uptake of cadmium and elevated docosapentaenoic acid content in Hepatocytes *in vitro*

Pavla Linhartova* and Sabine Sampels

Abstract

Background: Human hepatocellular cells Hep G2 were used to mimic and investigate the effect of the intake of cadmium (Cd^{2+}) contaminated fish on cytotoxicity, fatty acid (FA) and phospholipid class composition.

Methods: Cells were incubated with a combination of Cd^{2+} and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) fish specific FA.

Results: We measured a significant increased proportion of EPA and DHA in the treated cells compared to the control line confirming the uptake. While doses of 25 μM DHA showed to be toxic to the cells, repeated short term incubations (2 h) at lower doses resulted in an increased uptake of DHA. The resarzurin assay, evaluating cell viability, showed a significant decrease in cell viability between Cd^{2+} incubation time and, further, the pre-incubation with BSA-FA complex resulted in significantly increased cell viability. On the metabolic level, increased concentrations of EPA and DHA resulted in an increased proportion of docosapentaenoic acid (DPA) which indicated an increased metabolism. Also 24-h combined incubations of 5 μM Cd^{2+} and EPA and DHA showed a significant increase DPA in the total lipid fraction of the cells. In addition, incubation with 5 μM Cd^{2+} for 24 h also decreased the total cardiolipin (CL) fraction from the identified phospholipids.

Conclusions: We confirmed that the applied FA were taken up by the cells. A combination of EPA, DHA and Cd^{2+} influenced lysosomal integrity, cell viability and lipid metabolism in the cells. The most important finding is that DHA and EPA reduced the detrimental effect of Cd^{2+} on cell viability. The exact effects and kinetics behind our observations still need further evaluation.

Keywords: DHA, EPA, Fish lipids, Hep G2, Omega-3 fatty acids

Background

Balancing risks and benefits of fish consumption is an intensively discussed public health topic [1–3]. The health benefits from eating fish, partly attributable to omega-3 polyunsaturated fatty acids (n-3 PUFA), are well documented both for prenatal development and adult cardiovascular conditions [4, 5]. Due to their lipophilicity, fish take up and bioaccumulate heavy metals via feed and from the water, which in turn passes up the food chain into the human diet [6]. There have been no studies on the combined effects of FA and Cd^{2+} on cell cytotoxicity.

Cd^{2+} is an environmental pollutant which is taken up with drinking water and food, including seafood and fish. The TDI (daily tolerable intake) for Cd^{2+} was calculated according to the guidelines suggested by the Institute of Standard and Industrial Research of Iran (ISIRI). Based on ISIRI, the tolerable daily intake for Cd^{2+} is 1 μg/day kg of

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bw (body weight), [7]. Inorganic Cd^{2+} is a human carcinogen [8] and is classified as cancerogenic compound by the International Agency for Research on Cancer (IARC) [9]. Long-term exposure to low concentrations of Cd^{2+} result in accumulation in the liver and the kidneys (kidney cortex) where 30–60 % of ingested Cd^{2+} is deposited [10]. Cd^{2+} compounds have toxic effects on the kidney and are assumed to be neurotoxic. These effects could be due to oxidative stress, but also by different mechanisms that affect the cell membrane composition [11, 12]. Cd^{2+} acts as a catalyst during the formation of reactive oxygen species (ROS).

The long chain n-3 PUFA EPA and DHA have many metabolic functions in animals and humans. Mammals lack the Δ 15 desaturase that is essential for insertion of double bonds at n-3 and hence cannot synthesize the parent FA 18:3 n-3 [13]. In addition it has not been established whether mammals are able to elongate and desaturate 18:3 n-3 towards 20:5 n-3, 22:5 n-3 and 22:6 n-3 in significant amounts [14–16]. Therefore, these FA are regarded as essential for humans and they need to be included in the diet. They are vital nutrients and precursors of several metabolites, which are potent lipid mediators, known to be beneficial in the prevention and treatment of several diseases [17]. Fish are an important source of these FA and hence the consumption of fish (200 g portion) at least twice a week is recommended by several health organizations including FAO/WHO [18]. In the same report, the negative effects of eating contaminated fish are out weight against the benefits of the FA. However, it is unclear which exact mechanisms are affected by cadmium intake in relation to lipid metabolism and oxidative stress when it is simultaneously taken up with the long chain n-3 FA from fish.

The aim of the present study was therefore to evaluate the combined effects of the nutritional essential FA EPA and DHA from fish and Cd^{2+}. Our hypothesis took two aspects into account: Cd^{2+} is known to create oxidative stress in the cells, so one of the toxic effects could be the oxidation of important membrane or organelle FA or phospholipids, subsequently leading to cellular dysfunction or apoptosis. If contaminated fish are ingested, the long chain n-3 FA from the fish, which are the FA most easily oxidized, could prevent or decrease the impact of Cd^{2+} either by replacing the oxidized FA, or conversely by increasing the oxidative stress and thereby worsen the effects of the Cd^{2+}. Hepatocytes were selected for the investigation because the liver is a target organ for Cd^{2+}. A combination of DHA/EPA as 2/1 reflects the typical ratio in many fatty fish, common carp (*Cyprinus carpio*) for example, which is traditionally consumed in Czech Republic. In order to mimic the uptake of contaminated fish, we investigated the metabolic effects of cadmium (Cd^{2+}) on the cell line Hep G2 in combination with the long chain n-3 PUFA, eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) which are typical for fish [19, 20].

Methods

Caution

Inorganic cadmium chloride ($CdCl_2$, Cd^{2+}) is classified as a human carcinogen [21] it is hazardous, or potentially hazardous and should be handled with care.

Chemicals

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) supplied from Biochrom and Sigma-Aldrich (Berlin, Germany), were diluted in extra pure 98 % ethanol and bovine serum albumin (BSA) before transfer experiments. Hydrogen peroxide solution (30 %, Suprapurs) and nitric acid (65 %, Suprapur) were products of Merck (Darmstadt, Germany). Cadmium chloride was obtained from Aldrich, Germany. All other pro-analysis chemicals were obtained from Sigma-Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany). Trypsin, penicillin and streptomycin solutions were products of Sigma (Deisenhofen, Germany). Moreover, the culture dishes and the culture medium (MEM) for Hep G2 cells were obtained from Biochrom (Berlin, Germany).

Cell culture

Human hepatocellular cells (Hep G2, ATCC, No. HB-8065) were purchased from the American Type Culture Collection (ATCC, Manassas, V C, USA). Hep G2 cells were grown as a monolayer in culture dishes in Minimum Essential Medium Eagle (MEM) supplemented with FCS (10 %, v/v), non-essential amino acids (1 %, v/v), glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 μg/mL). The Hep G2 cultures were incubated at 37 °C with 5 % CO_2 in air with 100 % humidity. Cells were passaged every 3 days. The amount of 1.5 million cells were seeded on 10 cm (in diameter) sterile Petri dishes in 10 mL of sterile culture medium (MEM). Suspensions of Hep G2 cells were produced from confluent cultures using trypsin/EDTA solution. Before the transfer experiments, cells were three times sub-cultured to achieve a stable phenotype. For the transfer experiments, cells were seeded at a density of 66.7 cells per μL for 96 well plates and 1.5 Mio (million) per normal petri dishes (10 cm in diameter). Seeded Hep G2 were cultured for 24 h and 37 °C and subsequently prepared for pre-incubations and post-incubations with FA and Cd^{2+}. Hep G2 cells where used from passage Nr. 20 at least 3–4 weeks (till passage Nr. 35). Hep G2 cells can be used from the third passage to Nr. 130. Hep G2 cells were incubated with Cd^{2+} only, with Cd^{2+} and with BSA solved in PBS in order to evaluate if the BSA alone would have any effects

and finally with Cd^{2+} and the FA (EPA + DHA) as a BSA-FA complex.

EPA and DHA pre-incubations

Before incubation fresh stock solutions of FA diluted in extra pure 98 % EtOH were defrosted. The BSA-FA complex was prepared as follows: BSA was dissolved in PBS (phosphate buffer saline). EPA and DHA were dissolved in extra pure EtOH to a final volume of 50 μL. Then 20 μL of EPA and DHA solutions were added to 1 ml of a mixture of 0.1 M NaOH and BSA solution (1/5; v/v) each. The two solutions of FA were then combined and the pH was adjusted to 7.1 using 0.1 M HCl. After testing concentrations from 1–50 μM EPA and from 2–100 μM DHA, we chose concentrations for pre-incubations of liver human cells with 5 μM EPA+ 10 μM DHA for 2–48 h without changing the cell culture medium (MEM).

Cd^{2+} post-incubations

Fresh stock solutions of Cd^{2+} diluted in distilled sterile water (ddH_2O) were prepared before the transfer experiments. Hep G2 liver cells were post-incubated for 24 h or 48 h with Cd^{2+} stock solution with changing the cell culture medium (MEM). The range of Cd^{2+} concentrations were used from min. 0.25 μM to max. 20 μM.

Cytotoxicity and lysosomal integrity

The neutral red assay was used to assess the impact of the heavy metal on lysosomal integrity [22] using a plate reader (Invinite 200Pro, Tecan Group Ltd., Mannedorf, Switzerland). The levels of IC_{50} and IC_{70} were measured so as to indicate the percentage of control in the parameter of lysosomal integrity.

Cytotoxicity and cellular viability

Cellular viability, was measured with the resarzurin uptake assay [23] using a plate reader (Invinite 200Pro, Tecan Group Ltd., Mannedorf, Switzerland). The levels of IC_{50} and IC_{70} were measured so as to indicate percentage of control in the parameter of cell viability.

Pelleting of Hep G2 cells

Cells were seeded in number of 1.5 million cells per Petri dish to 10 mL MEM sterile culture medium. Seeded Hep G2 were cultured and treated with FA as a BSA-FA complex and Cd^{2+} for the appropriate times as described above. Subsequently cells were pelleted by trypsinising and centrifuging several times in PBS/FKS solution and frozen on −80 °C.

Fatty acid composition and phospholipid classes

The pelleted cells were re-suspended in buffer and the total lipids were extracted from cells according to Hara and Radin [24]. From these extracts, the composition of major lipid classes as well as phospholipid classes were evaluated via automated high performance thin layer chromatography (HPTLC), [24, 25]. For analyses of FA composition, methylation of total lipids was performed by using a combination of NaOH and BF_3 according to Appelqvist [26]. FA composition was then analyzed by GC on a BPX-70 50 m fused silica capillary column (id. 0.22 mm, 0.25 μm film thickness, SGE, USA) as described Sampels et al. [27]. Identification of FA and phospholipid classes was done by commercially available external standards (Nu-Check Prep, Inc. Elysian USA; Avanti Polar Lipids, Inc., Alabama, USA) quantification of the FA was done by internal standard (C 21:0, Nu-Check Prep, Inc. Elysian USA). Total fat and phospholipids and FA as % of total identified per million cells in μg were evaluated.

Determination of cadmium uptake

The content of cadmium chloride was analyzed via ICP-MS as described earlier [28]. Quantification was performed with authentic standards. Cd^{2+} concentration in μM in pelleted cells was analyzed.

Statistical analysis

All analyses were conducted in triplicate or quadruplicate. Normality and homogeneity of dispersions of studied values and comparisons were made by analysis of variance (two factorial ANOVA; factors: Cd^{2+} and FA) with subsequent post hoc Tukey's honest significant difference (HSD) test. The values were expressed as means ± SD ($n = 3$). All analyses were performed at a significance level of $p < 0.05$ using STATISTICA 9.0 for Windows.

Results

FA concentrations suitable for incubation trials

In order to establish suitable incubation concentrations of FA, we first incubated the cells with the individual FA ranging for EPA from 1 μM to 50 μM (Fig. 1a) and for DHA from 2 μM to 100 μM (Fig. 1b), respectively. For EPA, the highest 50 μM concentration was above toxic effects for the cells (Fig. 1a). Neither the level of IC_{50}, nor level of the IC_{70} was reached. No significant change on cell growth measured by lysosomal integrity was found for this FA. For DHA, significant changes were found only between control line (Hep G2) and cells treated with 25 μM DHA and higher DHA concentrations showed significant toxic effects on the cells (Fig. 1b). A significantly negative effect of DHA on Hep G2 cell growth was observed after *in vitro* for 24 h at concentrations 50 μM DHA (IC_{70}) and the IC_{50} was reached at the level of 76 μM DHA (Fig. 1b). Furthermore, the cells showed only 29.3 % vitality at a level of 100 μM DHA (Fig 1b). In a second step we evaluated

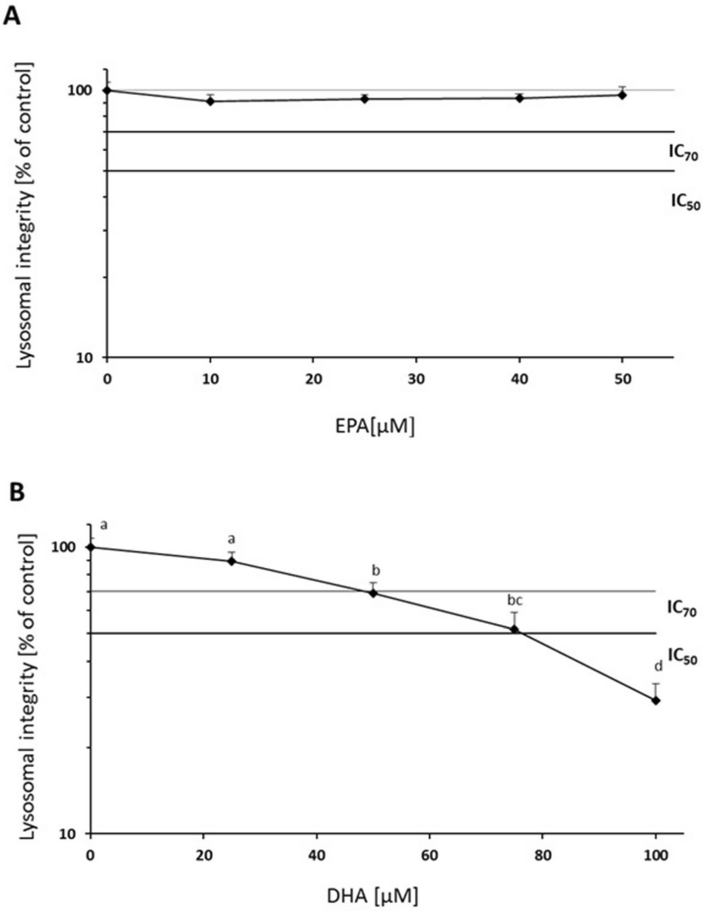

Fig. 1 Lysosomal integrity after *in vitro* pre-incubations of Hep G2 for 24 h with EPA (**a**) at nominal concentrations of 10, 25, 40 and 50 μM and DHA (**b**) at nominal concentrations of 25, 50, 75 and 100 μM compared to control cells. Data are presented as means ± SD, $n = 3$. Different letters denote significant difference between treatments (ANOVA, $p < 0.05$)

the effects of the incubation with a combination of EPA and DHA. The combination of 40 μM EPA+ 75 μM DHA resulted in a cell viability significantly below 50 % (18.9 % viable cells), while the combination of 10 μM EPA+ 20 μM DHA, was above the level IC_{70} (85.3 % vital cells) (Fig. 2), but was significantly lower compared to the control cells without added FA. As the level of DHA was still too high, we decreased the concentration to a combination of 5 μM EPA+ 10 μM DHA (Fig. 2). With these concentrations a cell viability of 96.9 % (EPA5 + DHA10) was reached. No cytotoxic effects of FA on cell growth in this combination with control line were found.

Cell viability

The resarzurin assay, evaluating cell viability, showed a significant correlation between Cd^{2+} incubation time and decreasing cell viability; an IC_{50} value of 6.6 μM and IC_{70} of 4 μM were measured after 24 h (Fig. 3a: Hep G2) and an IC_{50} value of 4.1 μM and IC_{70} of 3 μM after 48 h (Fig. 3b: Hep G2). Cell viability was significantly different in comparison to the control cells at 5 μM Cd^{2+} for both incubation times (Fig. 3ab).

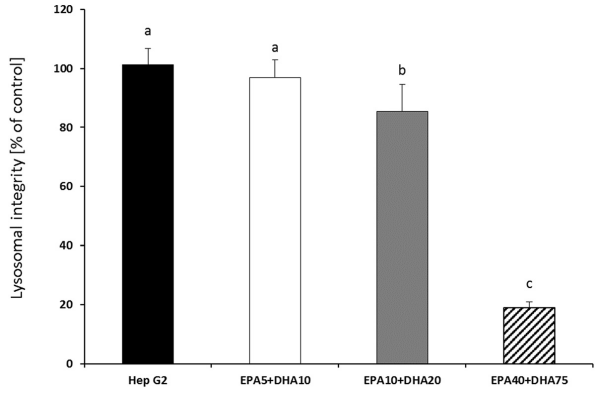

Fig. 2 Lysosomal integrity after *in vitro* pre-incubations of Hep G2 for 24 h with combined EPA+ DHA. Nominal concentrations of EPA5 + DHA10 μM, EPA10 + DHA20 μM and EPA40 + DHA75 μM compare to control cell line (Hep G2). Data are presented as means ± SD, $n = 3$. Superscript letters indicate significant differences between treatments (ANOVA, $p < 0.05$)

Pre-incubation with the BSA-FA complex resulted in significantly increased cell viability at concentrations starting at 5.5 μM Cd^{2+} (IC_{70}) for FA group in comparison with only 4 μM Cd^{2+} for control-Hep G2 (IC_{70}) and only 3.6 μM Cd^{2+} (IC_{70}: Hep G2 + BSA), after 24 h post-incubations with Cd^{2+} (Fig. 3a). No effects of BSA dissolved in PBS alone incubated with the cells (Hep G2 + BSA) were found compare to control (Hep G2), which demonstrated that the BSA can be used without affecting cell growth (Fig. 3ab).

Hep G2 lipid composition: phospholipids and fatty acids

Table 1 shows the contents of the long chain omega3-FA (EPA, DPA, DHA) in the cells with or without incubation of the FA for 24 h. A significant increase in the cells of both EPA and DHA was shown after the incubation (Fig. 4a and b). We demonstrated that after only two hours the maximum uptake of FA (Fig. 4b) was reached and there were no significant difference between 2, 24 or 48 h of FA incubation per million cells in Fig. 4a. Further we showed that repeated incubations were more effective. This was probably due to the totally increased substrate. After three replicated 2-h incubations with EPA or DHA (total 6 h of FA incubation of the cells with changing the medium every 2 h) proportions of FA in the cells were significantly increased compared to all other treatments and without toxic effects. When expressed as percentage of total fat, the increase of DPA was also significant after the incubation with FA; this suggests a synthesis from EPA toward DHA (Fig. 4b). When expressed as μg of fat per million (Mio.) cells, the increase of DPA was significant for only the 3 × 2-h incubation, which supports the hypothesis that an increased proportion of EPA will lead to an increased metabolism towards DHA (Fig. 4a). The level of EPA reached 1.31 μg per Mio. cells and the DHA level was almost eight times higher (2.33 μg per Mio. cells) compared to untreated cells (Fig. 4a). Moreover, EPA increased to 9.5 % and DPA to 3.3 % and the DHA level was almost three times higher (15.4 % of total identified FA than the untreated cells (Fig. 4b). In the control line, without FA incubation, both EPA and DPA showed levels of 0.6 % and DHA 2.7 % of total identified FA (Fig. 4b). Even though uptake had reached the maximum after 2 h, for practical reasons we decided to use 24-h pre-incubations for the subsequent FA and Cd^{2+} experiments.

Incubation with Cd^{2+} did not influence the content of EPA or DHA, (Table 1). However the subsequent incubation of the cells with the FA and 5 μM Cd^{2+} resulted in a significant increase of DPA.

There were no significant differences in phospholipid composition of the cells related to the pre-incubation with FA. Therefore, the data were combined to facilitate statistical evaluation. Figure 5 shows the phospholipid class composition in the Hep G2 after the various incubations. The phospholipids cardiolipin (CL) decreased significantly after incubation with 5 μM Cd^{2+} for 24 h. At this level of cadmium, only 12.1 % of CL were detected in cells compared to 14.9 % at 1 μM Cd^{2+} and 15.4 % without any cadmium treatment (Fig. 5). The decrease of CL between 1 μM and 5 μM incubations was significant ($p < 0.05$).

Uptake of Cd in relationship with FA

Figure 6 shows the result from Cd uptake, which was verified by ICP-MS. Three different concentrations of

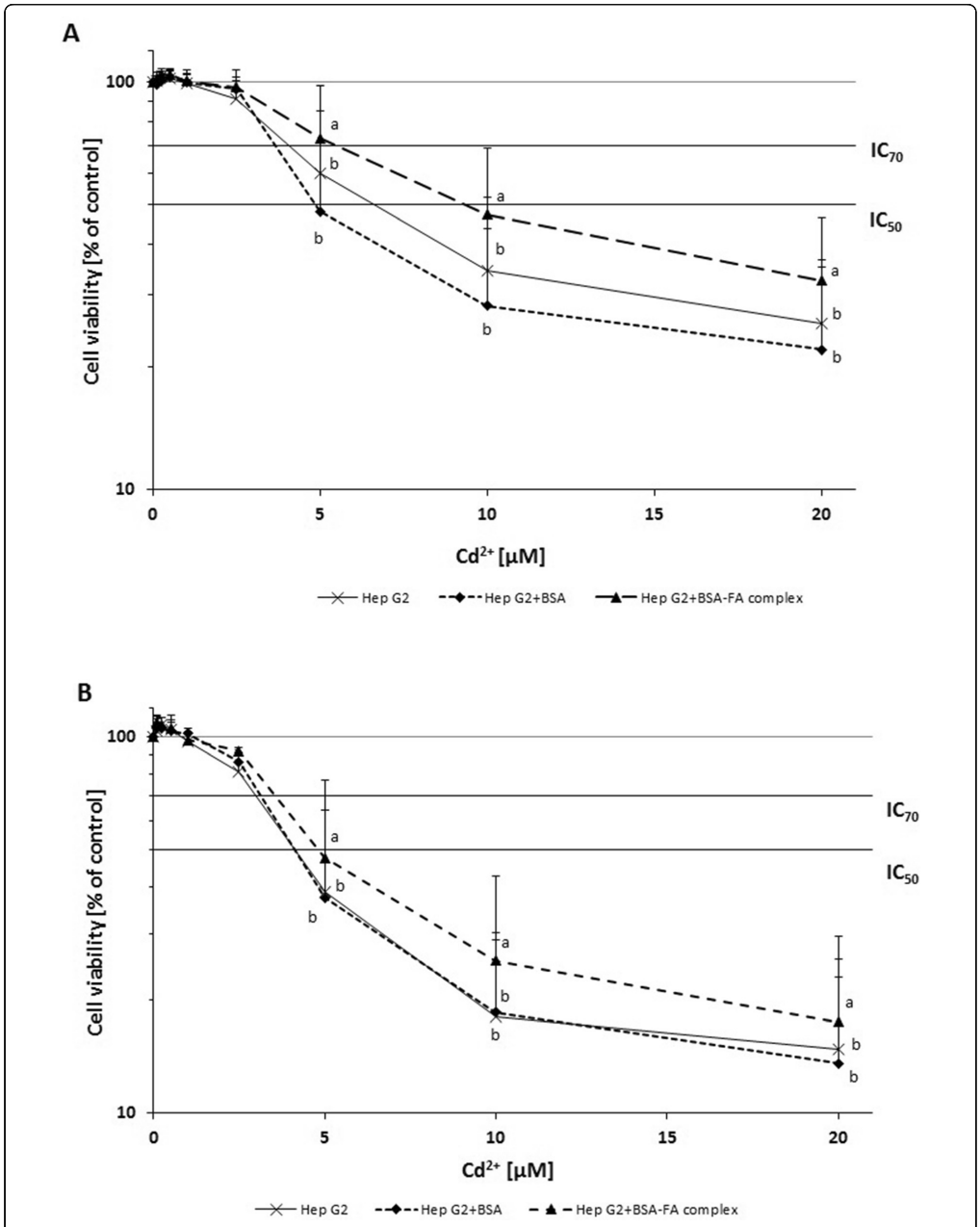

Fig. 3 Cell viability after *in vitro* pre-incubations of Hep G2 for 24 h with 5 μM EPA+ 10 μM DHA dissolved as BSA complex and 24 h (**a**) and 48 h (**b**) post-incubations with Cd^{2+} at nominal concentrations of 0.25, 0.5, 1, 2.5, 5, 10, 20 μM with changing culture (MEM) medium. Data are presented as means ± SD, $n = 3$. Different letters denote significant differences between treatments (two factorial, ANOVA, (FA, Cd^{2+}), $p < 0.05$)

Table 1 Fatty acid content in Hep G2 cells

μg FA per Mio cells	BSA-control no FA	BSA-FA (24 h)	BSA-control no FA Cd5 (24 h)	BSA-FA (24 h) Cd5 (24 h)
C20:5n-3	0.09 ± 0^{a}	0.37 ± 0.09^{b}	0.14 ± 0.05^{a}	0.59 ± 0.14^{b}
C22:5 n-3	0.10 ± 0.01^{a}	0.16 ± 0.03^{ab}	0.17 ± 0.07^{ab}	0.31 ± 0.06^{b}
C22:6n-3	0.40 ± 0.06^{a}	0.84 ± 0.08^{b}	0.57 ± 0.22^{a}	1.23 ± 0.19^{b}
SFA	5.61 ± 0.98	5.47 ± 0.66	7.24 ± 3.20	8.38 ± 2.19
MUFA	$8.19 + 0.98^{a}$	6.02 ± 0.33^{b}	8.65 ± 2.88^{ab}	7.48 ± 1.30^{b}
PUFA	$2{,}00 \pm 0.29^{a}$	2.60 ± 0.28^{b}	$2.84 + 1.08^{ab}$	4.01 ± 0.44^{b}
n-3	0.59 ± 0.07^{a}	1.38 ± 0.10^{b}	0.88 ± 0.34^{a}	2.13 ± 0.23^{b}
n-6	1.36 ± 0.26^{ab}	$1{,}20 \pm 0{,}19^{b}$	1.94 ± 0.76^{a}	1.81 ± 0.30^{ab}
n-6/n-3	2.28 ± 0.18^{a}	0.87 ± 0.08^{b}	2.21 ± 0.02^{a}	0.85 ± 0.08^{b}

FA content (% of total identified) in Hep G2 cells incubated with or without FA (24 h) and subsequently with or without 5 μM Cd^{2+} for 24 h. 'No FA' means cells were only incubated with BSA as control. Data are presented as means ± SD, $n = 3$. Different letters denote significant differences between treatments (two factorial ANOVA, (FA, Cd^{2+}), $p < 0.05$)

Abbreviations: *PC* L a-Phosphatidylcholine, *CL* Cardiolipin, *PA* L-a-Phosphatidic acid, and *EA* Phosphatidylethanolamine

Cd (1, 2.5 and 5 μM) were tested. One-half of pelleted cells was pre-incubated with FA for 24 h and all groups where then post-incubated with Cd^{2+} for the same time. The group incubated with the highest 5 μM Cd^{2+} and FA (5 μM EPA + 10 μM DHA), had a significantly higher uptake of cadmium chloride (457.6 365.8 μM), compared to the group not treated with FA (365.8 μM μM Cd^{2+}).

Discussion

In the present study we used a combination of EPA + DHA in a ratio 1:2 which is a proportion of FA typical for fatty fish such as common carp (*Cyprinus carpio*); carps are available on the local market year round [29]. Despite that fact that 60 μM DHA has been considered as physiological relevant [30], we found that the IC_{70} level had been already reached at 50 μM DHA. Our findings are in agreement with earlier results that DHA has cytotoxic effects on cancerogenic cells; similar toxic effects of DHA on cancerogenic neuroblastoma cells but not on non-transformed nervous tissue have been reported by Lindskog et al. [31]. These authors concluded that DHA counteracted cancer by causing apoptosis in the cancer cells. Juaudzus et al. [32] also showed that relatively high doses of DHA did not affect healthy cells. In the cell lines MIA PaCa-2, PANC-1 and CFPAC (human pancreatic cell lines), EPA was also shown to have an inhibitory effect of cell growth (IC_{50} 2.5-5 μM), [33]. In contrast, we found cytotoxic effects of DHA but not of EPA at concentrations of 50 μM. The mechanisms by which DHA acts differentially on cancerogenic versus normal healthy cells are still under discussion. In their review [34] describes the main mechanism to oxidative stress created by oxidation compounds derived from DHA. Due to their increased metabolism, cancer cells have increased natural levels of ROS and additional ROS can then not be counteracted as well as in healthy cells where the antioxidant mechanisms are still intact.

According to our results in the present setting, 5 μM EPA+ 10 μM DHA were the most suitable incubation concentrations for hepatocellular human liver cells (Hep G2; ATCC). We showed that the effect of EPA and DHA on the cells seems to be different, which should be evaluated further.

We showed that the incubated FA was taken up by the cells (Table 1), however, we found an increase of those FA only in the total lipid fraction (Table 1) and not in the subcellular lipid fractions (data not shown), indicating that an incubation time up to 48 h is probably too short for the FA to be incorporated into the cell structure.

When evaluating the incubation times, the maximum uptake already had been reached after only 2 h incubation with FA. After this, the proportion of the used FA did not significantly further. In contrast, Obermeier et al. [35] showed that the maximum levels of incorporation of AA, EPA, and DHA into U937 cell (human leukemic monocyte lymphoma cell line) was reached after 8 h. This finding suggests that different cell types might have different time optima for the uptake. Consequently, we decided to use 24-h incubation times for improved practical handling and to ensure a good repeatability.

In line with our results, Fujiyamafujiwara et al. [36] also found that incubation of Hep G2 cells with EPA resulted in a dose-dependent incorporation of EPA and a metabolism towards DPA but not DHA. The authors also found an increase of DHA, but a lower dose dependency, plus an increased amount of DPA in the cells after incubation with DHA, suggesting that DHA was ß-oxidized into DPA. These findings support our hypothesises that an increased proportion of substrate, in our case EPA, results in increased synthesis of the longer chain FA as well as a higher ß-oxidation of increased amounts of DHA into DPA.

In addition, we showed that repeated incubations are more effective. This is probably due to the totally increased

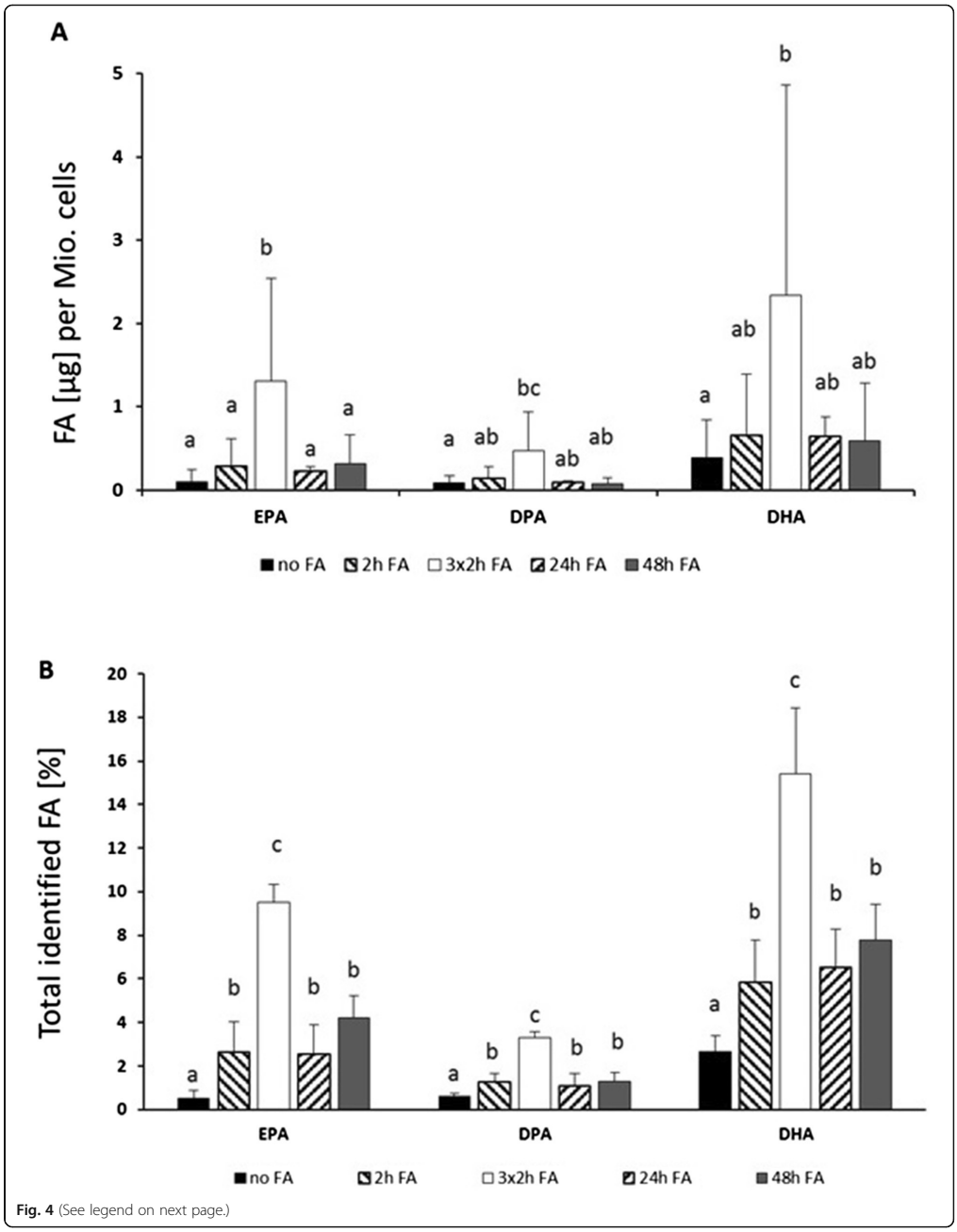

Fig. 4 (See legend on next page.)

(See figure on previous page.)
Fig. 4 Content of EPA, DPAn-3, DHA and total amount of FA after *in vitro* incubations of Hep G2 for 2, 3x2, 24 and 48 h with 5 μM EPA+ 10 μM DHA as BSA complex and different subsequent time of cell growth (30, 48 and 96 h), presented as μg of FA per Mio. cells (**a**) and total identified FA (%) content (**b**). 'No FA' means cells were only incubated with BSA as control. Data are presented as means ± SD, $n = 3$. Different letters denote significant differences of the respective FA between incubation times (ANOVA, $p < 0.05$)

substrate. In this way it seems to be possible to load the cells with greater amounts of EPA and DHA without the toxic effects caused by higher concentrated doses. This finding could be useful in future studies of the effects of elevated PUFA levels. On a metabolic level, the increased substrate also seems to result in a higher metabolism from EPA towards DPA and possibly DHA.

One of our hypotheses was that Cd^{2+} could cause a change in the cell lipid composition resulting in dysfunction and finally apoptosis. The results from the resazurin assay, with a significant correlation between Cd^{2+} incubation time and decreasing cell viability concurred with this, as the resarzurin method is based on the fact that normal viable and healthy cells reduce the blue resazurin to the pink resorfin. It is known that resazurin is effectively reduced in the mitochondria, making it useful also to assess mitochondrial metabolic activity. Hence we think that Cd^{2+} affects the mitochondrial functions. A reason for this could be oxidation of essential membrane lipids. An indication for this is that the highest used concentration of Cd^{2+} resulted in also decreased levels of cardiolipin (CL) in our study. CL is exclusively localized in the inner mitochondrial membrane and important for mitochondrial membrane functionality [37]. CL is especially sensitive to oxidation due to the high content including DHA [34]. Increased oxidation and subsequent decrease of CL has been connected to apoptosis [34] which is also inferred by our study.

In conclusion, our results, showed that a decrease in the proportion of CL together with a decreased cell viability, indicating that Cd^{2+} has an effect on cellular lipid composition and mitochondrial function.

Interestingly, the co-incubation of FA and Cd^{2+} resulted in a significantly higher uptake of Cd^{2+} at the highest concentrations of Cd^{2+} (5 μM), while the uptake seemed to reach a maximum level at 2.5 μM (Fig 6). This finding is in contrast to a study by Nostbakken et al., [38] evaluating the effect of EPA and DHA on methyl mercury (MeHg) uptake. In that study DHA decreased the uptake of MeHg in HEK293. In line with our results, DHA increased the uptake of the heavy metal and MeHg induced apoptosis in ASK. However, in the same study, EPA had an opposing effect and decreased the uptake of MeHg. Since we used a combination of these FA in our study, this could also mean that we had mixed increasing and decreasing effects on the uptake of Cd^{2+}. However, this hypothesis needs further evaluation.

Another interessting result from our study was that the combined incubation of the FA and Cd^{2+} at the

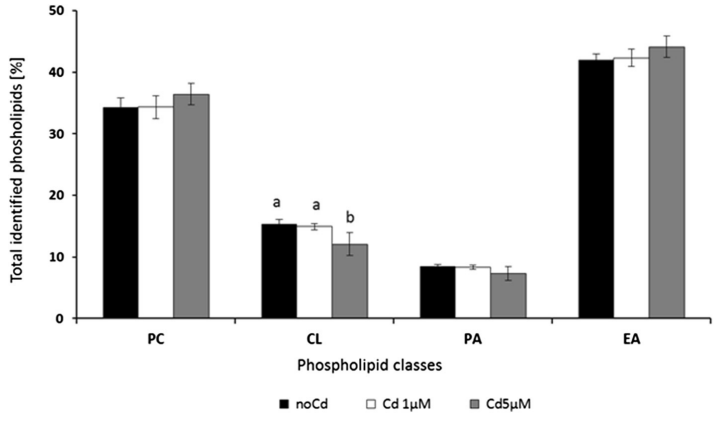

Fig. 5 Phospholipid class composition presented as percentage of total identified (means ± SD, $n = 3$) after *in vitro* pre-incubations of Hep G2 for 24 h with 5 μM EPA+ 10 μM DHA dissolved as BSA complex and 24 h post-incubations with/without Cd^{2+} at nominal concentrations of 1 and 5 μM with changing culture (MEM) medium. Data are presented as means ± SD, $n = 3$. Different letters denote significant differences between treatments (two factorial ANOVA, (FA, Cd^{2+}), $p < 0.05$)

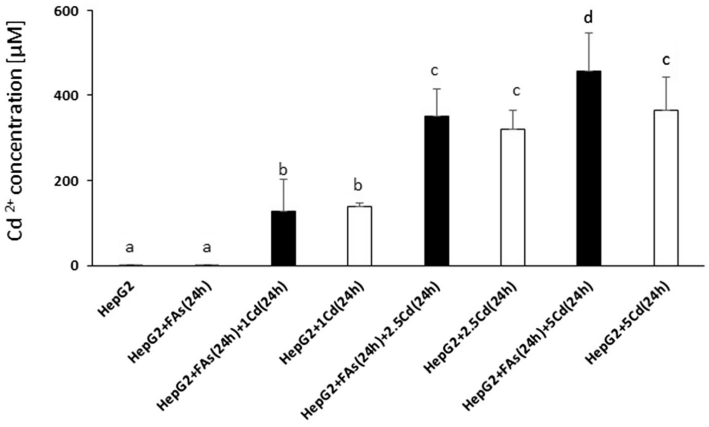

Fig. 6 Uptake of FA (24 h) and Cd^{2+} (1, 2.5 and 5 μM) for 24 h after *in vitro* incubations on Hep G2 cells with changing culture (MEM) medium. 'No FA' means cells were only incubated with BSA as control. Data are presented as means ± SD, $n = 3$. Different letters denote significant differences between treatments (two factorial ANOVA, (FA, Cd^{2+}), $p < 0.05$)

highest level increased the proportion od DPA in the cells. This indicates either an upregulated metabolism from EPA towards the longer chain products or an increased β-oxidation from DHA due to energy needs of the cells or oxidative stress. We hypothecize that Cd^{2+} has an enhancing effect on these processes. Therefore, the next step should be to evaluate various levels of oxidation and antioxidant response on the cells as well as the protein expression of the related elongases.

Conclusion

The findings of the present study showed that the applied FA were taken up by the cells and that subsequent incubation with Cd^{2+} did not decrease the contents of EPA and DHA. However, as a possible adverse effect, the combined incubation of FA and Cd^{2+} resulted in a significantly increased uptake of Cd^{2+} at the highest used levels. The possible toxic effects of this findings in vivo should be evaluated.

It should be highlighted that both FA (EPA and DHA) reduced the detrimental effect of Cd^{2+} on cell viability, which is the most relevant finding of present study with potentially important implications regarding fish consumption.

Further, the combined incubation of EPA and DHA and Cd^{2+} increased the levels of DPA in the total lipid content of the cells, which could either be a sign of increased metabolism from EPA to DPA or increased ß-oxidation from DHA to DPA induced by the cadmium. We also found that incubation with Cd^{2+} decreased CL, an essential phospholipid class in the mitochondria, indicating that part of the toxic effects of cadmium is related to mitochondria dysfunction. However, the reason for the increased DPA values needs to be investigated as well as the general oxidative stress parameters, which will be the subject of our further work.

Abbreviations

AA: arachidonic acid (C20:4 n-6); BSA: bovine serum albumin; BSA-FA: bovine serum albumin complex with fatty acids; Cd^{2+}: cadmium chloride; CL: cardiolipin; DHA: docosahexaenioc acid (C22:6 n-3); DPA: docosapentaenoic acid (C22:5 n-3); EA: phosphatidylethanolamine; EPA: eicosapentaenoic acid (C20:5 n-3); FA: fatty acids; MEM: Minimum Essential Medium Eagle; PA: L-α-phosphatidic acid; PBS: phosphate saline buffer; PC: L α-phosphatidylcholine; PUFA: polyunsaturated fatty acids..

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PL executed the cell culture work and the analyses related to cell viability and cadmium uptake and wrote major parts of the manuscript. Statistical evaluation was done together by both authors. SS was responsible for the study design, executed all lipid and fatty acid related analyses and wrote parts of the manuscript. All authors read and approved the final manuscript.

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CHAPTER 3

COMBINED INCUBATION OF CADMIUM, DOCOSAHEXAENOIC AND EICOSAPENTAENOIC ACID AFFECTING THE OXIDATIVE STRESS AND ANTIOXIDANT RESPONSE IN HUMAN HEPATOCYTES *IN VITRO*

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COMBINED INCUBATION OF CADMIUM, DOCOSAHEXAENOIC AND EICOSAPENTAENOIC ACID AFFECTING THE OXIDATIVE STRESS AND ANTIOXIDANT RESPONSE IN HUMAN HEPATOCYTES *IN VITRO*

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Short title: *Fatty acids, Cd^{2+}, ROS, Hep G2*

SUMMARY

Human hepatocellular cells Hep G2 were used to investigate the effects of the intake of contaminated fish on oxidative stress. Uptake of heavy metal contaminated fish was mimicked by incubating the cells with a combination of cadmium chloride (Cd^{2+}) as possible contaminant and a combination of eicosapentaenoic acid (EPA) and docosahexaenioc acid (DHA) as important fatty acids (FA) specific for fish. The main aim of this study was to determine the effects of these co-incubations (FA, Cd^{2+}) on lipid and protein oxidation. In addition we also evaluated the antioxidant response of the cells using two different methods (SOD and TAC). Pre-incubation with the chosen FA significantly reduced the oxidative stress caused by incubation with Cd^{2+}. We measured an increased level of carbonyl proteins (CP) in the cells pre-incubated with bovine serum albumin (BSA) and post-incubated with Cd^{2+}.
Key words: *EPA, DHA, fish lipids, heavy metal, ROS.*

1. INTRODUCTION

Pollution of the aquatic environment by heavy metals like cadmium poses a serious threat to the aquatic organisms including fish. In addition, subsequently and due to their lipophilic properties, heavy metals are bio-accumulated in the food chain and finally affect the consumers. The most anthropogenic sources of heavy metals are found in the industry, in addition to petroleum contamination and sewage disposal (Santos *et al.* 2005). Cd^{2+} is an environmental pollutant with a major intake via drinking water and food including seafood and fish. The main worldwide sources of cadmium are the phosphoric fertilizers used in crop farms (Mason 2002). Cd^{2+} concentrations in unpolluted natural waters are usually below 1 μg/l.

As fish are an important source of essential omega-3 fatty acids (FA) and therefore an integral part of the human diet, it is important to evaluate the possible effects of the consumption of contaminated fish. Omega-3 FA are the substrates for many hormone like substances that exert multiple beneficial effects in the human body, including anti-inflammatory actions by diminishing oxidative stress (Fukui *et al*. 2013). However, since the long chain polyunsaturated FA (PUFA) are also very prone to oxidation, this may lead to increased lipid peroxidation in the human body (Mori 2004). Several studies have shown that the accumulation of certain PUFA increase lipid peroxidation and the formation of lipid hydro peroxides in animals and humans (Cognault *et al.* 2000; Gago-Dominguez *et al.* 2005).

Cadmium (Cd^{2+}) is classified as a human carcinogen potent in a number of tissues, by International Agency for cancer research (IARC), (Waalkes 2003). Cd^{2+} acute intoxication leads to injuries of several organs, like liver, lungs or testes (Kasuya *et al.* 2000). Above that its chronic exposure is responsible to many human diseases as emphysema, end-stage renal failures, diabetic and renal dysfunction, deregulated blood pressure, osteoporosis, bone fractures or anemia (Jarup *et al.* 1998, Jin *et al.* 2004, Friberg *et al.* 1986). It also promotes the production of inflammatory cytokines (Maret and Moulis 2013). Important to know is that Cd has a high solubility and a huge bioaccumulation capacity in various aquatic species (Shreadah *et al*. 2015). However it also needs to be highlighted that despite enormous amount of research it is still not totally clear how Cd^{2+} induces cancer. There is clear evidence that multiple indirect mechanisms are involved in the tumor genesis, among which also oxidative stress has been mentioned by Hartwig (2010).

Oxidative stress can be defined as an imbalance between the systemic action of reactive oxygen species and the ability of biological systems to detoxify the reactive intermediates or repair the resulting damage in cells. Disturbances from normal redox conditions in cells can resort to toxic effects through the production of free radicals or peroxidases. These effects can damage all components inside of the cells, including proteins, lipids, or DNA (Sies 1995, 2007). It is known that Cd^{2+} can act as a catalyst in forming reactive oxygen species (ROS). It increases lipid peroxidation in addition it depletes antioxidants, glutathione and protein-bound sulfhydryl groups.

ROS is often involved in Cd^{2+} toxicology, as shown in different cell culture systems (Hart et al. 1999, He et al. 2008) and the acute toxicity of Cd^{2+} may result in advanced production of oxidative stress such as superoxide ion, hydrogen peroxide, and hydroxyl radicals (Bagchi et al. 1997; Liu and Jan 2000). Waisberg et al. (2003) also described that the expression of several stress response genes was induced by Cd^{2+}. The same authors also provide an overview of the effects of cadmium on various enzymes of the antioxidant system and concluded that this together with the increased production of ROS might explain the increase in lipid peroxidation and DNA damage in cells exposed to Cd^{2+} (Waisberg *et al.* 2003).

Cd^{2+} can cause inflammation in the liver and the activation of Kupffer cells, which have shown to be an important source for Cd^{2+}-induced inflammatory mediators of ROS such as IL-1β, TNF-α, IL-6, and IL-8. Several studies have shown a ROS production after exposure to Cd^{2+}, led to a reduction in cellular antioxidants and lowered cellular defense against oxidative stress (Kayama et al. 1995; Yamano et al. 2000). In the study of Yano and Marcondes (2005) the concentrations higher than 7.5 μM Cd^{2+} caused oxidation of lipids in skeletal muscle cells (myoblasts).

Knowing the capacity of Cd^{2+} to induce ROS production and the fact that the nutritional valuable long chain n-3 PUFA are very prone to oxidation, the aim of the present study was to investigate whether a combined exposure would increase the oxidative stress in the cells and thereby also alter the possible positive effects of the consumption of fish if that is contaminated. We chose eicospentaenoic acid (EPA) and docosahexaenoic acid (DHA) for a better understanding of the mechanism of nutritional important FA sensitive to oxidation. On the other hand, as DHA also has shown to have some anti-carcinogenic properties. For this reason we suppose that these FA may be helpful in the development of effective cancer chemotherapeutic strategies involving their use as potential anticancer adjuvants.

2. METHODS

2.1. Caution

Inorganic cadmium chloride ($CdCl_2$, Cd^{2+}) is classified as a human carcinogen (IARC 1993). This chemical is hazardous, or potentially hazardous and should be handled with care.

2.2. Chemicals

EPA and DHA supplied from Biochrom and Sigma-Aldrich (Berlin, Germany), were diluted in extra pure 98% ethanol and bovine serum albumin (BSA) before transfer experiments. Hydrogen peroxide solution (30%, Suprapurs) and nitric acid (65%, Suprapur) were products of Merck (Darmstadt, Germany). Cadmium chloride was obtained from Aldrich, Germany. All other pro-analysis chemicals were obtained from Sigma-Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany). Trypsin, penicillin and streptomycin solutions were products of Sigma (Deisenhofen, Germany). Moreover, the culture dishes and the culture medium (MEM) for Hep G2 cells were obtained from Biochrom (Berlin, Germany).

2.2.1. Cell Culture

Human hepatocellular cells (Hep G2, ATCC, No. HB-8065) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Hep G2 cells were grown as a monolayer in culture dishes in Minimum Essential Medium Eagle (MEM) supplemented with fetal bovine serum (FBS), (10%, v/v), non-essential amino acids (1%, v/v), glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml). The Hep G2 cultures were incubated at 37°C with 5% CO_2 in air with 100% humidity. The amount of 1.5 million cells were seeded on 10 cm (in diameter) sterile Petri dishes in 10 ml of sterile culture medium (MEM). Suspensions of Hep G2 cells were produced from confluent cultures using trypsin/EDTA solution. Before the transfer experiments, cells were three times thawed and sub-cultured to achieve a stable phenotype. For the transfer experiments, cells were seeded at a density of 1.5 Mio (million) per normal petri dishes (10 cm in diameter). Seeded Hep G2 were cultured for 24 h and subsequently prepared for following pre-incubations and post-incubations with FA and Cd^{2+}.

2.2.2. EPA and DHA pre-incubations

Before incubations fresh stock solutions of FA diluted in extra pure 98% EtOH were defrosted. The BSA-FA complex was prepared as follows: BSA was dissolved in PBS (phosphate buffer saline). EPA and DHA respectively were dissolved in extra pure EtOH to a final volume of 50µl and 20µl of these solutions were then added to 1ml of a mixture of 0.1 M NaOH and BSA solution (1/5; v/v) each. The two solutions of FA were then combined and the pH was adjusted to 7.4 using 0.1M HCl. After testing concentrations from 1-50 µM EPA and from 2–100 µM DHA (preliminary results) we chose concentrations for pre-incubations of liver human cells with 5 µM EPA+ 10 µM DHA for 24 h without changing MEM.

2.2.3. Cd^{2+} post-incubations

Fresh stock solutions of Cd^{2+} diluted in distilled sterile water (ddH_2O) were prepared before the transfer experiments. Hep G2 cells were post-incubated for 24 h with Cd^{2+} stock solution with changing the cell culture medium (MEM). The range of Cd^{2+} concentrations were used from min. 1 μM to max. 5μM.

2.2.4. Confirmation of uptake of cadmium and FA into the cells

The content of cadmium was earlier analysed via ICP-MS as described earlier. Quantification was performed with an authentic standard (Linhartova and Sampels 2015).

Uptake of FA was also earlier confirmed as described in Linhartova and Samples (2015).

2.3. Pelleting of Hep G2 cells

Cells were seeded in number of 1.5 million cells per Petri dish to 10 ml MEM sterile culture medium. Seeded Hep G2 were cultured and treated with FA as a BSA-FA complex and Cd^{2+} for the appropriate times as described above. Subsequently cells were pelleted by trypsinising and centrifuging several times in PBS/FBS solution and frozen on -80°C.

2.4. Oxidative stress and antioxidant response from pelleted cells

2.4.1. Extraction of pelleted samples

Pellets were unfrozen on room temperature. Immediately after thawing, the tubes with pelleted cells were put on ice. For analyses of pellets, we dissolved each pellet in 500 μl of extraction buffer (EB) and vortexed them well. Extraction buffer (EB) was prepared from phosphate buffer saline (PBS) containing 0.5mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF).

2.4.2. TBARS

For evaluation of thiobarbituric acid reactive substances (TBARS) (Lushchak *et al.* 2005, Lushchak 2011) each pellet (with FA/without FA and with/without Cd^{2+}, 1 and 5 μM Cd^{2+}) was dissolved in 500 μl EB solution. Afterwards 100 μl of sample dissolved in EB was taken for reaction process. 900 μl of 10% TCA in 0.2 M H_3PO_4 in total volume of 1 ml of solution was added to the sample. After centrifuging, the supernatant (upper phase containing lipids) was transferred to clean tubes and the volume of supernatant was equally divided to get blank and reagent samples. TBA solution or water was added to reagent and blank samples. A standard curve was prepared using tetraethylpropane (TEP). Finally all samples were incubated for 30 min., 60°C on a plate mover and the absorbance at 530 nm was measured on plate reader (Invinite 200Pro, Tecan Group Ltd., Mannedorf, Switzerland).

2.4.3. CP

Carbonyl Proteins (CP) as markers for protein oxidation were analyzed according to a method by (Lenz et al. 1989). 10% TCA in 0.2 M H_3PO_4 was added to each sample pellet and centrifuged (5000 x g, 10 min., 4°C), afterwards the supernatant was removed. Consequently EB was added and samples were vortexed well to resuspend the pellet. The obtained suspension was

divided equally to two tubes for each sample. After centrifugation (5000 x g, 10 min, 4°C) the supernatant was removed and 2,4-dinitrophenylhydrazine (10 mM DNPH) was added to one group of samples (CP sample), and 2M HCl was pipetted to the second group (blank sample). Samples were vortexed to re-suspend the pellets. Finally samples were left at 22°C in dark for 1 h. Then samples were centrifuged (5000 x g, 10 min, 4°C), supernatant was poured out and pellets were washed with ethanol-butyl acetate. After washing 6M guanidine-HCl was added, the pellets were homogenized and centrifuged to remove insoluble particles. At the end of this experiment the absorbance of supernatant was measured using plate reader (Invinite 200Pro, Tecan Group Ltd., Mannedorf, Switzerland) at 370 nm and the absorbance of total protein content was measured at 562 nm. The CP was counted according to the formula:

$CP = ((A_{370\ sample} - A_{370\ blank})/0.011)/220\ \mu l = ((A_{370sample} - A_{370blank})/0.011)/\ 0.22\ ml.$

2.4.4. SOD

Inhibition activity of superoxide dismutase (SOD), was evaluated with a commercially available assay Kit (19 160 SOD determination kit, Sigma Aldrich, St. Louis) according to the manufacturers description. The absorbance of blanks and samples was measured using a plate reader at 450 nm and the Inhibition rate (%) was counted according to formula:

Inhibition rate%= [(average blank 1-average blank 3)-(average sample- average blank 2)]/ (average blank 1- average blank 3).

2.4.2. TAC

Total antioxidant capacity (TAC) was analyzed using a commercial assay kit (Total Antioxidant Capacity Assay Kit, Sigma Aldrich, St. Louis) following the manufactures instructions. Pellets were dissolved in the same way was as described above for the previous kits. Supernatant was transferred from centrifuged samples to clean eppendorf tubes and put on ice. The 96 well-plate was prepared and firstly Trolox standart (0, 0.2, 0.4, 0.67, 0.8 and 1 mM) was pipetted. Continually pellet samples were pipetted (3 times each sample per well). Cu^{2+} reagent solution from the kit was added to each well and mixed with the samples or standards. The plate was incubated in dark at 22°C for 90 min. The absorbance of samples at 570 nm was measured using a plate reader (Invinite 200Pro, Tecan Group Ltd., Mannedorf, Switzerland) and the Trolox standart index was counted.

2.5. Statistical analysis

All analyses were conducted in triplicate. Normality and homogeneity of dispersions of studied values and comparisons were made by analysis of variance (two factorial ANOVA; factors: Cd^{2+} and FA) with subsequent post hoc Tukey's honest significant difference (HSD) test. The values were expressed as means ± SD (n=3). All analyses were performed at a significance level of $p < 0.05$ using STATISTICA 9.0 for Windows.

3. RESULTS

3.1. Cadmium and FA uptake

Final cadmium content in the cells was related to the incubation concentration. At an incubation level of 1 μM Cd^{2+} and final content of 127.18±75 and 138.45±8.27 μM Cd^{2+} /10^6 cells with and without FA (no significant difference) was reached, but significant differences were found between groups incubated with 5 μM Cd^{2+} and with/without FA, where final concentrations of 457.63±88.03 and 365.84±76.93 μM/10^6cells, respectively were found. At the 5 μM Cd^{2+} co-incubation with FA seemed to increase Cd^{2+}. Uptake of the FA into the cells was also evaluated. These results are further discussed in Linhartova and Sampels (2015).

3.2. Oxidative stress indices

Our results showed that, in the control group (0), the measured level of thiobarbituric acid reactive substances (TBARS) expressed as malondialdehyde was 8.93 nmol/10^6 cells (Fig. 1). No significant differences were found for cells incubated with BSA or FA or the combination of FA and Cd^{2+} (Fig. 1). However, a significantly higher level of TBARS was observed in Hep G2 cells exposed to only Cd^{2+} at concentrations 1 μM and above (13.79 nmol/10^6 cells and 16.89 nmol/10^6 cells for 1 and 5 μM Cd^{2+} respectively).

Similar results were found for protein oxidation (Fig. 2). Control and cells incubated with FA or with FA and Cd^{2+} showed no significant difference in CP while significant ($p < 0.05$) increased levels of CP was observed in group exposed to 1 μM Cd^{2+} (2.55 uM/ug) and 5 μM Cd^{2+} (4.25 uM/ug). However, cells exposed to BSA reached the level of CP to 2.12 uM/ug, which was an increase compare to control and FA groups.

3.3. Antioxidant responses

The antioxidant activity was assessed by total SOD and TAC activity. There was no significant difference in SOD level between cells in control (0) and groups incubated with BSA and FA or with 1 μM Cd^{2+} (Fig. 3). However, the antioxidant response was significantly enhanced in cells exposed to 5 μM Cd^{2+} (20.68 mU/10^6 cells).

Similar results were found for TAC (Fig. 4). Significant increased TAC values were observed in the cells exposed to 5 μM Cd^{2+}.

4. DISCUSSION

The level of TBARS and CP were used as indicatives of the extent of lipid oxidation (LO) and protein oxidation (PO), respectively. Cd^{2+} alone in concentrations from 1 μM induced oxidative stress. However, our results show a clear effect of the used FA in keeping both lipid and protein oxidation at levels comparable to control even when Cd^{2+} is present. In opposite incubation with only Cd^{2+} without the FA resulted in increased oxidation. This indicates to a protective effect of the chosen FA against oxidative stress caused by Cd^{2+}. Also the results from the antioxidant response parameters point towards a protective effect of the FA, even if a significant increase of the antioxidant response was only visible at the highest used Cd^{2+} concentrations. However also here the co-incubation with FA resulted in similar low values of antioxidant response as in the non-exposed cells.

The increased CP values for the cells incubated with BSA only could be caused by the de facto higher protein availability in these cells from the free BSA used for incubation. In the cells incubated with FA as a BSA-FA complex, BSA was bound to the FA, which might result in a different reactivity and lower availability for oxidation.

Although the antioxidant system apparently responds to the increased ROS production, it seems that the antioxidant capacity of Hep G2 cells exposed to Cd^{2+} (without FA) is not sufficient to prevent cell damage. The antioxidant response, as shown by TAC and SOD assays, had significantly different values between groups treated with FA and with cadmium chloride only. The present study clearly demonstrated that Hep G2 cells are highly susceptible to oxidative stress induced by the environmental pollutant Cd^{2+}.

In opposite to our results Qu et al. (2005) found that the exposure of rat liver cells to a low-dose (1.0 μM) of Cd^{2+} after 28 weeks of continuous heavy metal exposure did not produce ROS. However this was a long term exposure, and already (Waisberg et al. 2003) showed in his review a possible adaptive effect against oxidative stress during long term exposure. On the other hand and in line with our results ROS production was evident after acute exposure of liver cells to higher doses of Cd (between10–50 μM Cd), which are higher Cd^{2+} concentrations, but obviously human cells are more prone to oxidative stress than animal cells (Qu et al. 2005). This suggests that the effects of acute and chronic exposure to Cd^{2+} might differ and need a further evaluation.

Only few studies have investigated whether ROS are generated as the reaction to Cd^{2+} in Hep G2 cells and if this is responsible for inducing oxidative stress. The research of Lawal and Ellis (2010) showed a toxic effect of 5, 10 and 50 μM Cd^{2+}, in three human different cell lines, (hepatocellular HepG2, astrocytoma (1321N1) and embryonic kidney (HEK 293) human cell lines). However significant increase of malondialdehyde (TBARS) and antioxidant enzymes activities were found in all three mentioned cell groups only after exposure to 50 μM Cd^{2+}, which differs from our data, where we found ROS production already after exposure to ten time lower Cd^{2+} concentrations. More in line with our results, another study showed that already lower doses of Cd^{2+} (10-20 μM) produced ROS in MCF-7 breast cancer cells (Matsuoka and Igisu 2001).

Many studies using different supplements like N-acetyl cysteine, vitamin E, vitamin C, and selenium have shown that these substances can enhance body anti-oxidant machinery, by for example decreasing Cd^{2+} -induced oxidative stress in kidney, liver, and testes, with improved cellular and tissue functions in many *in vitro* and *in vivo* studies (Shaikh et al. 1999; Sen Gupta et al. 2004; Zhou et al. 2009).

The results need to be confirmed in some kind of *in vivo* model, but our studies show that human cells *in vitro* assays may provide means of evaluating the effects of human environmental pollutants in relationship with nutrients as FA on the human body. This is necessary as usually the contaminants are not entering the human body alone but usually together with some food, which might result in some interaction, as shown in the present study.

5. CONCLUSIONS

Our results showed a positive effect of FA (5 μM EPA and 10 μM DHA) against oxidative stress in the Hep G2 cells. It can be concluded, that antioxidant defense system, lipid peroxidation and oxidative damages are positively affected by the FA pre-incubations with the chosen FA in the determined doses. The combination of EPA and DHA seem to have a potential to decrease oxidative stress caused by Cd^{2+} in concentrations from 1 μM in hepatocytes. Further research is needed to investigate the mechanism and the relationship between oxidative stress and FA uptake by liver cells.

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Abbreviations: BSA: bovine serum albumin; BSA-FA: bovine serum albumin complex with fatty acids; Cd^{2+}: cadmium chloride; DHA: docosahexaenioc acid (C22:6 n-3); EPA: eicosapentaenoic acid (C20:5 n-3); FA: fatty acids; MEM: Minimum Essential Medium Eagle; PBS: phosphate saline buffer; PUFA: polyunsaturated fatty acids.

Competing interests: The authors declare that they have no competing interests.

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Figures:

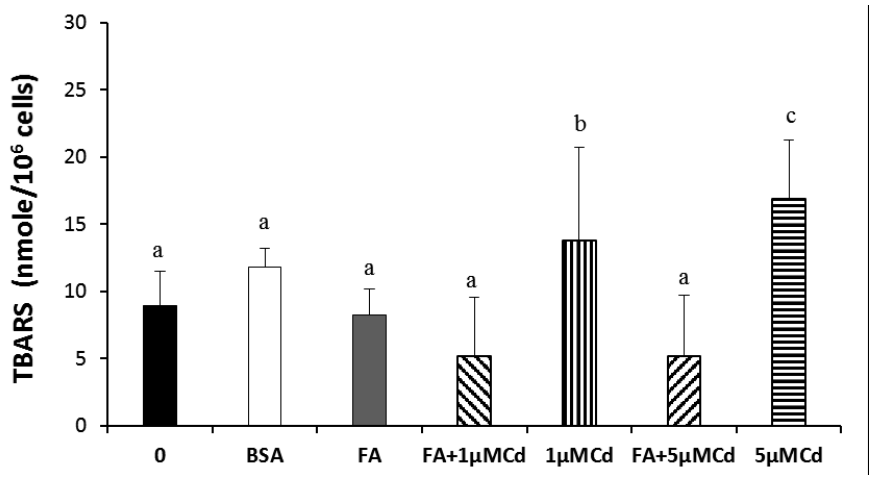

Fig. 1. *Effects of BSA, FA and Cd^{2+} on TBARS in Hep G2 cells. Data represent mean values ± SD, n=3. Different letters indicate significant differences among samples (two factorial, ANOVA, FA and Cd^{2+}, p <0.05).*

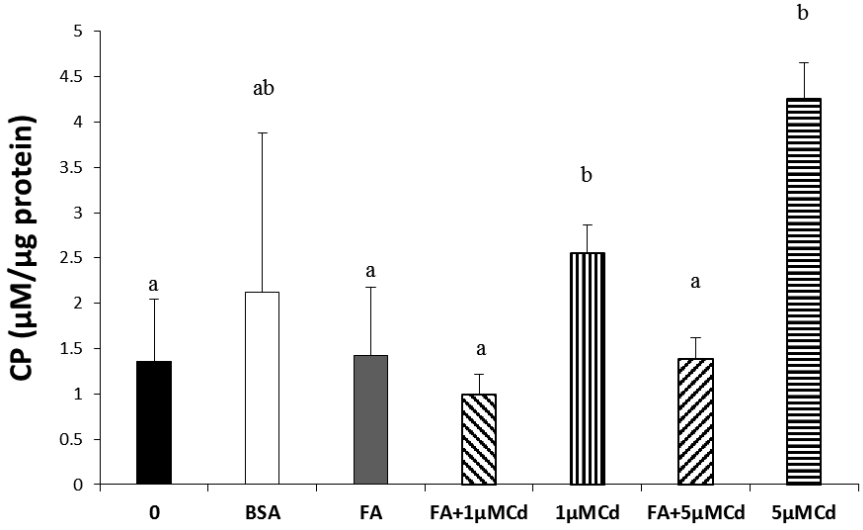

Fig. 2. *Effects of BSA, FA and Cd^{2+} on CP in Hep G2 cells. Data are presented as means ± SD, n=3. Different letters indicate significant differences among samples (two factorial, ANOVA, FA and Cd^{2+}, p <0.05).*

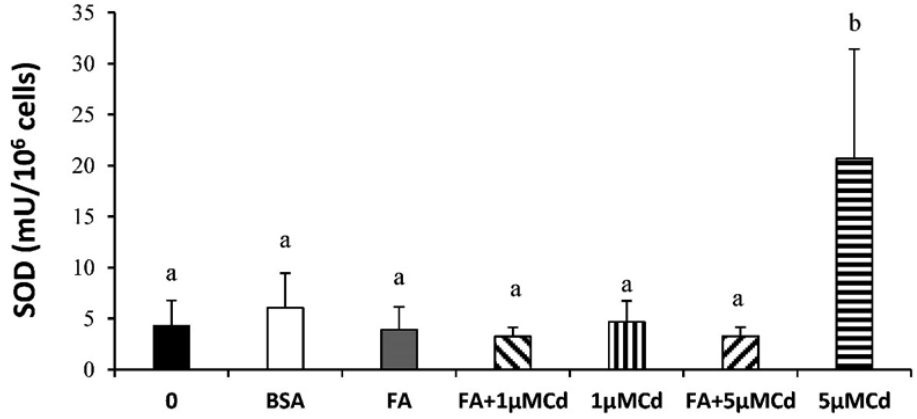

Fig. 3. *Effects of BSA, FA and Cd^{2+} on SOD activity in Hep G2 cells. Data represent mean values ± SD, n=3. Different letters indicate significant differences among samples (two factorial, ANOVA, FA and Cd^{2+}, p <0.05).*

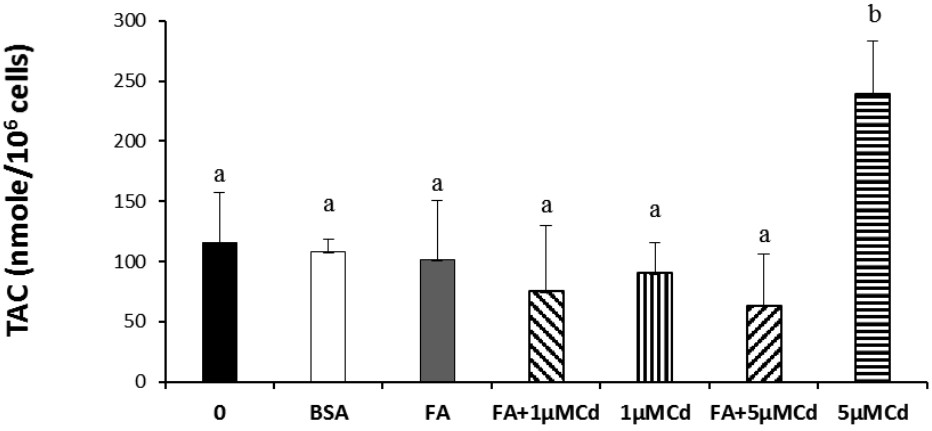

Fig. 4. *Effects of BSA, FA and Cd^{2+} on TAC activity in Hep G2 cells. Data are presented as means ± SD, n=3. Different letters indicate significant differences among samples (two factorial, ANOVA, FA and Cd^{2+}, p <0.05).*

CHAPTER 4

THE *IN VITRO* EFFECT OF DUROQUINONE ON FUNCTIONAL COMPETENCE, GENOMIC INTEGRITY, AND OXIDATIVE STRESS INDICES OF STERLET (*ACIPENSER RUTHENUS*) SPERMATOZOA

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The *in vitro* effect of duroquinone on functional competence, genomic integrity, and oxidative stress indices of sterlet (*Acipenser ruthenus*) spermatozoa

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ABSTRACT

The sturgeon is a highly endangered fish species mostly due to over-fishing, habitat destruction, and water pollution. Duroquinone (derivative of 1,4-benzoquinone) is a xenobiotic compound widespread in the environment. The effect of duroquinone on motility, DNA integrity, and oxidative stress indices in sterlet, *Acispenser ruthenus*, spermatozoa was investigated. Sterlet sperm was exposed for 2 h to duroquinone at concentrations of 25, 50, 100, and 150 μM. Spermatozoa motility, velocity, and ATP content were significantly decreased with exposure to duroquinone. The level of DNA damage significantly increased at concentrations of 50 μM and above. Oxidative stress indices (lipid peroxidation and content of carbonyl proteins) and superoxide dismutase (SOD) activity increased significantly with increasing concentrations of duroquinone. Oxidative stress in sterlet spermatozoa induced by duroquinone was shown to impair spermatozoa DNA integrity, motility parameters, and the antioxidant defense system. Spermatozoa motility, content of carbonyl proteins, and SOD activity were shown to be sensitive biomarkers, exhibiting strong responses to low concentrations of the xenobiotic. Results also suggested that fish spermatozoa *in vitro* assays may provide a simple and efficient means of monitoring residual pollutants in the aquatic environment.

1. Introduction

Over the past 20 years it has been established that fish are capable of a wide variety of biotransformation reactions (Li et al., 2012; Thomas et al., 1998). Recent studies on the biotransformation of xenobiotic chemicals in fish have focused on the specific metabolites produced, since these metabolic reactions affect distribution, accumulation, and toxicity of chemicals (Hulak et al., 2008; Mikula et al., 2009). Xenobiotics are also known to enhance production of reactive oxygen species (ROS) (Livingstone, 2001) that can attack a variety of biological macromolecules such as DNA, proteins, and lipids, leading to oxidative damage (Kelly et al., 1998). Increased levels of ROS are believed to be involved in male infertility associated with lipid peroxidation and oxidative stress, which affect sperm physiology and integrity (Sikka et al., 1995).

Duroquinone is a potentially hazardous xenobiotic compound receiving recent attention. Duroquinone is a derivate of 1,4-benzoquinone which is well soluble in water, and, at low concentrations (50–150 μM), was shown to induce cytotoxic effects through ROS production (Lemaire et al., 1994; Zhou et al., 2006).

A wide variety of quinones (Smith et al., 1985), including duroquinone, enter freshwater and marine ecosystems from anthropogenic sources. Quinones are widespread in the environment and form an important class of toxic metabolites generated by the metabolism of phenols and related compounds, including phenol, 1-naphthol, and diethyl stilbesterol. They occur both naturally and as water pollutants at concentrations to 480 μg/l (Usenko, 2012). One of the more common types of quinones is benzoquinone and its derivatives. Among them, duroquinone is of prime interest due to its ability to induce mitochondrial Ca^{2+} release through H_2O_2 generation by redox cycling followed by NADPH oxidation and gluthatione oxidation (Moore et al., 1987). Hence if the antioxidant system is robust, duroquinone has only a minimal cytotoxic effect, as shown in rat hepatocytes (Moore et al., 1987), and may be more damaging to cell types lacking protective cytoplasmic enzymes.

Reproductive success is a key factor in determining species survival. Water pollution can decrease the quality of gametes of aquatic organisms, which in turn may affect fertilization success, hatching of embryos, and subsequent survival of offspring. The gametes of most fish species are usually released into water, where they can be directly exposed to manmade compounds capable of interrupting cellular metabolism at various levels. Since spermatozoa movement is a major prerequisite for successful fertilization, assessment of motility and velocity may be a sensitive and accurate bio-indicator of aquatic pollution (Li et al., 2010a).

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The sturgeon (Acipenseridae) is among the oldest living vertebrates and is classified as endangered fishes by many international organizations. Their population has drastically decreased, mostly due to over-fishing and destruction of natural habitat, as well as to water pollution (Birstein and DeSalle, 1998). For a large species such as sturgeon, it is logistically difficult and costly to conduct toxicity evaluations on broodstock-sized animals (Tashjian et al., 2006). In the present study we used sterlet *Acipenser ruthenus* as a model to investigate potential adverse effects of duroquinone on spermatozoa in sturgeon. *A. ruthenus* is a common Eurasian sturgeon, that has undergone significant decline, but local populations survive in most parts of its distribution range (rivers draining to the Black, Azov, and Caspian Seas; Siberia from Ob eastward to the Yenisei drainages; and Danubian estuaries). The sterlet is an attractive model for biological studies of sturgeon because of its freshwater status, its small size and therefore low cost of maintenance, and its early sexual maturation. Sterlet spermatozoa differ from those of teleost fish (Billard, 1986) in the presence of an acrosome (Psenicka et al., 2007) with acrosin- and trypsin-like activities (Ciereszko et al., 1994, 1996), as well as in size and behavior (Cosson et al., 2000). The presence of acrosomal reactions in sturgeon spermatozoa means differing enzyme activity from teleosts (Ciereszko et al., 1996). The differences in sperm morphology and biochemistry between chondrostean and teleost fish species may translate to different reactions to environmental stimuli.

The aim of this study was to investigate how spermatozoa physiology is affected by short-term (2 h) *in vitro* exposure of sterlet sperm to environmentally relevant concentrations of duroquinone, by analyzing DNA integrity, oxidative stress indices (lipid peroxidation – LPO and protein carbonylation – CP), and antioxidant enzyme (superoxide dismutase – SOD) activity of sturgeon sperm, as well as spermatozoa motility and velocity. The effect of duroquinone on intracellular ATP level was also investigated.

2. Materials and methods

2.1. Broodstock handling and collection of gametes

Sperm of sterlet was obtained from six males (age: 6–7 years; body weight: 1.5 ± 0.2 kg; body length: 55 ± 9.3 cm) reared in the aquaculture facility of the Research Institute of Fish Culture and Hydrobiology at the University of South Bohemia. Spermiation was stimulated by intraperitoneal injection of carp pituitary powder dissolved in 0.9% NaCl solution at doses of 4–5 mg/kg of body weight, 48 h before sperm collection. Sperm was collected in 250 ml cell culture containers and kept on ice (0–4 °C) until processing. Sperm concentration of each male was estimated microscopically (Olympus BX 41) at 20× using a Burker cell hemocytometer.

2.2. Chemicals

All chemicals used were analytical grade and purchased from Sigma (St. Louis, MO, USA). The stock solution of duroquinone (DQ) was dissolved in 96% ethanol and diluted with distilled water to obtain a stock solution of 10 mg/ml.

2.3. Sperm dilution and exposure

Sturgeon sperm was diluted with an immobilization medium (20 mM Tris, 30 mM NaCl, 2 mM KCl, pH 8.5) to obtain a spermatozoon concentration of 5×10^7 cells/ml. We use the standard concentration of spermatozoa per egg in sterlet to ensure fertilization success. Sperm sub-samples ($n = 6$) were then exposed for 2 h to environmentally realistic DQ concentrations of 25, 50, 100, and 150 μM. A control group was exposed to immobilization medium with 1% ethanol equal to the amount of ethanol in the experimental samples. Each experimental condition was conducted in duplicate.

2.4. Sperm motility and velocity

Spermatozoa velocity and percent of motile spermatozoa were determined after triggering motility under dark-field microscopy (Olympus BX 50, Japan) (20× objective magnification). For triggering, sperm was diluted in activation medium (10 mM NaCl, 1 mM CaCl, 10 mM Tris pH 8.5) at a dilution ratio of 1:5000. To avoid sperm sticking to the microscope slide, Pluronic 0.2% (w/v) (Sigma–Aldrich, USA) was added to the swimming solution. Spermatozoa motility was recorded with a CCD video camera (SONY DXC-970MD, Japan) mounted on the microscope, illuminated with a stroboscopic flash (ExposureScope®, Czech Republic). Analysis of sperm motility was done in triplicate for each sample.

Velocity and motility were assessed at 10, 30, 60, 90, and 120 s post-activation. The successive positions of the video recorded spermatozoa heads were analyzed from video frames using Olympus MicroImage software (Version 4.0.1. for Windows with a special macro by Olympus C & S). Velocity and percent motility were calculated from spermatozoa head positions on five successive frames with three colors (frame 1 red, frames 2–4 green, and frame 5 blue). Twenty to 40 spermatozoa were counted for each frame. Spermatozoa that moved were visible in three colors, while non-moving spermatozoa were white. The percent of motile spermatozoa was calculated from the number of white and red cells. Spermatozoa velocity was calculated at μm/s based on length traces of spermatozoa from blue to green and red heads, calibrated for magnification.

2.5. Assessment of DNA damage

The alkaline single-cell gel electrophoresis (Comet) assay followed the methods of Li et al. (2008). Unless otherwise stated, molecular grade DNAse-free reagents (Sigma Aldrich, USA) were used throughout. Microscope slides (OxiSelectST; Cell Biolabs, Inc. USA) were used for the assay, with each slide prepared in the following manner: 100 μl of sperm (5×10^7 cells/ml) was diluted in 5 ml of PBS. Diluted samples (200 μl) were mixed with 700 μl of 0.8% NuSieve GTG low melting temperature agarose (OxiSelectST; Cell Biolabs, Inc. USA). Finally, 55 μl of this mixture was added to the slide, and the agarose was allowed to solidify for 1 h. Slides were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10% of 10× Lysis Solution [OxiSelect™ Comet Assay Kit], 10% DMSO pH 10). The slide was then subjected to enzyme treatment with proteinase K (20 mM Tris–HCl, 1 mM $CaCl_2$, and 50% glycerol, pH 7.4) and incubated for 12 h at 32 °C. After the proteinase K solution was drained, the slides were immersed in a horizontal gel tank filled with running buffer, and electrophoresis was carried out for 20 min at 35 V and 170 mA. The slides were drained well, dehydrated by dipping into absolute ethanol for 5 min, and air-dried for storage. Fifty μl of Vista Green DNA Staining Solution (OxiSelectST; Cell Biolabs, Inc. USA) was added to each agarose spot, and the slide was examined using an Olympus BX50 fluorescence microscope at 20× magnification. One hundred cells were scored for each sample, and the captured images were analyzed using CometScore image analysis software (TriTek Corporation, USA). Tail length (measured from the middle of the head to the end of the tail) and tail DNA content (% tail DNA) were measured. Percent of DNA in tail and Olive tail moment were measured using the following methods:

$$\text{Tail DNA}\% = 100 \times \text{Tail DNA intensity/Cell DNA intensity}$$

$$\text{Olive tail moment} = \text{Tail DNA}\% \times \text{Tail moment length}.$$

2.6. Oxidative stress indices and antioxidant activity

The sperm samples were centrifuged at 13,000*g* at 4 °C for 10 min, and the pellet, in an ice bath, was homogenized using a Sonopuls HD 2070 ultrasonicator (Bandelin Electronic, Berlin, Germany) with potassium phosphate buffer (50 mM KPi-buffer pH 7.0, 0.5 mM EDTA, 0.1 mM PMSF) to obtain density of 5×10^8 cells/ml. The homogenate was divided into two portions, one for measuring TBARS and CP, and a second centrifuged at 12,000*g* for 30 min at 4 °C to obtain the post-mitochondrial supernatant for other antioxidant enzymes activity studies.

The TBARS method described by Zhou et al. (2006), and Lushchak et al. (2005) was used to evaluate sperm LPO. The TBARS concentration was calculated by absorption at 535 nm and a molar extinction coefficient of 156 mM cm^{-1}. The content of TBARS was expressed as nanomoles per 10^8 cells. Carbonyl derivatives of proteins (CP) were detected by reaction with 2,4-dinitrophenylhydrazine (DNPH) according to the method described by Lenz et al. (1989). The amount of CP was measured spectrophotometrically at 370 nm using a molar extinction coefficient of 22 mM cm^{-1} and was expressed as nanomoles per 10^8 cells.

Total superoxide dismutase (SOD) activity was determined by the method of Marklund and Marklund (1974). The homogenized samples were centrifuged at 12,000*g* at 4 °C for 30 min. The SOD activity was assessed spectrophotometrically at 420 nm. One unit of SOD activity is defined as the amount of the enzyme needed to exhibit 50% dismutation of the superoxide radical per min. Activity was expressed in international units (or milliunits) mU per 10^8 cells.

2.7. ATP bioluminescence assay

ATP content in spermatozoa was determined after 2 h of exposure to DQ using the bioluminescence method described by Boryshpolets et al. (2009). Sperm samples were added to a boiling extraction medium consisting of 100 mM Tris–HCl, pH 7.75 and 4 mM EDTA. After boiling for 2 min at 100 °C, samples of the sperm suspension were centrifuged at 12,000*g* for 20 min. ATP content in the supernatants was evaluated by bioluminescence, using a Bioluminescence Assay Kit CLS II (Roche Diagnostics GmbH, Germany). Luminescence was read with a multifunctional microplate reader Infinite M200 (Tecan, Austria). ATP content was expressed as nmol ATP/10^8 spermatozoa.

2.8. Statistical analysis

Normality and homogeneity of dispersions in studied values were tested using Shapiro–Wilkox and Levene tests. Statistical comparison was made by analysis of variance (ANOVA) with subsequent post hoc Tukey's honest significant difference (HSD) test. The values were expressed as mean ± SEM ($n = 6$). The observed relationships among the parameters were confirmed and quantified according to Spearman's test. Values for motility and velocity at 10 s post-activation were used. The *t*-test was used to establish whether the correlation coefficients were significantly different from zero. All analyses were performed at a significance level of $p < 0.05$ using STATISTICA 9.0 software for Windows.

3. Results

3.1. Spermatozoa motility and velocity

A significant effect of DQ on sperm motility was observed at each tested post activation time (df = 4, $p < 0.05$, Fig. 1). The duration of spermatozoa motility decreased with increasing DQ concentrations. In the control, 38.3% of spermatozoa were motile at 120 s post-activation, while 25.3% were motile in the group exposed to 150 μM of DQ. A significant positive correlation ($r = 0.67$, $p < 0.05$) was found between percent of motile spermatozoa and ATP content (Table 1). A similar trend was observed for spermatozoa velocity. Typically, at 10 s post-activation the spermatozoa velocity in the control group reached 200 μm/s compares to 168 μm/s for spermatozoa exposed to 150 μM DQ (Fig. 2). Fig. 3 shows swimming behavior of sterlet spermatozoa after activation at tested times post-activation for DQ concentrations and in controls. A C-shaped flagellum was observed at 25 and 50 μM DQ at 60 s post-activation and for 150 μM at 10 s post-activation, and when motility ceased. In the control, the flagellum remained straight when motility ceased at 120 s post-activation.

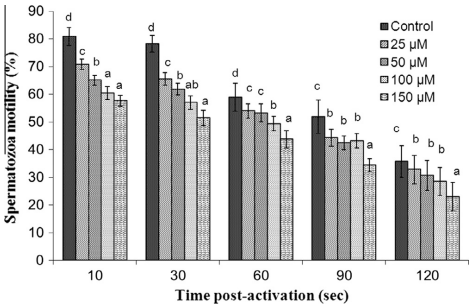

Fig. 1. Sterlet (*A. ruthenus*) spermatozoa motility after *in vitro* exposure to duroquinone at nominal concentrations (25, 50, 100, and 150 μM). Data are presented as means ± SEM, $n = 6$. Different letters denote significant difference between treatments.

3.2. Evaluation of DNA damage

An illustration of the outcome of a typical assay for DNA damage is presented in Fig. 4. The level of DNA fragmentation in spermatozoa exhibited a significant dose related response to DQ after 2 h of *in vitro* exposure (Figs. 5 and 6). The level of DNA fragmentation, expressed as the mean Olive tail moment, after *in vitro* exposure of spermatozoa to concentrations over 50 μM of DQ was significantly greater than in the control (df = 4, $p < 0.05$) (Fig. 6). Fig. 5 shows the effects of *in vitro* exposure to DQ on the percent of tail DNA. The spermatozoa exposed to the highest concentration of duroquinone (150 μM) showed DNA fragmentation of almost 8%, as compared with 1–2% for the controls. A significant positive correlation was found between percent of DNA in tail and CP ($r = 0.66$, $p < 0.05$), as well as between Olive tail moment and CP ($r = 0.72$, $p < 0.05$; Table 1). A significant negative correlation ($r = -0.69$, $p < 0.05$) was found between percent of DNA in tail and ATP (Table 1).

3.3. Oxidative stress indices and antioxidant response

To verify the presence of oxidative imbalance induced by duroquinone, LPO levels (as indicated by spermatozoa TBARS level) and CP levels were measured in all groups (Fig. 7A and B). LPO level significantly (df = 4, $p < 0.05$) increased upon exposure to DQ at 50 μM and greater, and LPO production was 1.96-fold that of the control value when exposed to 150 μM. Compared to the control, the level of CP significantly increased upon exposure to 25 μM DQ (df = 4, $p < 0.05$). The SOD activity, an indicator of antioxidant activity in

Table 1
Correlation coefficients among the parameters measured in *Acipenser ruthenus* spermatozoa after 2 h exposure to different concentrations of duroquinone. The *t*-test is used to establish significance of the correlation between pairs of parameters.

	Velocity	Motility	TBARS	SOD	CP	ATP	% DNA tail	Olive moment
Velocity	1.00							
Motility	0.07	1.00						
TBARS	0.16	−0.06	1.00					
SOD	0.74**	−0.19	0.06	1.00				
CP	0.46	−0.53	0.32	0.71	1.00			
ATP	−0.12	0.67*	0.10	−0.11	−0.43	1.00		
Tail DNA %	0.45	−0.53	−0.14	0.38	0.66*	−0.69*	1.00	
Olive moment	0.54	−0.52	−0.06	0.50	0.72*	−0.67*	0.98**	1.00

* $p < 0.05$.
** $p < 0.01$.

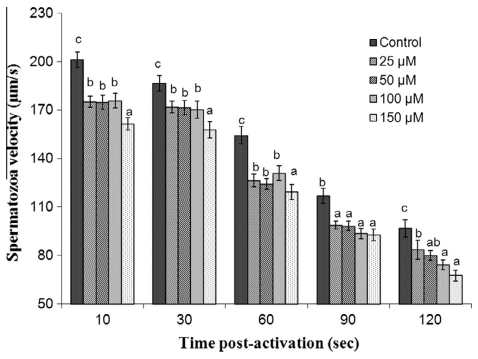

Fig. 2. Sterlet (*A. ruthenus*) spermatozoa velocity (μm s^{-1}) after *in vitro* exposure to duroquinone at nominal concentrations (25, 50, 100, and 150 μM). Data are presented as means ± SEM, $n = 6$. Different letters denote significant difference between treatments.

fish spermatozoa, increased significantly in all treatment groups compared to the control (df = 4, $p < 0.05$, Fig. 8) and reached the maximum value (threefold the control value) when exposed to the highest (150 μM) concentration of DQ.

3.4. Evaluation of ATP content

To further investigate the potential mechanism underlying the decrease in sperm quality, the intracellular ATP content in spermatozoa was analyzed. The level of ATP in control spermatozoa was 68 nmol ATP/10^8 spermatozoa. Significant differences (df = 4, $p < 0.05$) in intracellular ATP levels were observed with exposures at 100 and 150 μM DQ, with the lowest ATP content at 45 nmol ATP/10^8 spermatozoa (Fig. 9).

4. Discussion

As a first step toward understanding the harmful effects of duroquinone on sterlet sperm, an *in vitro* spermatozoa motility assay was analyzed using a computer-aided motion-analysis system. Spermatozoa motility and velocity decreased significantly in all treatment groups compared to the control. The percent motile spermatozoa was reduced in a dose-dependent manner, while reduction of spermatozoa velocity was dose-dependent only at 120 s post activation. This is in agreement with a previous study in common carp *Cyprinus carpio* (Zhou et al., 2006). Duroquinone was associated with damage to the spermatozoa flagella, and atypical flagella positions were observed during spermatozoa movement as well as when motility ceased. It is not known whether the observed flagella positions were due to damage of the plasma membrane or of the axoneme. The plasma membrane plays an important role in the initiation of sperm motility via hypo-osmotic signals (Li et al., 2009, 2012), and modification of the plasma membrane can significantly alter spermatozoa movement.

It is known that non-estrogenic as well as estrogenic organic compounds, such us zearalenone, BPA, and estradiol (Thomas et al., 1998) can bind to the spermatozoa membrane receptors and upregulate spermatozoa motility. However, the opposite, a reduction of motility, can also be observed when xenobiotic compounds, such as duroquinone, affect spermatozoa energy (intracellular ATP content). We observed a decreasing trend in intracellular ATP during *in vitro* treatment as well as a significant positive correlation between intracellular ATP content and spermatozoa motility. ATP is the main source of energy required for axonemal beating in fish spermatozoa (Perchec-Poupard et al., 1998; Rurangwa et al., 2002). In mammalian spermatozoa there is evidence that intracellular ATP concentrations decrease upon exposure to oxidative stress (Cummins et al., 1994). ATP depletion by ROS has been explained by inactivation of mitochondrial ATP synthase or by activation of the nuclear enzyme poly (ADP-ribose) polymerase-1 (PARP). Activation of PARP as a consequence of ROS-induced DNA damage, which causes excessive consumption of substrates such as nicotinamide adenine dinucleotide (NAD+) and ATP, has been shown to induce cell death by either apoptosis or necrosis (Jagtap and Szabó, 2005). Duroquinone as a potent redox cycler (Lemaire and Livingstone, 1997) was reported to induce ROS production in carp spermatozoa (Zhou et al., 2006) with a subsequent decline of spermatozoa motility.

In addition to negatively affecting spermatozoa motility, ROS have long been implicated in the lipid peroxidation of spermatozoa membranes because of their relatively high polyunsaturated fatty acid (PUFA) content (Li et al., 2009). The lipid peroxidation cascade is initiated when reactive oxygen species attack PUFA in the sperm cell membrane (Storey, 1997). As a consequence of lipid peroxidation, the plasma membrane loses the fluidity and integrity. In the present study, fish spermatozoa treated with duroquinone showed increased levels of LPO. Enhanced LPO may indicate an increased generation of oxygen free radicals, and has been associated with mid-piece abnormalities and decreased spermatozoa quality (Chitra et al., 2003).

Protein carbonylation is a result of protein oxidation. It is well known that different types of ROS induce damage in different cell structures (Dalle-Donne et al., 2003). Thus the nature of ROS determines the type of oxidative stress biomarker to be used. However, the use of protein CO groups as markers may have some advantages over lipid peroxidation products, since the formation of protein-bound CO groups seems to be a common phenomenon of

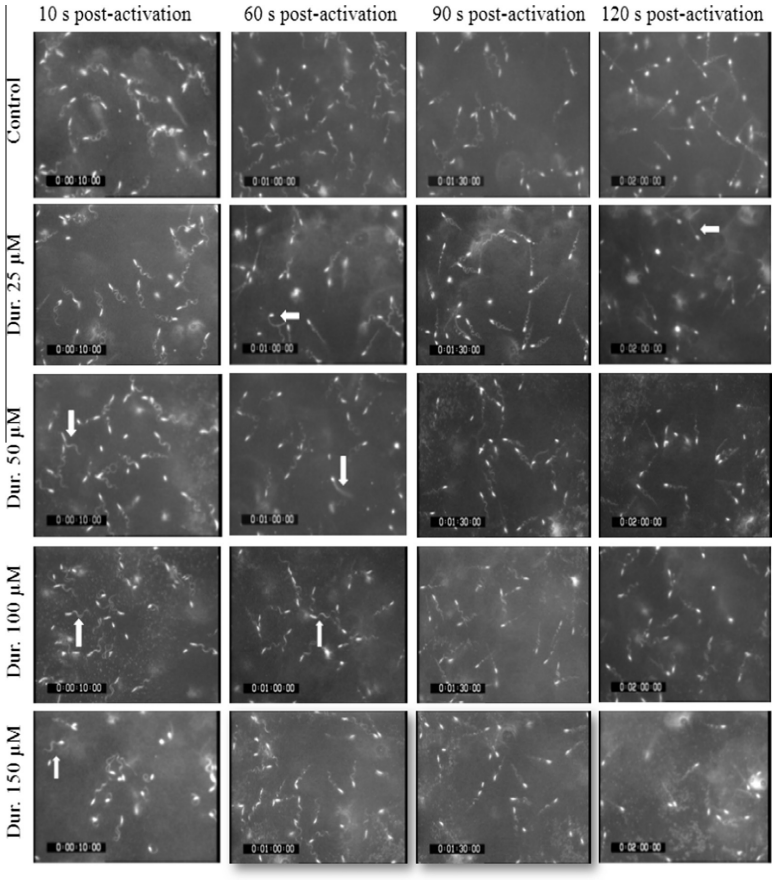

Fig. 3. *In vitro* effects of duroquinone on *Acipenser ruthenus* spermatozoa flagella. Beat frequency and number of waves along the flagellum decreased with increasing concentration of DQ. Different flagellar positions were observed during spermatozoa movement as well as when motility ceased. The white arrows indicate changes in flagellar shape, such as decrease in beat frequency and occurrence of a C-shape, with various concentrations of duroquinone compared to control.

protein oxidation and because of the relatively early formation and stability of oxidized proteins (Dalle-Donne et al., 2003). Reactive oxygen species directly attack proteins and lead to the formation of carbonyl. The formation of CP produces structural changes and decreased catalytic activity in enzymes and ultimately results in, owing to increased susceptibility to protease action, breakdown of proteins by proteases (Almroth et al., 2009). In the present study, all tested concentrations of duroquinone significantly increased CP level after short-term exposure, and marked increases in CP level were readily observed at lower concentrations. Our results also suggested that CP was more sensitive than LPO as an indicator of oxidative stress in sterlet spermatozoa. This finding, as well as the absence of a correlation between TBARS level and CP, may suggest direct influence of DQ on spermatozoa proteins, not mediated by lipid peroxidation. Thus, CP may be one of the most sensitive biomarkers of DQ-induced oxidative stress in sterlet spermatozoa.

To counteract the damaging effects of ROS, a variety of antioxidant enzymes are present in spermatozoa (Chitra et al., 2003; Li et al., 2010a,b,c). Superoxide dismutase is considered the first line of defense against deleterious effects of oxyradicals in the cell by catalyzing the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen (Li et al., 2009). In the present study a significant enhancement of SOD activity at the lowest concentration of DQ (25 µM) as well as significant correlation between spermatozoa velocity and SOD content was observed. It is likely that the enhancement of SOD activity was an adaptive response to toxicant stress and served to neutralize the impact of increased ROS generation.

The integrity of spermatozoa DNA is affected by ROS (Fraczek and Kurpisz, 2005; Menezo et al., 2007), which induce damage associated with poor semen quality, low fertilization rate, impaired implantation, increased abortion rate, and even an elevated incidence of disease in offspring (Lewis and Aitken, 2005). In general

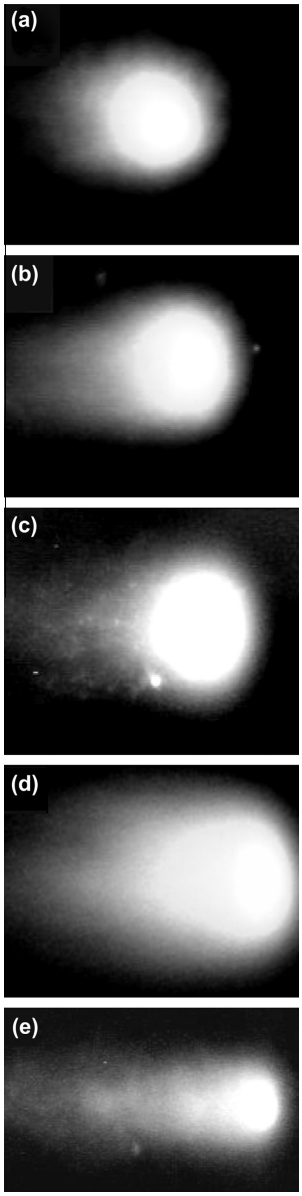

Fig. 4. Examples of results from a typical comet assay. Five levels of DNA damage in sterlet (*A. ruthenus*) spermatozoa: (a) no damage, % tail DNA < 5% (control); (b) slight damage, % tail DNA < 15% (25 μM of duroquinone); (c) moderate damage, % tail DNA < 50% (50 μM of duroquinone); (d) higher damage, % tail DNA < 60% (100 μM of duroquinone); and (e) highest damage, % tail DNA > 60 % (150 μM Dur.).

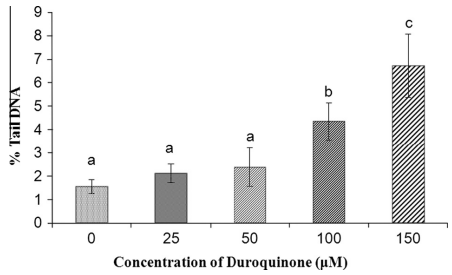

Fig. 5. Percent tail DNA (% DNA in tail measured in comet assay) after *in vitro* exposure of sterlet (*A. ruthenus*) spermatozoa to duroquinone at nominal concentrations (25, 50, 100 and150 μM). Data are presented as means ± SEM, $n = 6$. Different letters denote significant difference between treatments.

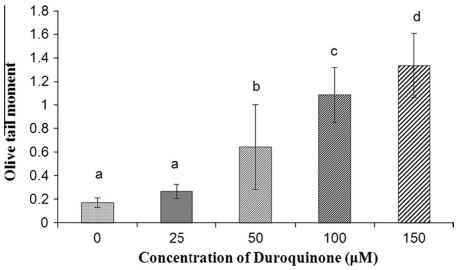

Fig. 6. Olive Tail Moment of the spermatozoa after *in vitro* exposure of sterlet (*A. ruthenus*) spermatozoa to duroquinone at nominal concentrations (25, 50, 100, and 150 μM). Data are presented as means ± SEM, $n = 6$. Different letters denote significant difference between treatments.

it is not only the DNA damage, that can affect spermatozoa, but DNA modifications can be generated by chemical reactions (Box et al., 2001) leading to mutations in the embryo (Kopeika et al., 2004). However, DNA strand breaks are usually considered the most damaging type of DNA modification. In the present study, using the comet assay, we were able to demonstrate that the short-term exposure of sterlet spermatozoa to DQ caused a dramatic increase in DNA damage expressed by Olive moment and percent of tail DNA. Levels of CP were found to be significantly positively correlated with Olive moment and % tail DNA. High concentrations of ROS were necessary to induce DNA damage (Figs. 5–8). With these data we can speculate that an antioxidant system is present in sterlet sperm that is capable of partially neutralizing ROS attack. High concentrations of uric acid, an important antioxidant in seminal plasma, have been reported in rainbow trout (*Oncorhynchus mykiss*), yellow perch (*Perca flavescens*), and common carp (*C. carpio*), potentially protecting spermatozoa against oxidative damage (Ciereszko et al., 1999). Further studies are needed to reveal mechanisms responsible for antioxidant defenses in sterlet sperm.

The results of the present study clearly demonstrated that duroquinone can induce oxidative stress in sterlet spermatozoa *in vitro*, resulting in accumulation of LPO and CP together with the inhibition of intracellular ATP content and DNA damage. These oxidative responses were associated with reduced spermatozoa quality, as measured by spermatozoa motility and velocity. The use of sperm *in vitro* assays may provide a simple and efficient means for eval-

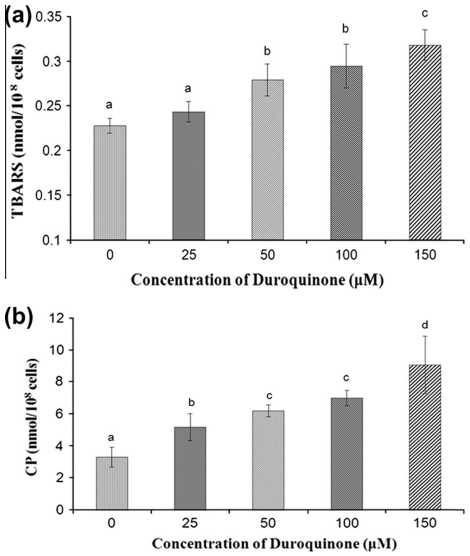

Fig. 7. Oxidative stress indices [TBARS (A) and CP (B)] following *in vitro* exposure of sterlet (*A. ruthenus*) spermatozoa to duroquinone at nominal concentrations (25, 50, 100, and 150 μM). Data are presented as means ± SEM, $n = 6$. Different letters denote significant difference between treatments.

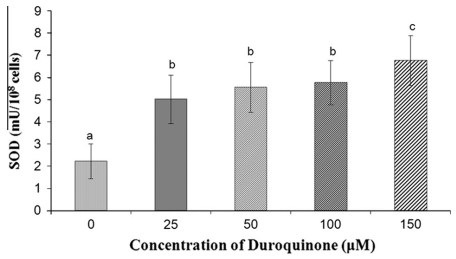

Fig. 8. Superoxide dismutase (SOD) activity levels following *in vitro* exposure of sterlet (*A. ruthenus*) spermatozoa to duroquinone at nominal concentrations (25, 50, 100, and 150 μM). Data are presented as means ± SEM, $n = 6$. Different letters denote significant difference between treatments.

uating the effects of pollutants on sturgeon in the aquatic environment. The data reported here showed for the first time that the most sensitive biomarkers of DQ-induced oxidative stress in sterlet spermatozoa are spermatozoa motility, protein carbonylation, and SOD activity. Compared to spermatozoa of common carp (Zhou et al., 2006), spermatozoa of sterlet showed lower sensitivity to DQ-induced DNA damage, but were highly susceptible to protein oxidation leading to significant motility reduction. Further research is needed to investigate the precise mechanisms involved and the relationship between motility and oxidative stress in sturgeon sperm as well as its consequences on fertilization.

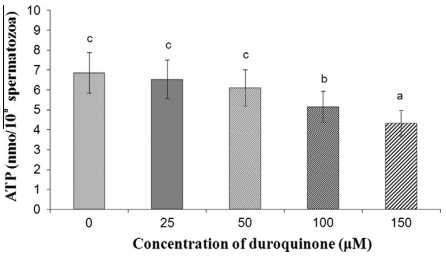

Fig. 9. Intracellular ATP content of sterlet (*A. ruthenus*) spermatozoa after 2 h exposure to duroquinone. Data are presented as means ± SEM, $n = 6$. Different letters indicate significant differences between treatments (ANOVA, $p < 0.05$).

Conflict of interest

We declare that we have no financial or non-financial competing interests.

Acknowledgements

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CHAPTER 5

EFFECTS OF TETRABROMBISPHENOL A ON DNA INTEGRITY, OXIDATIVE STRESS, AND STERLET (*ACIPENSER RUTHENUS*) SPERMATOZOA QUALITY VARIABLES

Linhartova, P., Gazo, I., Shaliutina-Kolesova, Hulak, M., Kaspar, V., 2015. Effects of tetrabrombisphenol A on DNA Integrity, oxidative stress, and sterlet (*Acipenser ruthenus*) spermatozoa quality variables. Environ. Toxicol. 30, 735–745.

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Effects of Tetrabrombisphenol A on DNA Integrity, Oxidative Stress, and Sterlet (*Acipenser ruthenus*) Spermatozoa Quality Variables

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ABSTRACT: The sperm of sterlet (*Acispenser ruthenus*) was used to investigate the effect of the xenobiotic tetrabrombisphenol A (TBBPA) on sperm quality variables (ATP content, spermatozoa motility, and velocity), DNA integrity, and oxidative stress indices. Sperm was diluted to obtain a spermatozoa density of 5×10^8 cells/mL and exposed for 2 h to final concentrations of TBBPA (0.5, 1.75, 2.5, 5, and 10 μg/L). The oxidative stress indices, including lipid peroxidation, carbonyl derivatives of proteins, and antioxidant activity were significantly higher with increased concentrations of TBBPA. There was significantly less intracellular ATP in sperm samples at TBBPA concentrations of 2.5 μg/L and above. Spermatozoa velocity and percent motile sperm were significantly lower at each sampling time post-activation compared to controls. DNA damage expressed as percent DNA in Tail and Olive Tail moment was significantly higher with exposures ≥2.5 μg/L TBBPA. The results demonstrated that TBBPA and other xenobiotics can induce reactive oxygen species stress in fish spermatozoa, which could impair the sperm quality, DNA integrity, ATP content, and the antioxidant defense system. This study confirmed that fish spermatozoa can be used in *in vitro* assays for monitoring residual pollution in aquatic environments. © 2014 Wiley Periodicals, Inc. Environ Toxicol 30: 735–745, 2015.

Keywords: DNA damage; comet assay; reactive oxygen species; spermatozoa motility

INTRODUCTION

Artificial propagation and rearing of sturgeon has been implemented along with the expanded development of fish culture in several European countries, since 1971 (Chebanov and Billard 2001). Major challenges in sturgeon culture include their late sexual maturation and high sensitivity to water quality. Native sturgeon stocks are highly endangered because of over-fishing, habitat loss, and water pollution and are on the International Union for Conservation of Nature Red List of Threatened Species. The length of time required for sexual maturation in wild sturgeon provides a longer period for xenobiotic accumulation in reproductive organs, leading to their gradual deterioration (Tashjian et al., 2006; Gharaei et al., 2008). The present study used sterlet (*Acipenser ruthenus*) as a model organism because of its low cost, high survival, small size, earlier sexual maturation, and its freshwater habitat.

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Anthropogenic activity, including industrial development, has lead to increasing accumulation of water-soluble pollutants and toxic substances. One of the more common xenobiotics in surface waters is tetrabromobisphenol A (TBBPA), a brominated flame retardant and derivative of bisphenol A. The estimated annual worldwide use was 119,600 metric tons in 2001, of which 11,600 metric tons were used by European industries, mainly in the production of plastics for electronic applications. TBBPA is emitted into the environment and can be detected in trace concentrations in the atmosphere, hydrosphere, soil, and sediments. Sellström and Jansson (1995) reported TBBPA concentrations to 34 ng/g dry weight upstream and 270 ng/g dry weight downstream of the industry, which were found in surficial sediments. Casually, Abdallah et al. (2008) measured concentrations of TBBPA (average, min, and max) in UK homes of 16, 9, and 22 pg/m^3, in offices of 16, 4 and 33 pg/m^3, in public microenvironments of 26, 17 and 32 pg/m3, and outdoors of 0.8, 0.7 and 0.9 pg/m3. The concentration of TBBPA detected in soil samples from China was 25.2±2.7 ng/g (Peng et. al., 2007).

TBBPA was the subject of an eight-year evaluation under the EU risk assessment procedure that reviewed over 460 studies. TBBPA will be regulated under the chemical policy of the European Union: registration, evaluation, and authorization of chemicals (REACH) and is in the process of being classified in the EU as an R50–53 substance, indicating toxicity to aquatic organisms. TBBPA is acutely toxic to fish at low concentrations. LC50s reported include 0.51 mg/L (0.9 μM) for bluegill sunfish, 0.40 mg/L (0.7 μM) for rainbow trout (WHO/IPCS 1995), and a relatively high 3 mg/L (5.5 μM) for zebrafish (Lee et al., 1993). TBBPA is known to enhance production of reactive oxygen species (ROS) (Livingstone, 2001) that attack a variety of biological macromolecules such as DNA, proteins, and lipids, leading to oxidative damage (Kelly et al., 1998). Gametes of most fish species are particularly susceptible to oxidative injury because of lack of protective cytoplasmic enzymes as well to high polyunsaturated fatty acid content in spermatozoa plasma membranes (Drevet, 2006). As fish spermatozoa are motile for only 1–2 min (Cosson, 2010), negative effects of xenobiotics on sperm traits can significantly decrease the probability of fertilization (Zhou et al., 2006).

The primary objective of this study was to determine effects of short-term *in vitro* exposure to environmentally relevant concentrations of TBBPA on physiology of sterlet spermatozoa through analysis of their motility and velocity, DNA integrity, and intracellular ATP content. In addition, oxidative stress in spermatozoa was assessed through measurement of lipid peroxidation (LPO) and carbonyl derivatives of proteins (CP), and the antioxidant response was evaluated by superoxide dismutase activity (SOD).

MATERIAL AND METHODS

Broodstock Handling and Collection of Gametes

Sperm of sterlet was obtained from 6 to 7 year-old males (body weight: 1.5 ± 0.2 kg; body length: 55 ± 9.3 cm) in the aquaculture facility of the Research Institute of Fish Culture and Hydrobiology at the University of South Bohemia, Vodnany, Czech Republic. Fish were maintained according to the EU harmonized Animal Welfare Act, and the principles of animal care and national law 246/1992 Animal Welfare were followed. Prior to hormonal stimulation, fish were kept in tanks with water temperatures of 14–15°C. Spermiation was stimulated by intramuscular injection of a suspension of carp pituitary in physiological saline (0.9% NaCl) at 4–5 mg/kg of body weight. At 48 h postinjection, sperm was collected from the urogenital papilla by insertion of plastic catheter (5–7 mm diameter) connected to a 20 mL syringe. Samples were kept on ice (0–4°C) until processing. Sperm contaminated by mucus, feces, urine, or water was excluded. The spermatozoa concentrations of all samples were estimated microscopically (Olympus BX 41) at 20× using a Burker cell hemocytometer.

Sperm Dilution and Exposure

Sperm from six individual males was centrifuged separately at 300 × *g*, 4°C for 30 min, seminal plasma decanted, and the concentrated spermatozoa were diluted in immobilizing medium (20 mM Tris, 30 mM NaCl, 2 mM KCl, pH 8.5) to obtain a density of 5 × 10^8 cells/mL. Tetrabrombisphenol A (TBBPA) [2,2′,6,6′-Tetrabromo-4,4′-isopropylidenediphenol or 2,2-bis(3,5-dibrom-4 hydroxyfenyl) propane; empirical formula: $C_{15}H_{12}Br_4O_2$; MW: 543.85) (≥99%; Sigma-Aldrich, USA)] was dissolved in 96% ethanol and diluted in distilled water to obtain a stock solution of 0.1 g/L. The spermatozoa sub-samples ($n = 6$) were exposed to environmentally relevant concentrations of 0.5, 1.75, 2.5, 5, and 10 μg/L of TBBPA at 4°C for 2 h. Samples exposed to immobilizing medium with 0.5% ethanol were used as a negative control. Each experimental condition was duplicated and for final results the mean value of obtained results was calculated.

Sperm Motility and Velocity

Spermatozoa velocity (μm/s, only motile sperm) and percent motile spermatozoa (%) were determined by dark-field microscopy (Olympus BX 50, Japan) (20× objective magnification) after triggering motility in activating medium (AM) (10 mM NaCl, 1 mM $CaCl_2$, 10 mM Tris, pH 8.5) at a dilution of 1:5000. Pluronic 0.2% (w/v) (Sigma-Aldrich, USA) was added to the swimming solution to avoid spermatozoa sticking to the microscope slide. Motility was recorded with a CCD video camera (SONY DXC-970MD, Japan) mounted on the microscope, illuminated with a stroboscopic flash

(ExposureScope®, Czech Republic). Motility was recorded in triplicate for each sample using a video-recorder (SONY DVD Recorder-1000MD, Japan).

Velocity and motility were assessed at 10, 30, 60, 90, and 120 s postactivation. The positions of sperm heads were analyzed from video frames using Olympus MicroImage software (Version 4.0.1. for Windows with a macro by Olympus C & S). Percentage of motile spermatozoa and velocity were calculated from spermatozoa head positions on five successive video frames indicated by three colors (frame 1 red, frames 2–4 green, and frame 5 blue) as described by Rodina et al. (2007). Nonmotile spermatozoa were white. Twenty to 40 spermatozoa were counted in each frame, and the percentage of motile spermatozoa was calculated from the number of white and red cells. Spermatozoa velocity was calculated as μm/s from length of traces of spermatozoa from red to green to blue heads, calibrated for magnification.

Assessment of DNA Damage

DNA integrity was assessed using the comet assay or single cell gel electrophoresis assay following the method described by Li et al. (2008). Unless otherwise stated, molecular grade DNAse-free reagents (Sigma Aldrich, USA) were used. Microscope slides (OxiSelectST; Cell Biolabs, INC. USA) were prepared in the following manner: 100 μL of sample (5×10^8 cells/mL) were diluted in 5 mL of PBS (Ca^{2+} and Mg^{2+} free). The diluted samples (200 μL) were mixed with 700 μL of 0.8% NuSieve GTG low melting temperature agarose (OxiSelect), 50 μL of the mixture was added to the slide, and the agarose was allowed to solidify for 1 h at 4°C. Slides were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10% of 10x Lysis Solution [OxiSelect^{TM} Comet Assay Kit], 10% DMSO pH 10) for 1 h. Finally, slides were subjected to treatment with proteinase K (20 mM Tris-HCl, 1 mM $CaCl_2$ and 50% glycerol, pH 7.4) and incubated overnight at 32°C. Slides were drained and immersed in a horizontal gel tank filled with running buffer, and electrophoresis was carried out for 20 min at 35 V and 170 mA. Slides were transferred from the electrophoresis chamber to a clean container with prechilled DI H_2O for 2 min. This washing procedure was done three times. Slides were drained well, dehydrated by dipping into absolute ethanol for 5 min, and air-dried for storage. Prior to the analysis, 50 μL Vista Green DNA Staining Solution (OxiSelectST) was added to each agarose spot and analyzed with an Olympus BX50 fluorescence microscope at 20× magnification. One hundred cells were scored for each sample, and the captured images were analyzed using CometScore image analysis software (TriTek Corporation, USA). Tail length (measured from the middle of the head to the end of the comet tail) and % tail DNA (content of DNA in comet tail) were measured. Percent DNA in tail and Olive Tail Moment were calculated with the following formulae:

$$\text{Tail DNA \%} = 100 \times \text{Tail DNA Intensity} / \text{Cell DNA Intensity}$$

$$\text{Olive Tail Moment} = \text{Tail DNA \%} \times \text{Tail Moment Length}.$$

Oxidative Stress Indices

The sperm samples were centrifuged at 13 000 × *g* at 4°C for 10 min, and the pellet was homogenized in an ice bath using a Sonopuls HD 2070 ultrasonicator (Bandelin Electronic, Berlin) with potassium phosphate buffer (KPi-buffer). The homogenate was divided into two portions, one for measuring thiobarbituric acid reactive substances (TBARS) and carbonyl derivatives of proteins (CP) levels, and the other centrifuged at 12,000 × *g* for 30 min at 4°C to obtain the postmitochondrial supernatant for investigation of antioxidant enzyme activity. The TBARS method (Li et al., 2010) was used to evaluate sperm LPO. Concentration of TBARS was calculated by absorption at 535 nm and a molar extinction coefficient of 156 mM/cm. The TBARS content was expressed as nmol/10^8 cells. Carbonyl derivatives of proteins were detected by reaction with 2, 4-dinitrophenylhydrazine (Lenz et al., 1989). The amount of CP was measured spectrophotometrically at 370 nm using a molar extinction coefficient of 22 mM/cm and was expressed as nmol/10^8 cells.

Superoxide dismutase activity (SOD)

Total SOD activity was determined by the method of Marklund and Marklund (1974) based on the autoxidation of pyrogallol. The prepared semen samples were diluted in phosphate buffer (50 mM KPi-buffer pH 7.0, 0.5 mM EDTA, 0.1 mM PMSF) to obtain spermatozoa density of $5x10^8$ cells/mL. The homogenized samples were centrifuged at 12,000 × *g* at 4°C for 30 min. The SOD activity was assessed spectrophotometrically at 420 nm and expressed as the amount of enzyme per milligram of protein. One unit of SOD activity is defined as the amount of the enzyme necessary to produce 50% dismutation of the superoxide radical per min.

ATP Content

ATP content in spermatozoa was determined by the bioluminescence method described by Boryshpolets et al. (2009). Sperm samples were diluted in 5 mL of medium containing 100 mM Tris-HCl, pH 7.5, and 4 mM EDTA. After boiling 2 min at 100°C samples were centrifuged at 12,000 × *g* for 20 min. The supernatant was collected and stored at −80°C. A Bioluminescence Assay Kit CLS II (Roche Diagnostics GmbH, Germany) was used to determine ATP content. Luminescence was assessed with an Infinite M200

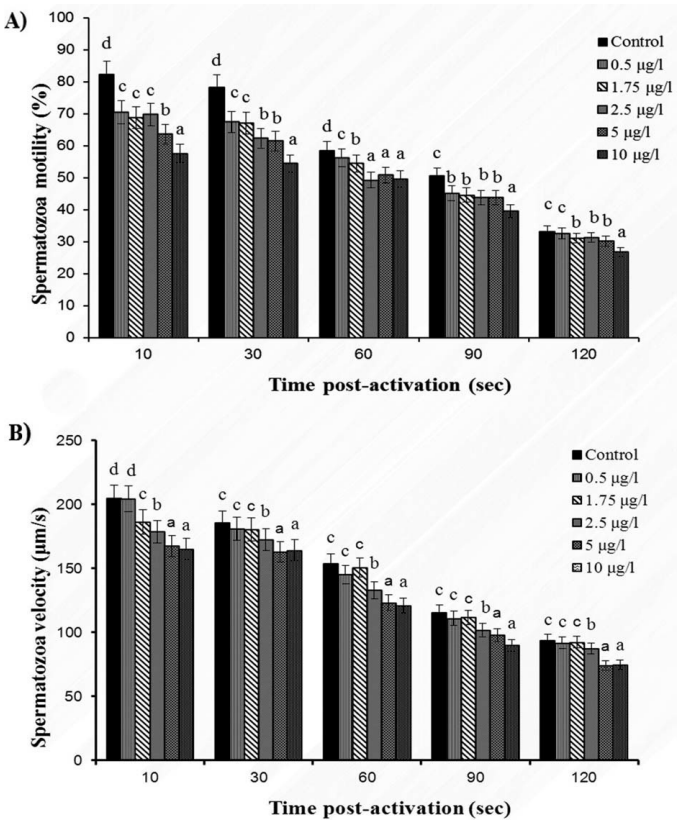

Fig. 1. Effects of tetrabrombisphenol A (TBBPA) on spermatozoa motility (A) and velocity (B) after in vitro exposure of Acipenser ruthenus spermatozoa at nominal concentrations of 0.5, 1.75, 2.5, 5 and 10 µg/L. Data are presented as means ± SEM, $n = 6$. Superscript letters indicate significant differences among samples at the same time post-activation (ANOVA, $p < 0.05$).

multifunctional microplate reader (Tecan, Austria). ATP content was expressed as nmol ATP/10^8 spermatozoa.

Statistical Analysis

All analyses were conducted in triplicate. Normality and homogeneity of dispersions of studied values were tested using Shapiro–Wilk and Levene tests. Comparisons were made by analysis of variance (ANOVA) with subsequent post hoc Tukey's honest significant difference (HSD) test. The values were expressed as means ± SEM ($n = 6$). The observed relationships among the parameters were confirmed and quantified according to the Spearman's test. All analyses were performed at a significance level of $p < 0.05$ using STATISTICA 9.0 for Windows.

RESULTS

Spermatozoa Motility and Velocity

A significant effect of TBBPA on spermatozoa motility and velocity after 2 h exposure to TBBPA *in vitro* was observed. Reduced spermatozoa motility and velocity compared to the control was observed at 10, 30, 60, 90, and 120 s postactivation [df = 4, $p < 0.05$, Fig. 1(A,B)]. At 10 and 30 s postactivation, spermatozoa motility was significantly ($p < 0.05$) lower in all tested concentrations compared with controls. At 120 s postactivation 33.2% of spermatozoa were motile in the control, compared to 26.7% motility in the 10 µg/L TBBPA exposure. No significant difference in spermatozoa velocity at 10 s postactivation was observed between the

TABLE I. Correlation coefficients among the parameters measured in *Acipenser ruthenus* spermatozoa after 2 h exposure to TBBPA

	Motility	Velocity	Tail DNA	Olive tail moment	TBARS	CP	SOD	ATP
Motility	1							
Velocity	0.29*	1						
Tail DNA	−0.19	−0.02	1					
Olive Tail moment	−0.13	−0.02	0.83*	1				
TBARS	−0.35*	−0.12	0.05	0.04	1			
CP	−0.36*	−0.01	0.29*	0.02	0.37*	1		
SOD	−0.19	−0.12	0.24	0.04	0.21	0.39*	1	
ATP	0.52*	0.57*	−0.14	−0.09	−0.39*	−0.26	−0.36*	1

The *t*-test used to establish significance of the correlation between pairs of parameters (* $p < 0.05$).

control and the sample exposed to 0.5 μg/L of TBBPA, [Fig. 1(B)]. Velocity at 10 s postactivation was 165.2 μm/s in spermatozoa exposed to 10 μg/L of TBBPA. At 120 s postactivation, the velocity of the control was 93.7 μm/s, whereas, with exposure to 5 and 10 μg/L of TBBPA, velocity was significantly lower at 74.7 μm/s. A significant negative correlation ($r = -0.35$, $p < 0.05$) between percent of motile spermatozoa and TBARS level was observed (Table I). A significant positive correlation ($r = 0.52$, $p < 0.05$) was found between ATP content and motility of spermatozoa. Figure 2 shows swimming **behavior** of sterlet sperm after activation in control media and TBBPA at 10, 60, 90, and 120 s postactivation. We observed changes in flagella movement and the C shaped flagella typical of dead spermatozoa as well as sperm with very low velocity, mainly at 90 and 120 s postactivation.

DNA Fragmentation

An illustration of the assay for DNA damage is presented in Figure 3. Comet images were used to evaluate the following parameters: % of DNA in tail (% tail DNA = 100 × tail DNA Intensity/Cell DNA Intensity) and Olive tail moment (Olive Tail Moment = % Tail DNA × Tail Moment Length). The level of DNA fragmentation in spermatozoa exposed to TBBPA was measured by CometScore software [Figs. 4(A,B)]. Comet assay analysis indicated a higher level of DNA fragmentation in spermatozoa nuclei exposed to TBBPA (1.75–10 μg/L) compared to control. Figure 4(A) shows the effects of *in vitro* exposure to TBBPA on percentage of DNA in tail. Sperm exposed to the highest concentration of TBBPA (10 μg/L) exhibited DNA fragmentation of almost 3.8% compared to 1.5% for the control sample. The level of DNA fragmentation, expressed as the mean Olive tail moment, was significantly increased ($df = 4$, $p < 0.05$) compared to control with exposure to concentrations 2.5 μg/L of TBBPA and above [Fig. 4(B)]. Spearman's correlation test revealed significant correlation between % DNA in tail and Olive tail moment ($r = 0.83$, $p < 0.05$) and CP level ($r = 0.29$, $p < 0.05$) (Table I).

Oxidative Stress Indices and Antioxidant Response

The LPO and CP levels were measured to investigate oxidative imbalance induced by TBBPA (Figs. 5 and 6). TBARS level was used as an indicator of LPO. The results showed that, in the control samples, TBARS were approximately 0.16 nmol/10^8 spermatozoa (Fig. 5). A significantly higher level of LPO was observed in spermatozoa exposed to TBBPA at concentrations 5 μg/L and higher. The highest concentration of TBBPA (10 μg/L) was associated with a TBARS level of 0.33 nmol/10^8 spermatozoa. TBBPA was associated with a significant increase ($p < 0.05$) the CP level compared to control at all tested concentrations. However, there was no significant difference in CP level among spermatozoa exposed to 0.5–5 μg/L of TBBPA (Fig. 6). A significantly higher level ($p < 0.05$) of CP (12.4 nmol/10^8) was observed in samples exposed to 10 μg/L TBBPA compared to 5 μg/L and lower concentrations of TBBPA. In controls, the value was 2.7 nmol/10^8. There was significant positive correlation between levels of TBARS and CP ($r = 0.37$, $p < 0.05$) (Table I). Significant negative correlations were found between motility of spermatozoa and TBARS level ($r = -0.35$, $p < 0.05$) and CP level ($r = -0.36$, $p < 0.05$) as well as between TBARS level and ATP content ($r = -0.39$, $p < 0.05$) (Table I).

The SOD activity was used as an indicator of antioxidant activity in fish spermatozoa exposed to TBBPA *in vitro*. SOD activity increased significantly in all treatments as compared to the control ($df = 4$, $p < 0.05$, Fig. 7). Increase was gradual and in a dose-dependent manner.

ATP Content

The ATP content was analyzed to assess the decline in spermatozoa quality. Mean intracellular ATP content in the control was 6.8 nmol ATP/10^8 spermatozoa (Fig. 8). Significant differences ($df = 4$, $p < 0.05$) in intracellular ATP content were found with exposure to 2.5, 5, and 10 μg/L of TBBPA. The lowest level of ATP found was 3.1 nmol ATP/10^8 spermatozoa with the TBBPA concentration of 10 μg/L. ATP content in spermatozoa correlated significantly with

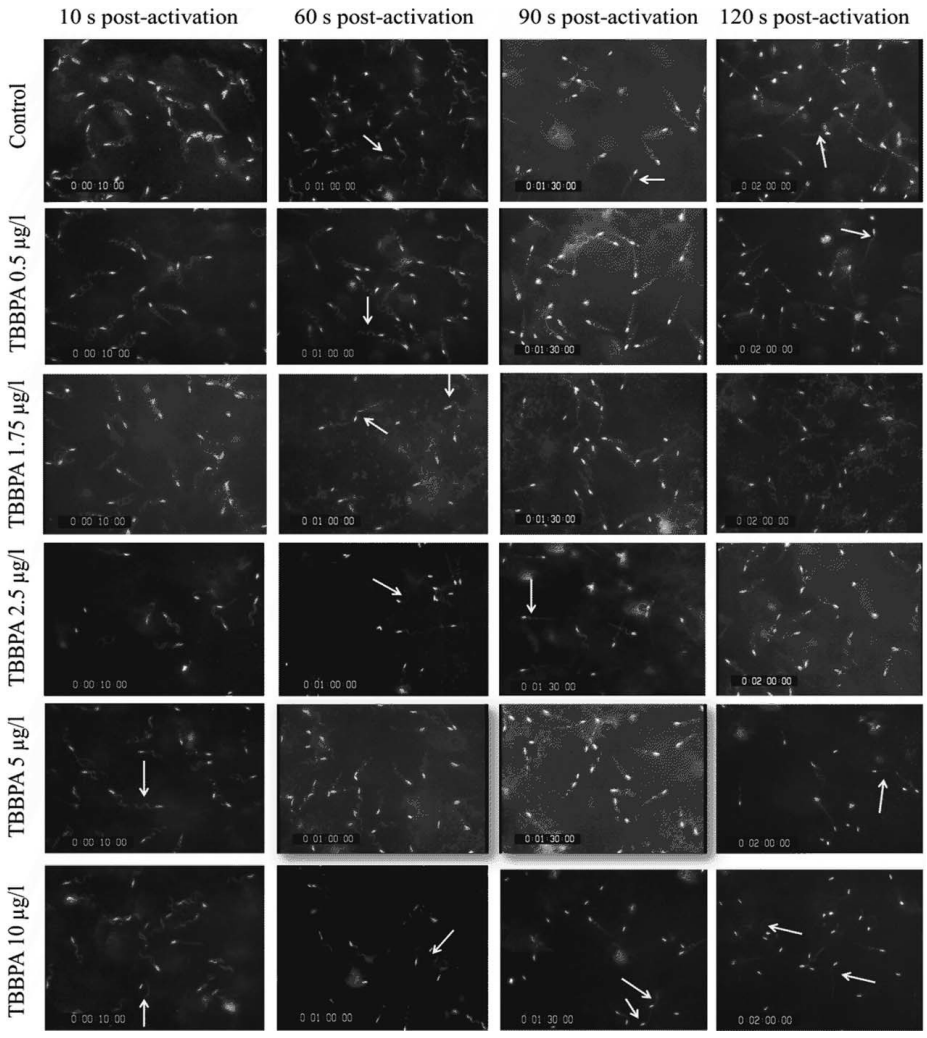

Fig. 2. Swimming behavior of Acipenser ruthenus spermatozoa. Flagella movement was recorded under dark field microscopy with stroboscopic illumination at 10, 60, 90 and 120 s post-activation of spermatozoa.

spermatozoa motility ($r = 0.52$, $p < 0.05$) and velocity ($r = 0.57$, $p < 0.05$), TBARS ($r = -0.39$, $p < 0.05$), and SOD activity ($r = -0.36$, $p < 0.05$) (Table I).

DISCUSSION

TBBPA can induce reproductive dysfunction in wildlife and humans (Grizard et al., 2007). Toxicity screening using animal sperm is considered a simple and valid method, as its use does not require expensive sterile cell culture conditions (Rurangwa et al., 2002). In this study, we attempted to confirm and quantify a negative effect of TBBPA on sperm motility parameters, DNA integrity, ATP metabolism, and the antioxidant defense system. Previous studies (Lahnsteiner et al., 2005), as well as our initial results, suggested an impact of environmentally relevant levels of TBBPA on the fish reproduction.

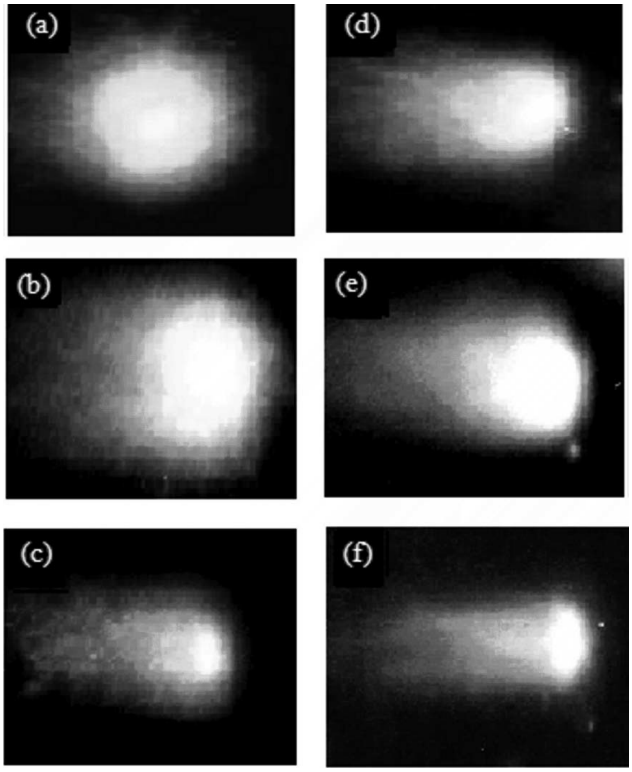

Fig. 3. Example of figures from Comet assay of Acipenser ruthenus spermatozoa exposed to differing concentrations of tetrabrombisphenol A (a– control, b– 0.5 μg/L, c– 1.75 μg/L, d– 2.5 μg/L, e– 5 μg/L, f– 10 μg/L). The different degrees of DNA damage in spermatozoa are arranged in six classes: a- no damagecontrol, % of DNA in Tail < 5%; b- small damage-%DNA in Tail < 15 % (0.5 μg/L of TBBPA); c –smaller damage- % of DNA in Tail <30 % (1.75 μg/L of TBBPA); d-medium damage-% of DNA in Tail < 50 % (2.5 μg/L of TBBPA); e- higher damage - % DNA in Tail <50 % (5 μg/L of TBBPA) and f- the highest damage- % of DNA in Tail >60% (10 μg/L of TBBPA).

Nonestrogenic as well as estrogenic organic compounds (TBBPA, BPA, and estradiol) can bind to sperm membrane receptors and reduce spermatozoa motility (Thomas et al., 1998). The effect of TBBPA on sperm energy is another consideration. ATP, as the energy source essential for axonemal beating in fish spermatozoa (Perchec-Poupard et al., 1998; Rurangwa et al., 2002), is a key factor in spermatozoa motility, and decrease in ATP is associated with decreased spermatozoa movement. As demonstrated by de Lamirande and Gagnon (1992), spermatozoa motility may be the most sensitive indicator of oxidative stress, as high levels of ROS inhibit one or more enzymes of oxidative phosphorylation and/or glycolysis, limiting the generation of ATP. Thus chemically induced stress may inhibit ATP generation. Spermatozoa movement is primarily dependent on initial ATP content, plasma membrane potential, and Ca^{2+} signalling for triggering flagellar beating (Li et al., 2009; Cosson 2010). Therefore any modification of these key elements can significantly modify spermatozoa movement. Our results showed a significant positive correlation between intracellular ATP content and spermatozoa motility.

Spermatozoa DNA damage assessment has gained attention as a sperm quality marker (Peknicova et al., 2002). Comet assay demonstrated that short-term exposure of sterlet spermatozoa to TBBPA results in a dramatic increase in DNA damage expressed by two parameters: Olive tail moment and percent of DNA in comet tail. Significant positive correlations were observed between the level of CP and Olive tail moment. This is in accordance with studies on human spermatozoa, showing that the DNA damage can be

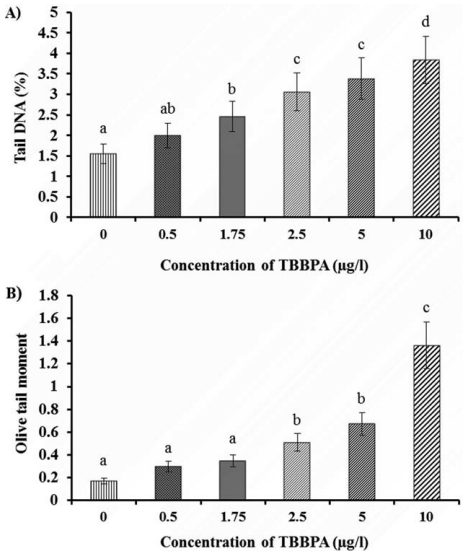

Fig. 4. Percent DNA in tail (A) (the % of DNA in tail measured in comet assay) after in vitro treatment of Acipenser ruthenus sperm in nominal concentrations (0.5, 1.75, 2.5, 5 and 10 μg/L). Data are presented as means ± SEM, $n = 6$. Different letters denote significant difference between treatments. Olive Tail Moment (B) of the spermatozoa after in vitro treatment of Acipenser ruthenus spermatozoa in nominal concentrations (0.5, 1.75, 2.5, 5, and 10 μg/L).

associated with oxidative stress and LPO (Chen et al., 1997; Aitken et al., 1998). In addition to DNA damage such as strand breaks, there is a possibility that other DNA modifications occur, for example, oxidization of nitrogen bases without generating strand breaks, as well as antioxidant responses, can induce mutations in the embryo (Kopeika et al., 2004). Environmental TBBPA can become a substantial problem, responsible for a dramatic increase of DNA fragmentation in gametes of animals with external fertilization. It may affect future reproduction of sturgeon and participate in their gradual disappearance from natural waters.

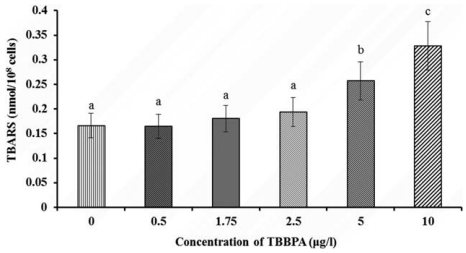

Fig. 5. Effect of tetrabrombisphenol A (TBBPA) on TBARS in Acipenser ruthenus spermatozoa. Data represent mean values ± SEM. Different letters indicate significant differences among samples (ANOVA, $p < 0.05$).

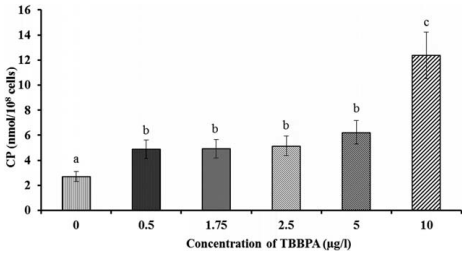

Fig. 6. Effect of tetrabrombisphenol A (TBBPA) on CP in Acipenser ruthenus spermatozoa. Data represent mean values ± SEM. Data are presented as means ± SEM, $n = 6$. Different letters denote significant difference between treatments.

In recent study oxidative stress indices, motility and velocity parameters were measured in TBBPA-exposed to sterlet spermatozoa. Sterlet spermatozoa were capable of being activated at 90% to 100% motility immediately upon transfer to swimming medium. Differences in motility and velocity appeared after 2 h exposure of sperm to TBBPA. A decreasing trend of both motility and velocity was observed with increasing concentrations of TBBPA. Our results are in accordance with studies on brown trout (*Salmo trutta*), on perch (Perca fluviatilis L.) and on goldfish (Carassius auratus L.) that showed lower motility and velocity in spermatozoa exposed to environmentally relevant concentrations of 1.7, 2.4 and 5.0 g/L BPA (Lahnsteiner et al., 2005), 0.12, 0.25, 0.5, 1.5, and 2 mM BPA (Hatef et al., 2010) and 0.6, 4.5 and 11.0 mg/L of BPA (Hatef et al., 2012), both *in vivo* and *in vitro*. High spermatozoa motility is essential for successful

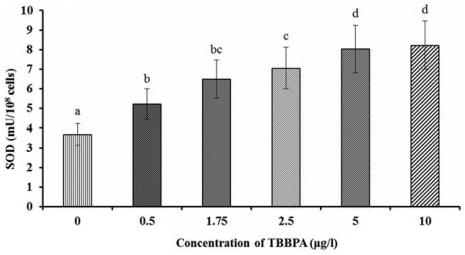

Fig. 7. Effect of tetrabrombisphenol A (TBBPA) on SOD activity in Acipenser ruthenus spermatozoa. Data represent mean values ± SEM. Data are presented as means ± SEM, $n = 6$. Different letters denote significant difference between treatments.

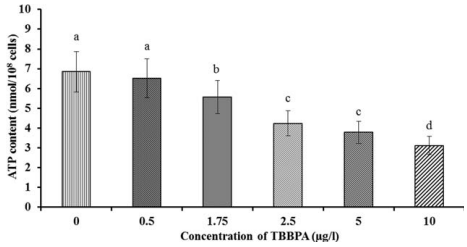

Fig. 8. Effect of TBBPA on intracellular ATP content in Acipenser ruthenus spermatozoa after 2 h exposure to TBBPA. Data are presented as means ± SEM, $n = 6$. Different letters indicate significant differences between treatments (ANOVA, $p < 0.05$).

reproduction of fish and other animals, and LPO is important for aquatic organisms, because they normally contain a higher amount of highly unsaturated fatty acid (HUFA) than other animals. HUFA is a major contributor to the loss of cell function under oxidative stress (Storey, 1996). LPO is usually assessed by TBARS in fish (Oakes and Van der Kraak, 2003). Our results showed a significantly increasing trend in TBARS following 2 h incubation of spermatozoa samples with concentrations of TBBPA >5 µg/L.

Protein carbonylation is a result of protein oxidation. In this study we observed that CP significantly increased with increasing concentrations of TBBPA.

It has been demonstrated that TBBPA can induce production of ROS in cell cultures (Radice et al., 1998; Reistad et al., 2005). The effect of ROS on spermatozoa is well described and characterized in mammals. It may cause LPO of spermatozoa membranes; damage to midpiece, axonemal structure, and DNA; as well as malfunction of capacitation and acrosomal reaction. ROS can lead to the loss of spermatozoa motility and infertility (Gagnon et al., 1991, Sikka 2001). Toxic effects of ROS on fish spermatozoa have been reported in several species (Dietrich et al., 2005; Li et al., 2010). Measures of lipid peroxidation, protein carbonylation, and antioxidant response give indirect evidence that TBBPA may induce ROS production in fish spermatozoa. A significant correlation was found between levels of DNA fragmentation and CP, suggesting that DNA damage to spermatozoa exposed to TBBPA may be associated with oxidative stress. Studies on zebrafish (*Danio rerio*) have demonstrated that the presence of environmental TBBPA in concentrations up to 1.5 µM is detrimental to fish reproduction (Kuiper et al., 2007). TBBPA and related compounds, can induce ROS production and lead to oxidative damage, which may be a major cause of impaired fish reproduction.

It can be concluded that environmentally relevant concentrations of TBBPA (0.5, 1.75, 2.5, 5, and 10 µg/L) have the potential to induce oxidative stress in spermatozoa leading to reduced motility, DNA integrity, and ATP content. Although the antioxidant system apparently responds to increased LPO and CP, it seems that the antioxidant capacity of spermatozoa is not sufficient to prevent cell damage. Our results suggest that the loss of spermatozoa motility and velocity with 2 h *in vitro* exposure of sterlet sperm to TBBPA was induced by oxidative stress that impaired cellular metabolism of spermatozoa. TBBPA was shown to decrease both motility and velocity, but the mechanism or mechanisms of action on spermatozoa are not clear. There is a possibility of action through damage to the plasma membrane or the oraxonemal apparatus, or through depletion of ATP content.

The present study clearly demonstrated that sturgeon spermatozoa are highly susceptible to oxidative stress induced by environmental pollutants. Accumulation of products of LPO as well as increased carbonylation of proteins in spermatozoa provided evidence of TBBPA-induced oxidative stress. We observed reduced spermatozoa quality, as measured by decrease in spermatozoa motility and velocity, DNA damage, and inhibition of ATP content. Spermatozoa *in vitro* assays may provide an efficient means of evaluating the effects of human-synthesized environmental pollutants on sturgeon. Further research is needed to investigate the mechanism and the relationship between oxidative stress and motility in sturgeon sperm, as well as consequences of these effects on fertilization ability.

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CHAPTER 6

IN VITRO EFFECTS OF BISPHENOL A ON THE QUALITY PARAMETERS, OXIDATIVE STRESS, DNA INTEGRITY AND ADENOSINE TRIPHOSPHATE CONTENT IN STERLET (*ACIPENSER RUTHENUS*) SPERMATOZOA

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In vitro effects of bisphenol A on the quality parameters, oxidative stress, DNA integrity and adenosine triphosphate content in sterlet (*Acipenser ruthenus*) spermatozoa

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ABSTRACT

Among endocrine disruptors, the xenoestrogen bisphenol A (BPA) deserves particular attention due to widespread human exposure. Besides hormonal effects, BPA has been suspected to be responsible for adverse effect on reproductive ability of various species. In the present study the effect of BPA on the quality parameters, oxidative stress, the DNA integrity and intracellular ATP content of sterlet (*Acipenser ruthenus*) spermatozoa were investigated *in vitro*. Fish spermatozoa were exposed to concentrations of BPA possibly occurring in nature (0.5, 1.75, 2.5, 5 and 10 μg/L) for 2 h. Results revealed that BPA significantly decreased spermatozoa motility and velocity of spermatozoa at concentration of BPA 2.5–10 μg/L. Significant positive correlation ($r = 0.713$, $P < 0.05$) was found between percent motile spermatozoa and ATP content. Oxidative stress was observed at concentrations 1.75–10 μg/L, as reflected by significantly higher levels of protein and lipid oxidation and superoxide dismutase activity. Intracellular ATP content of spermatozoa decreased with increasing concentrations of BPA. A dramatic increase in DNA fragmentation expressed as percent tail DNA (2.2% ± 0.46) and Olive tail moment (0.37 ± 0.09 arbitrary units) was recorded at concentrations of 1.75 μg/L and above. The present study confirms that concentrations of BPA that can be encountered in nature are capable to induce oxidative stress, leading to impaired sperm quality, DNA fragmentation and intracellular ATP content.

1. Introduction

Bisphenol A (BPA), a xenobiotical compound used to harden polycarbonate plastics and epoxy resin, is the focus of a growing number of research studies and legislative actions. BPA is a one of endocrine disrupting chemicals that can act as a xenoestrogen modulating the endocrine pathways via a receptor-mediated process (Hatef et al., 2012). Trace BPA exposure has been shown to disrupt the endocrine system and trigger a wide variety of disorders, including chromosomal and reproductive system abnormalities (Lahnsteiner et al., 2005; Schug et al., 2011). The results of recent novel studies using low-dose approaches and examining different endpoints describe subtle effects in laboratory animals at very low concentrations (Lahnsteiner et al., 2005; Hatef et al., 2012). Some of these low-dose studies are potentially of concern for the environment because the identified effective concentration levels are similar to some current environmental levels to which sensitive aquatic organisms may be exposed (Mikula et al., 2009; Martí et al., 2011). Moreover, during the past decade, toxicity tests involving fish gametes have received broad attention (Li et al., 2010a, 2010b). It was shown that *in vitro* assays did not accurately reflect the *in vivo* potency of the test compounds, but, on the other hand, they can serve as an important tool in investigation of mechanisms of xenobiotic's action (Segner et al., 2003).

Sturgeon species have been classified as endangered fishes by many international organizations, and the populations of these species have drastically decreased mostly due to over-fishing, destruction of their natural habitat, as well as water pollution (Havelka et al., 2011). However, the rapid development of industry, intensification of agriculture and growth of municipal economies led to a discharge of very large quantities of pollutants into the natural sturgeon habitats. The most common pollutants that occurred in the natural sturgeon habitats are oil products, phenols, and in the North Caspian Sea, detergents. Among these pollutants, the occurrence of BPA in natural sturgeon habitats has been frequently documented (Grund et al., 2011). Concentrations of BPA in surface waters were shown to vary significantly from 0.5 ng/L up to 21 μg/L (Belfroid et al., 2002). For surface waters in Europe, the median and 95th percentile concentrations of BPA were reported to be <0.006 μg/L and <5.1 μg/L respectively (Arnold et al., 2013). Acute toxicity levels, defined as the concentration at which half

Abbreviations: BPA, bisphenol A; LO, lipid oxidation; CP, carbonyl proteins; TBARS, thiobarbituric acid reactive substances; SOD, superoxide dismutase activity.

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of the organisms survive (LC_{50} values), range from 1000 to 20 000 μg/L (Staples et al., 2002). Though lethal doses normally does not occur in surface waters, it is well known that lower concentrations of BPA can act as an estrogen agonist in mammals and fish *in vitro* and *in vivo* (Christiansen et al., 2000; Yokota et al., 2000; Segner et al., 2003). On the other hand, several studies demonstrated the adverse effect of BPA on growth, sexual differentiation and reproductive dysfunctions in several fish species such as Japanese medaka *Oryzial latipes* (Yokota et al., 2000) or swordtail fish *Xiphophorus helleri* (Kwak et al., 2001). Additionally, numbers of xenobiotics are known to enhance production of reactive oxygen species (ROS) (Livingstone, 2001) that can attack a variety of biological macromolecules such as DNA, proteins and lipids, leading to oxidative damages. Due to the fact that, BPA is frequently occurring in sturgeon habitats, the present study was conducted to explore the mechanisms of action of BPA on sperm performance by using sterlet (*Acipenser ruthenus*) as a model organism. Since the quality of fish sperm is a major factor contributing to successful reproduction, measurement of motility of spermatozoa could provide a sensitive and accurate bio-indicator of aquatic pollution (Tashjian et al., 2006). Though the range of BPA concentrations used in the present study exceed typical concentrations found in European rivers, similar and even higher amounts of BPA were found in surface waters (Staples et al., 2000; Belfroid et al., 2002). Thus concentrations of BPA from 0.5 to 10 μg/L we refer to as environmentally relevant.

In the present study we investigated the effect of short-term *in vitro* exposure (2 h) to BPA on sterlet (*Acipenser ruthenus*) sperm with respect to spermatozoa motility parameters, ATP content, DNA integrity and oxidative stress indices.

2. Materials and methods

2.1. Broodstock handling and collection of gametes

Sperm of sterlet (*Acipenser ruthenus*) was obtained from six different males (age: 6–7 years old; body mass: 0.5–2 kg; body length: 55 ± 9.3 cm) raised in the aquaculture facility of the Research Institute of Fish Culture and Hydrobiology at the University of South Bohemia, Vodnany, Czech Republic. Prior to hormonal stimulation, fish were kept in tanks with water temperature varying between 14 and 15 °C. Spermiation was stimulated by intramuscular injection of carp pituitary powder dissolved in 0.9% (w/v) NaCl solution at doses of 5 mg/kg of body mass, 48 h before sperm collection. Semen was collected from urogenital papilla by aspiration through a plastic catheter (5–7 mm diameter) connected to a 20 mL syringe. Sperm contaminated by mucus, feces, or water was excluded. Sperm samples were stored on ice (0–4 °C) until processing. Sperm concentrations of each of six males were estimated microscopically (Olympus BX 41) at 20× using a Burker cell hemocytometer.

2.2. Sample preparation

BPA [2,2-Bis (4-hydroxyphenyl) propane; empirical formula: $C_{15}H_{16}O_2$; MW: 228.29), (≥99%; Sigma-Aldrich, USA)] was dissolved in 96% ethanol and diluted with distilled water to obtain a stock solution of 0.1 g/L. Stock solutions were prepared daily. Sperm from six individual males was centrifuged at 300 *g*, 4 °C for 30 min to remove seminal plasma and diluted with an immobilization medium (IM) (20 mM Tris, 30 mM NaCl, 2 mM KCl, pH 8.5) to obtain spermatozoa densities of 5×10^8 cells/mL: this is equivalent to the standard concentration of spermatozoa used for sterlet fertilization conditions. The sperm sub-samples were then exposed for 2 h (at 4 °C) to final concentrations of 0.5, 1.75, 2.5, 5 and 10 μg/L of BPA. Control group was exposed to immobilization medium with 0.5% of ethanol since this solvent was used to dissolve BPA. Each experimental condition was duplicated.

2.3. Spermatozoa motility assessment

Spermatozoa velocity (μm s^{-1}, taking into account only motile sperm) and percentage of motile sperm cells (%) were determined after triggering sperm motility under dark-field microscopy (Olympus BX 50, Japan) (20× objective magnification). For motility triggering, sperm was diluted in activation medium (AM) (10 mM Tris, 10 mM NaCl, 1 mM $CaCl_2$, pH 8.5) at a dilution ratio of 1:5000. To prevent spermatozoa from sticking to the microscope slide, 0.25% (w/v) of pluronic (Sigma-Aldrich) was added to the solution. Spermatozoa motility was recorded with a CCD video camera (SONY SSCDC50AP, Japan) mounted on a microscope. The successive positions of the heads of spermatozoa were measured from five successive frames using a video recorder (SONY SVHS, SVO-9500 MDP, Japan), and analyzed with a micro image analyzer (Olympus Micro Image 4.0.1. for Windows). The trajectory of sperm movement was recorded and traced by overlapped five successive frames. Spermatozoa motility and velocity were calculated as described by Rodina et al. (2007). Measurement of spermatozoa motility for each sample was performed in triplicate.

2.4. Assessment of DNA damage

In the present study DNA integrity was assessed using the Comet assay, or single cell gel electrophoresis assay following the method described by Li et al. (2008). Unless otherwise stated, molecular grade, DNAse-free reagents (Sigma Aldrich, USA) were used throughout. Microscope slides (OxiSelectST; Cell Biolabs, INC. USA) were used for the assay, and each slide was prepared in the following manner. The 100 μL of sperm sample was diluted in 5 mL of PBS (phosphate buffer solution; Ca^{2+} and Mg^{2+} free) to obtain a sperm density of 1×10^7 cells mL^{-1}. Diluted samples (200 μL) were mixed with 700 μL of 0.8% NuSieve GTG low melting temperature agarose (OxiSelectST; Cell Biolabs, INC. USA). Finally, a 55 μL of this mixture was then added to the slide, and the agarose was allowed to gelify for 1 h at 4 °C. The slides were then immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10% Lysis Solution (OxiSelect ™ Comet Assay Kit], 10% DMSO, pH 10) at 4 °C. Thereafter, the slides were subjected to enzymatic treatment with proteinase K (1 mg/mL in 2.5 M NaCl, 100 mM EDTA, 10% DMSO, pH 7.4) and incubated over night at 32 °C. After the proteinase K solution was drained, the slides were immersed in a horizontal gel tank filled with freshly made electrophoresis buffer (90 mM Tris Base, 90 mM Boric acid, 2.5 mM EDTA). Electrophoresis was carried out for 20 min at 35 V and 170 mA. Slides were then carefully transferred from the electrophoresis chamber to a clean container with pre-chilled distilled water for 2 min. The washing procedure was repeated three times. The slides were drained well, dehydrated by dipping into absolute ethanol for 5 min and air-dried for storage. Prior to the analysis, 50 μL of Vista Green DNA Staining Solution (OxiSelectST; Cell Biolabs, INC. USA) was added to each agarose spot of the slide and analyzed with an Olympus BX50 fluorescence microscope with a 20× magnification lens. A total of 100 cells were scored for each sample and the captured images were analyzed using CometScore image analysis software (TriTek Corporation, USA). Tail length (measured from the middle of the head to the end of the tail) and tail DNA content (Tail DNA %) were measured. Percentage of DNA in tail and Olive tail moment were calculated using the following formulas:

$$\text{Tail DNA\%} = 100 \times \text{Tail DNA intensity/Cell DNA intensity}$$

$$\text{Olive tail moment} = \text{Tail DNA\%} \times \text{Tail moment length.}$$

2.5. Oxidative stress indices

Sperm samples were centrifuged at 5000 × g at 4 °C for 10 min. The supernatant was carefully collected and discarded. The spermatozoa pellet was diluted with 50 mM potassium phosphate (KPi) buffer, pH 7.0, containing 0.5 mM EDTA to obtain a spermatozoa concentration of 5×10^8 cells/mL, then homogenized in an ice bath using a Sonopuls

HD 2070 ultrasonicator (Bandelin Electronic, Berlin, Germany). The homogenate was divided into two: one part for measuring thiobarbituric acid reactive substances (TBARS) and carbonyl derivatives of proteins (CP), and a second part that was centrifuged at 12 000 *g* for 30 min at 4 °C to obtain the post mitochondrial supernatant for the antioxidant enzyme activity assay.

The TBARS method described by Li et al. (2010a, 2010b) was used to evaluate sperm lipid oxidation (LO). Its concentration was calculated by absorption at 535 nm and a molar extinction coefficient of 156 mM/cm. The content of TBARS was expressed as nano-moles per 10^8 cells. CP were detected by reaction with 2,4-dinitrophenylhydrazine according to the method described by Lenz et al. (1989). The amount of CP was measured spectrophotometrically at 370 nm using a molar extinction coefficient of 22 mM/cm, expressed as nmol per 10^8 cells.

2.6. Superoxide dismutase activity (SOD)

Total SOD (EC 1.15.1.1) activity was determined by the method of Marklund and Marklund (1974). This assay depends on the autoxidation of pyrogallol. The activity of SOD in supernatant was assessed spectrophotometrically at 420 nm. One unit of SOD activity is defined as the amount of the enzyme needed to exhibit 50% dismutation of the superoxide radical per min. Activity was expressed in international units (or milli-units) mU per 10^8 cells.

2.7. Evaluation of ATP content

ATP content in spermatozoa was determined after 2 h exposure to BPA using bioluminescence method described by Boryshpolets et al. (2009). Sperm samples were diluted in 5 mL of boiling medium containing 100 mM Tris–HCl, pH 7.5 and 4 mM EDTA. After boiling for 2 min at 98 °C, samples were centrifuged at 12 000 g for 20 min. Supernatant was collected and stored at −80 °C before analysis. A Bioluminescence Assay Kit CLS II (Roche Diagnostics GmbH, Germany) was used for determination of ATP content. Luminescence was recorded with a multifunctional micro-plate reader Infinite M200 (Tecan, Austria). ATP content was expressed as nmol ATP/10^8 spermatozoa.

2.8. Data analysis

All measurements were conducted in triplicate. Normality and the homogeneity of variance of all data were first tested with the Kolmogorov test and the Bartlett test, respectively. Data obtained from the Comet assay were log-transformed if necessary. Values are expressed as means ± SEM (n = 6) and analyzed by factorial ANOVA. Statistical comparison was made by analysis of variance (ANOVA) followed by Tukey's HSD test for each analyzed parameter. The relationships among the parameters were quantified according to the Spearman's correlation tests. All analyses were performed at a significance level of 0.05 using STATISTICA 9.0 software for Windows.

3. Results

3.1. Spermatozoa motility and velocity

Sperm quality parameters (motility and velocity) were determined after 2 h exposure to BPA *in vitro*. Spermatozoa motility and velocity were significantly ($P < 0.05$) affected by BPA at 10, 30, 60, 90 and 120 s post activation (Fig. 1, A and B). At 10 and 30 s post-activation, spermatozoa motility significantly ($P < 0.05$) decreased in all tested

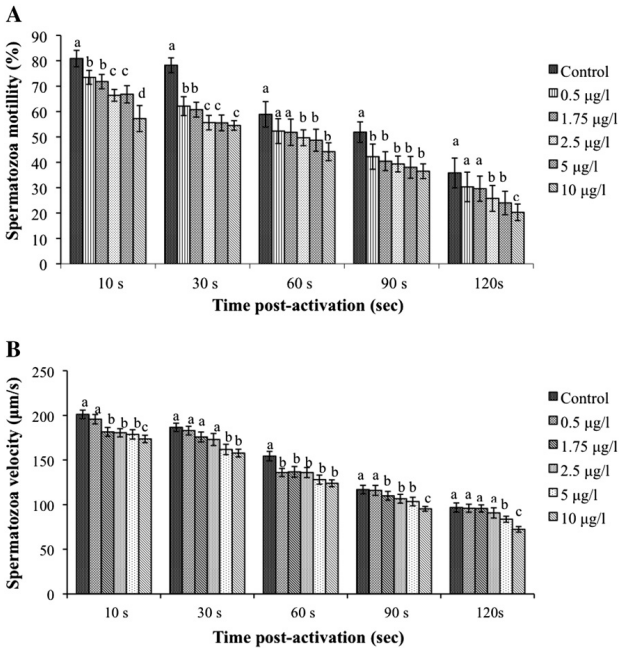

Fig. 1. *In vitro* effects of bisphenol A (BPA) on spermatozoa motility (A) and velocity (B) in *Acipenser ruthenus*. Data are presented as means ± SEM. Superscript letters indicate significant differences among samples at the same time post-activation (ANOVA, $P < 0.05$).

Table 1
Correlation coefficients among the parameters measured in *Acipenser ruthenus* spermatozoa after 2 h exposure to bisphenol A (BPA). Spermatozoa motility and velocity were included at 10 s post-activation only. The t-test is used to establish significance of the correlation between pairs of parameters.

	Motility	Velocity	Tail DNA %	Olive tail moment	TBARS	CP	SOD	ATP
Motility	1.00							
Velocity	0.29*	1.00						
%DNA in tail	−0.12	0.02	1.00					
Olive tail moment	−0.19	0.02	0.96*	1.00				
TBARS	−0.55*	−0.07	0.38*	0.39*	1.00			
CP	−0.8*	−0.21*	0.17	0.22	0.66*	1.00		
SOD	−0.74*	−0.18	0.02	0.05	0.46*	0.71*	1.00	
ATP	0.78*	0.18	−0.11	−0.16	−0.48*	−0.61*	−0.56*	1.00

P* < 0.05.

concentrations in comparison with control. Significant ($P < 0.05$) differences between tested concentrations were observed as well. At 90 s post-activation, a significant decrease ($P < 0.05$) in spermatozoa motility was observed at all tested concentrations in comparison with control. However, no statistical difference ($P > 0.05$) was observed between treatments. At 60 and 120 s post-activation, no statistical significance ($P > 0.05$) was observed between control and concentrations of 0.5–1.75 μg/L, whereas, significant decline of spermatozoa motility was observed at 2.5–10 μg/L. Moreover, significant positive correlation ($r = 0.713$, $P < 0.05$) was found between percent of motile spermatozoa at 10 s post-activation and ATP content (Table 1). Fig. 2 illustrates changes in flagella movement after exposure of spermatozoa to BPA.

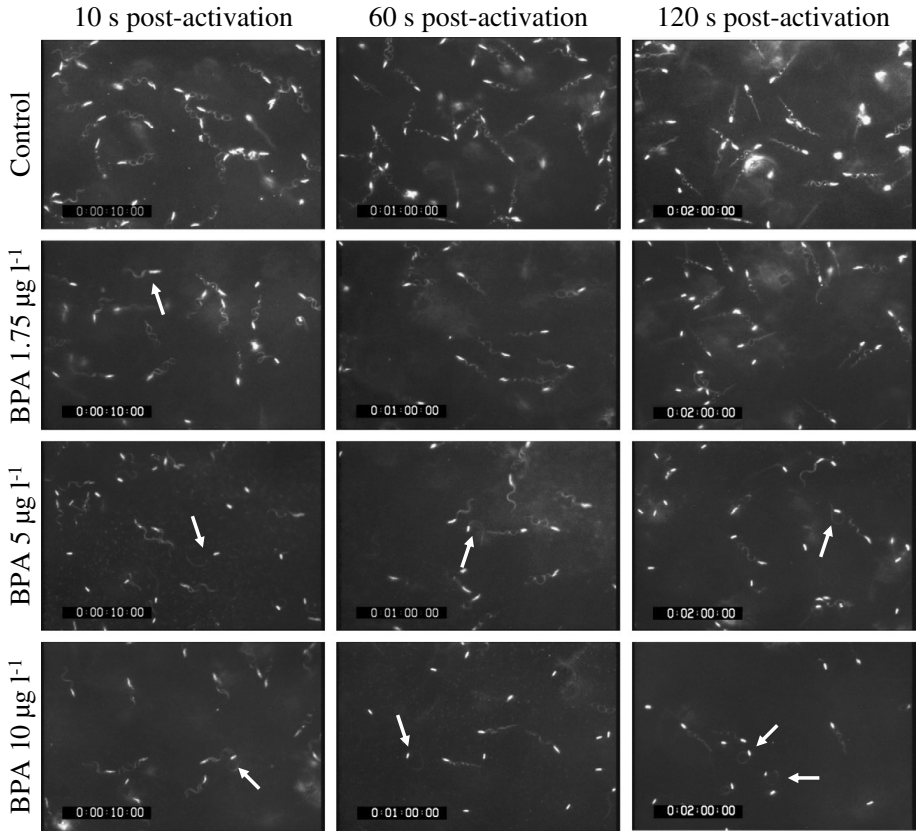

Fig. 2. Swimming behavior of *Acipenser ruthenus* spermatozoa incubated with four concentrations of bisphenol A (BPA) (0, 1.75, 5 and 10 μg/L). Flagella movement was recorded under dark field microscopy with stroboscopic illumination at 10, 60, and 120 s post-activation of spermatozoa. Beat frequency and number of waves along the flagella decreased with increasing concentration of BPA. Arrows indicate various forms of flagella detected after sperm exposure to BPA *in vitro*.

The beat frequency and number of waves along the flagellum were significantly lower at the highest concentration of BPA compared to control. Atypical C-shaped flagella were observed.

Estimation of spermatozoa curvilinear velocity (VCL) showed a normal distribution and ANOVA showed that: at 10 and 90 s post-activation, spermatozoa velocity significantly decreased at 1.75, 2.5, 5 and 10 μg/L. At 30 s post-activation, velocity significantly decreased at 5 and 10 μg/L. At 60 s post-activation, a significant decrease in spermatozoa velocity was observed at all tested concentrations. At 120 s post-activation, a significant decrease of sperm velocity was observed at 5 and 10 μg/L.

3.2. DNA fragmentation

An illustration of the outcome of this assay for DNA damage is presented in Fig. 3. Comet assay analysis indicated a dramatic increase in DNA fragmentation in spermatozoa nuclei after 2 h exposure to BPA at concentrations of 1.75–10 μg/L compared to controls ($P < 0.05$; Fig. 4, A and B). In addition, the highest BPA concentration (10 μg/L) was associated with 6% of DNA fragmentation. There were also significant correlations between % DNA in Tail and TBARS level ($r = 0.38$, $P < 0.05$) as well as between % DNA in Tail and Olive tail moment ($r = 0.96$, $P < 0.05$) (Table 1).

3.3. Oxidative stress indices and antioxidant response

The level of TBARS and CP were used as indicatives of the extent of LO and protein oxidation, respectively. The results showed that, in the control group, the measured level of TBARS was typically around 0.16 nmol/10^8 spermatozoa (Fig. 5). A significantly higher level of LO was observed in spermatozoa exposed to BPA at concentrations 2.5 μg/L and above. The highest concentration of BPA (10 μg/L) enhanced the level of TBARS to 0.3 nmol/10^8 spermatozoa. There was no significant difference in CP level between spermatozoa in control and group exposed to 0.5 μg/L of BPA (Fig. 6). However, significant ($P < 0.05$) increase in the level of CP was observed in groups exposed to concentrations higher than 1.75 μg/L. The maximum detected level of CP was 15.3 nmol/10^8 in spermatozoa exposed to 10 μg/L of BPA. The antioxidant activity was assessed by total SOD activity. There was no significant difference in SOD level between spermatozoa in control and groups exposed to 0.5 – 1.75 μg/L of BPA (Fig. 7). However, the antioxidant response was significantly enhanced in spermatozoa exposed to BPA at concentrations 2.5 μg/L and higher. Total SOD activity increased from 3.2 mU/10^8 of spermatozoa in control to 7.8 mU/10^8 of spermatozoa at 10 μg/L of BPA. In addition, Spearman's correlation revealed significant correlations between TBARS and CP level ($r = 0.66$, $P < 0.05$) and SOD ($r = 0.46$, $P < 0.05$) (Table 1).

3.4. ATP content

The ATP level was evaluated as an additional parameter that can explain quality decline of fish spermatozoa. The intracellular ATP content in control group was detected at 68 nmol ATP/10^9 spermatozoa (Fig. 8). After 2 h of exposure to BPA at concentrations higher than 2.5 μg/L, the ATP level significantly ($P < 0.05$) declined. The lowest

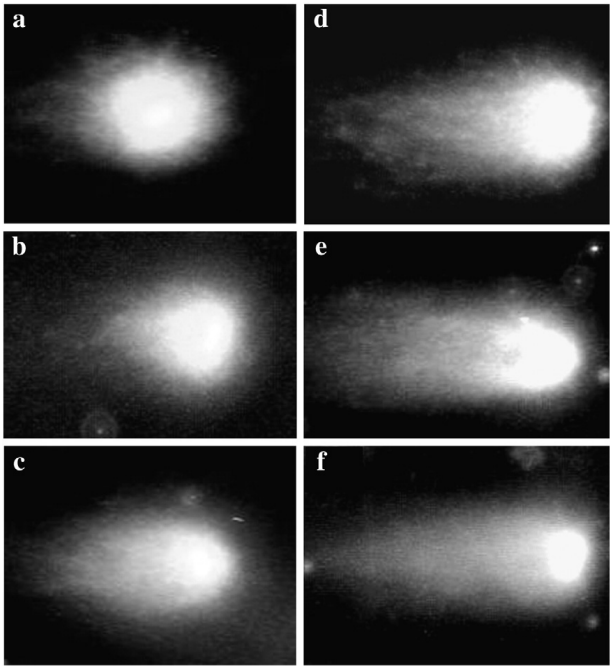

Fig. 3. Examples from Comet assay of *Acipenser ruthenus* spermatozoa exposed to differing concentrations of bisphenol A (a - control, b - 0.5 μg/L, c - 1.75 μg/L, d - 2.5 μg/L, e - 5 μg/L, f - 10 μg/L). Relative changes in DNA fragmentation are represented by an increasing amount of DNA in the comet tail. Typical shape of the comet tail indicates that no double strand breaks appears after spermatozoa exposure to BPA.

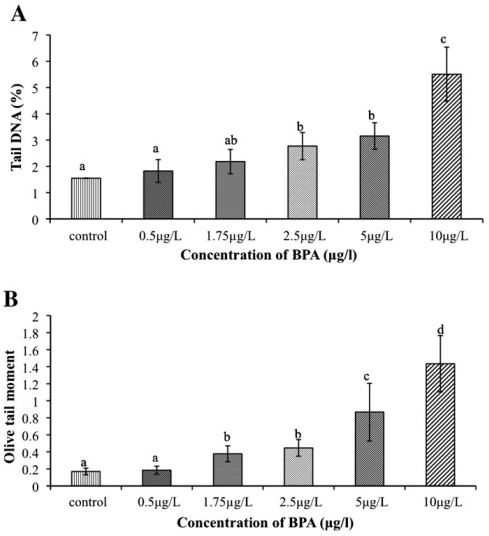

Fig. 4. Influence of bisphenol A (BPA) on DNA fragmentation in *Acipenser ruthenus* spermatozoa measured by Comet assay as a percentage of DNA in the tail (A) and Olive tail moment (B). Data represent mean values ± SEM. Different letters indicate significant differences among samples (ANOVA, $P < 0.05$).

ATP content was around 50 nmol ATP/10^9 spermatozoa observed at BPA concentrations 10 µg/L.

4. Discussion

Environmental contaminants have been shown to induce reproductive abnormalities in both wildlife and humans (Chitra et al., 2003; Hulak et al., 2008; Jana and Sen, 2012). Recent data suggest that environmentally realistic, meaning those that can be found in nature, levels of xenobiotics such as BPA may have an impact on the reproduction of fish in the wild (Lahnsteiner et al., 2005). Number of studies pointed out that fertilization and hatching rates were decreased following *in vivo* fish exposure to BPA (Staples et al., 2002). However, *in vitro* tests, in contrast to tests conducted *in vivo*, can provide valuable information about mechanisms of action of xenoestrogenes such as BPA on fish gametes. In the present study we explored the effect of

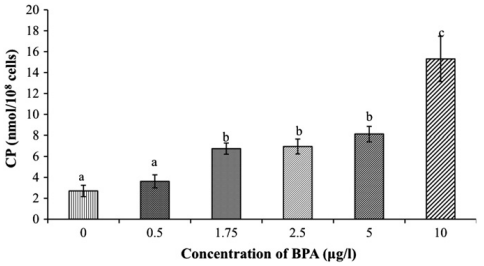

Fig. 6. Effect of bisphenol A (BPA) on carbonyl proteins (CP) level in *Acipenser ruthenus* spermatozoa. Data represent mean values ± SEM. Different letters indicate significant differences among samples (ANOVA, $P < 0.05$).

BPA on sperm performance by using sterlet as a model organism. The obtained results demonstrated that BPA could induce oxidative stress in fish spermatozoa *in vitro*, which results in accumulation of LO and CP, together with the modification of antioxidant system activity. These oxidative responses were associated with spermatozoa quality depression, as measured by decrease in the values of spermatozoa motility and velocity. BPA treatment also resulted in depletion of ATP metabolism and significant DNA fragmentation.

Sperm motility is a key prerequisite determining the quality and fertilizing ability of semen. Fish spermatozoa differ from that of mammals in being immotile on ejaculation, attaining motility only on contact with surrounding water, and having an extremely short period of motility, typically ranging from several seconds to minutes (Li et al., 2009; Cosson, 2010) depending on species. Previous studies have shown that different factors from external medium can induce decreased spermatozoa motility associated with a reduction of fertilization rate (Zhou et al., 2006; Dietrich et al., 2012). Therefore the fertilization ability strongly depends on the capacity of spermatozoa to move rapidly during the short time frame allowed to contact the egg after release (Kaspar et al., 2007, 2008). In the present study, spermatozoa motility and velocity were significantly reduced after *in vitro* BPA treatment in all tested concentrations, compared to the control. When sperm was exposed to increasing concentrations of BPA, the reduction in these two analyzed parameters was shown to be dose-dependent. These results are in agreement with several other studies on brown trout (*Salmo trutta*) and goldfish (*Carassius auratus auratus*) that showed lower spermatozoa motility and velocity in the sperm exposed to environmentally realistic concentrations of BPA either *in vivo* or *in vitro* (Lahnsteiner et al., 2005; Hatef et al., 2010, 2012).

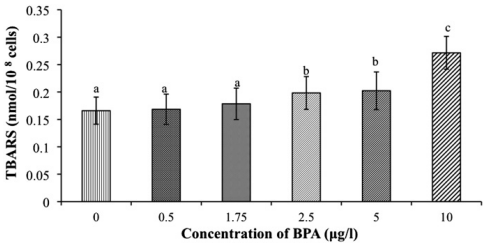

Fig. 5. Effect of bisphenol A (BPA) on thiobarbituric acid reactive substances (TBARS) level in *Acipenser ruthenus* spermatozoa. Data represent mean values ± SEM. Different letters indicate significant differences among samples (ANOVA, $P < 0.05$).

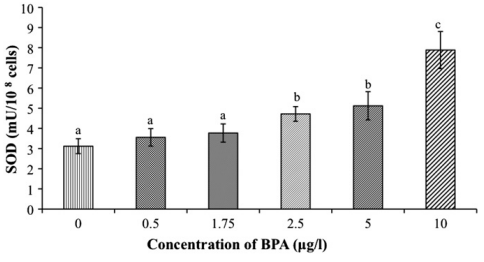

Fig. 7. Effect of bisphenol A (BPA) on superoxide dismutase (SOD) activity in *Acipenser ruthenus* spermatozoa. Data represent mean values ± SEM. Different letters indicate significant differences among samples (ANOVA, $P < 0.05$).

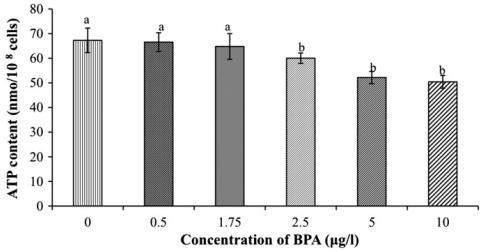

Fig. 8. Influence of bisphenol A (BPA) on ATP content in *Acipenser ruthenus* spermatozoa. Data represent mean values ± SEM. Different letters indicate significant differences among samples (ANOVA, $P < 0.05$).

The reduction of spermatozoa motility after BPA treatment may be explained in several ways. As have been shown by de Lamirande and Gagnon (1992) spermatozoa motility may be the most sensitive indicator of oxidative stress, because high levels of ROS inhibit one or more enzymes of oxidative phosphorylation and/or glycolysis, limiting the generation of ATP. In the present study, fish spermatozoa treated with BPA showed increased levels of LO and CP indicating that oxidative stress has been induced by BPA. In addition, results of this study indicated decreasing trend in intracellular ATP concentration during *in vitro* treatment as well as a positive significant correlation between intracellular ATP content and spermatozoa motility. Spermatozoa movement is primarily dependent on initial ATP content, plasma membrane potential for triggering the flagellar beating and Ca^{2+} signaling (Li et al., 2009; Cosson, 2010). Therefore any modification of these three key elements can significantly modify spermatozoa movement capacity. In addition our results showed that, BPA was capable to damage the sperm flagella, and variations of the flagella shape due to BPA were observed during spermatozoa movement as well as after termination of motility period. Based on the above-mentioned facts, it's likely that, oxidative stress induced by BPA resulted in production of ROS, leading to impaired motility and velocity via mitochondrial respiration.

Lipid peroxidation is particularly dangerous for aquatic animals, since the latter normally contain greater amounts of highly unsaturated fatty acids than found in other species. LO has been reported to be a major contributor to the loss of cell function under oxidative stress (Storey, 1997) and has usually been indicated by TBARS in fish (Oakes and Van der Kraak, 2003). Our results showed that TBARS levels were greater in spermatozoa incubated with BPA at concentrations 2.5 μg/L and higher. Thus increased levels of LO can trigger the loss of membrane integrity, causing increased cell permeability, enzyme inactivation, and structural damages of DNA (Domínguez-Rebolledo et al., 2010). On the other hand, the CP is a result of protein oxidation. The formation of CP is irreversible, causing conformational changes, decreased catalytic activity in enzymes, and ultimately resulting in higher susceptibility of proteins to breakdown by proteases (Zhang et al., 2008). In the present study, we observed that short-term incubation with BPA led to increase in CP level. Therefore based on our results, we hypothesize that another way to explain motility parameters decline could be the loss of membrane integrity as well as enzyme inactivation caused by oxidative stress.

In order to cope with the oxidative damage, organisms have evolved multiple systems of antioxidant defense, including endogenous enzymatic and non-enzymatic antioxidants (Li et al., 2009, 2010a, 2010b). SOD is considered the first line of defense against deleterious effects of oxyradicals in the cell by catalyzing the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen (Li et al., 2012). In the present study a significant enhancement of SOD activity at concentration 2.5 μg/L of BPA and above was observed. It is likely that the enhancement of SOD activity was an adaptive response to toxicant stress and served to neutralize the impact of increased ROS generation.

In recent years, sperm DNA damage assessment has gained special attention as an important sperm quality indicator, linked with sperm fitness as well as with offspring quality (Peknicova et al., 2002; Meeker et al., 2010). Most of the reported data indicate that DNA damage is correlated with a reduction in fertilization ability, with development failure after fertilization and offspring problems (Kopeika et al., 2004). In the present study the comet assay has been used to evaluate DNA damage associated with *in vitro* exposure of sterlet spermatozoa to BPA. Using the comet assay, we were able to demonstrate that the short-term exposure of sterlet spermatozoa to BPA caused a dramatic increase in DNA damage expressed by Olive moment and percentage of tail DNA. Moreover, significant positive correlations have been found between level of CP and Olive moment and % of Tail DNA. Our results are in good agreement with those published in humans, where several researchers have reported that the DNA damage in human spermatozoa is associated with membrane lipid peroxidation (Chen et al., 1997; Potts et al., 2000) and oxidative stress (Aitken et al., 1998). Therefore, it is likely that oxidative stress induce by BPA treatment is the major factor responsible for dramatic increase of DNA fragmentation observed in the present study.

5. Conclusions

The results of the present study demonstrated that environmentally relevant concentrations of BPA can induce oxidative stress in fish spermatozoa *in vitro*, which results in accumulation of LO and CP, together with the significant inhibition of ATP metabolism, leading to significant reduction of sperm quality. Based on the obtained results, the use of sperm *in vitro* assay may provide a novel and efficient mean for evaluating the effect of xenobiotics in aquatic environment on sturgeon.

Acknowledgments

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CHAPTER 7

GENERAL DISCUSSION

The health benefits of seafood are well recognized and fish and its products are increasingly advocated as functional foods (Gormley, 2006). Many health benefits are attributable to n-3 FA (Kinsella, 1986) and low caloric content of fish (Kris-Etherton et al., 2002). The intake of n-3 FA has been associated with protective effects against cardiac arrhythmias (preventing heart disease), lowering cholesterol levels, as well as decreasing levels of depression and rheumatoid arthritis (Swanson et al., 2012; Leaf et al., 1996). In contrast, fish and seafood that have been exposed to pollution can contain several xenobiotics (as for example cadmium, DQ, TBBPA, BPA and others). Bioavailability on the one hand, and potential toxicity on the other, remain unclear. Thus, the main goal of this thesis was to study the effects of different pollutants, like heavy metals (Cd^{2+}: Chapters 2 and 3) and other xenobiotics (DQ, TBBPA and BPA: Chapters 4, 5 and 6) and to investigate the effects of pollution on water organism (especially fish) and the resultant impact on human as the final comsumers.

The study of Guan et al. (2015) examined the relationship between eating seafood and levels of Cd^{2+} in the blood of 252 seafood consumers in Long Island (New York). They found no changes in blood after regular seafood intake, but they did find an association between salmon intake and blood Cd^{2+}, however, salmon were eaten more often than other seafood. Other studies have identified an increase in Cd^{2+} levels in certain types of fish (Andreji et al., 2006; Li et al., 2013; Sissener et al., 2012). It is important to mention that tobacco smokers have higher blood Cd^{2+} levels than non-smokers (Boonprasert et al., 2011; Madeddu et al., 2011). The study of Ju et al. (2012) reported that eating seafood is one source of human exposure to Cd^{2+} where increase in levels may be significant (Storelli and Barone, 2013). However, Hellberg et al. (2012) suggest that seafood may not be the major factor of Cd^{2+} exposure. Consequently, the American Heart Association suggests eating fish at least two times per week so as to increase the intake of n-3 FA (Kris-Etherton et al., 2002). However, there are still questions relative to the risks versus benefits of eating fish. Therefore, one of the aims of the present thesis was to evaluate whether EPA and DHA could have a protective effect against oxidative stress and cytotoxicity induced by Cd^{2+} (Linhartova and Sampels, 2015, Chapter 2). This is pertinent to the Czech Republic where fish consumption is reltively low and where waters and associated fishes are contaminated with xenobiotics (Valova et al., 2010).

It is also important to consider that Cd^{2+} will enter the body along with other nutrients, therefore it is important to evaluate their combined effects on human nutrition and health issues. We chose to test Cd^{2+}, at levels comparable to open waters, in combination with FA (ratio 1:2 of EPA: DHA), thereby simulating consumption of contaminated fish but also with the valuable nutritional compounds. FA should have both anti-inflammatory and anti-oxidative effects as shown in the study of Kaur et al. (2008) (Chapters 2 and 3). Furthermore, EPA and DHA has purported anticancer activity (Shirota et al., 2005; Lim et al., 2009). The level of FA selected is typical for certain fatty fish such as common carp, which is a traditional freshwater fish eaten in Czech Republic available year-round in the local markets (Meyer et al., 2014; Domingo, 2007). Hep G2 cells were used as a model system *in vitro* to study changes in lipid metabolism caused by FA and Cd^{2+} co-incubations. Hep G2 cells were chosen, since liver is the target organ affected by Cd^{2+}. Moreover the benefits of the Hep G2 cell line for *in vitro* studies are known compared to primary hepatocytes that are difficult to obtain, and using them for *in vitro* studies is more complicated (Wilkening et al., 2003). The main objective was to observe vital/toxic and metabolic changes in liver cells being incubated with select FA and heavy metal. We hypothesized that a change in lipid metabolism might indicative of an unidentified mechanism of causative effects of Cd^{2+} for apoptosis and cancer. However, our

studies only investigated the basic effects, and further studies will be needed to apply our results.

The most suitable concentrations (95–100% viability) for the liver cells (Hep G2) were related to the co-incubation with 5 μM EPA and 10 μM DHA for at least 2–24h (Chapter 2). Higher concentrations of FA were also tested; 50 μM DHA and 50 μM EPA were incubated separately for 24h in the cells. Only 70% of cells survived at 50 μM DHA and neither IC_{50} nor IC_{30} for EPA were reached (Linhartova and Sampels, 2015, Chapter 2). In contrast to our results, Di Nunzio et al. (2011) reported that 60 μM DHA was physiological relevant. However, our results agree with those of Lindskog et al. (2006). They showed reduced survival after 72h incubation of DHA to cancerogenic cells with IC_{50} ranging from 25–75 μM and similar toxic effects were shown for DHA on cancerogenic neuroblastoma cells, but not on non-transformed nervous tissue; they concluded that DHA counteracted cancer by causing apoptosis in the cancer cells (Lindskog et al., 2006). Moreover Jaudszus et al. (2013) showed that relatively high doses (100 μM) of EPA and DHA did not affect viability of human healthy cells (leukocytes). The review of Merendino et al. (2013) deals with a problem of oxidative stress created by oxidation compounds which were derived from DHA. They concluded that due to their increased metabolism, cancer cells have naturally increased levels of ROS, and therefore ROS can not be counteracted as efficiently as in healthy cells where the antioxidant mechanisms are intact. Higher doses of EPA (50 μM) had no effect on the viabiality of the hepatoma cells (Chapter 2). Schumann et al. (2012) investigated the effect of FA on the uptake in a human epithelial kidney cell line NLZ9 and found that saturated FA were taken up more rapidly and in higher proportions than unsaturated ones. No difference in uptake due to time (3h versus 72h) was found, which agrees with our study. However we found significant higher contents of EPA and DHA after several repetitions of FA incubations (3 times 2h) (Chapter 2). Obermeier et al. (1995) achieved the maximal incorporation of AA, EPA, and DHA into U937 cell (human leukemic monocyte lymphoma cell line) after 8h, with no repeated incubations. We showed that the maximal proportion of EPA and DHA was taken up by the cells after an incubation time of 2h (Chapter 2).

We could also identified some changes in FA composition in the chosen cell line (Hep G2). The combined incubation of both FA (EPA + DHA) and 5μM Cd^{2+} significantly increased the proportion of docosapentaenoic acid (DPA) in the cells. Our data showed that EPA increased to 9.5%, DPA to 3.3% compared to 0.6% of total FA in control group. Moreover the DHA level was almost five times higher (15.4% of total identified FA compared to 2.7% in the untreated control cells). This increased DPA level could suggest either an upregulated metabolism from EPA towards the longer chain products, or an increased β-oxidation from DHA to DPA due to energy needs of the cells (Linhartova and Sampels, 2015, Chapter 2). Our findings agree with Fujiyamafujiwara et al. (1992), where incubation with EPA resulted in a dose-dependent incorporation in to the Hep G2 and an increased metabolism towards DPA but not DHA.

Additionally, phospholid classes and their metabolism changes were checked in our study. In mitochondria especially two phospholipid classes, cardiolipin (CL) and sphingomyelin (SM), were important for membrane functionality. Changes in mitochondrial CL have been found in various tumor cells in mice brain (Kiebish et al., 2008). The authors also reviewed changes in CL FA composition, as well as change of phospholipid and FA composition in different neural tumors; they suggested that these changes could be due to variable factors causes such as FA metabolism, ROS and FA transport. We found no significant differences in the phospholipid composition of the cells related to the pre-incubation with only FA (Chapter 2). Another interesting result was that the FA concentration did not cause oxidative stress to Hep G2, while Merendino et al. (2013) showed that higher doses of DHA can create ROS in cancer cells, due to their increased metabolism and the increased natural levels of ROS. We

showed that lower doses of FA (5 μM EPA + 10 μM DHA) did not provoke the proliferation of ROS in Hep G2 cells. No significant differences of ROS were found for the cells incubated with or without FA for 24h (Chapter 3).

Many studies are currently focusing on the problem of Cd^{2+} induction of ROS, which is thought to be an important mechanism in Cd^{2+} carcinogenesis (Waisberg et al., 2003; Valko et al., 2006; Mates et al., 2010), either through oxidative stress or through the inhibition of oxidative DNA damage repair (Waisberg et al., 2003). Lawal and Ellis (2010) showed toxic effects of 5, 10 and 50 μM Cd^{2+} in three human cell lines, namely: a human hepatoma cell line (Hep G2), a human astrocytoma cell line (1321N1) and a human embryonic kidney cell (HEK 293). Significant increases in all three cell lines especially after exposure to 50 μM Cd^{2+} (10 times higher Cd^{2+} concentration compared with our study); they found DNA damage, lactate dehydrogenase leakage, malondialdeyde content and antioxidant enzymes activities. Matsuoka et al. (2001) found signs of oxidative stress in the used cells (MCF-7 breast cancer cells) at lower concentrations of 10–20 μM Cd^{2+}. In contrast to our results in the liver cell line Hep G2, we found ROS production started at the lower Cd^{2+} concentrations of 1 μM (Linhartova et al., 2016, Chapter 3). However, a significantly higher level of ROS (measured by TBARS and CP methods) was observed in hepatocellular cells exposed to Cd^{2+} (without FA pre-incubations) at concentrations of 1 and 5 μM, in comparison to the control cells (not incubated with FA or Cd^{2+}) where the level of TBARS (expressed as malondialdehyde) was almost two times lower. Surprisingly, ROS were not significantly increased in Hep G2 cells exposed to FA for 24h and subsequently incubated with 1 or 5 μM Cd^{2+} (24h). Moreover, we did not find significant differences for the antioxidant activity from the SOD level between the control cells and cells incubated with FA, nor with/without 1 μM Cd^{2+}. However, the antioxidant response measured as total antioxidant capacity (TAC), was significantly enhanced in cells exposed to 5 μM Cd^{2+}, but not in groups pre-incubated with FA and post-incubated with 5 μM Cd^{2+}. Significantly increased TAC values were observed only in groups treated with 5 μM Cd^{2+} (Chapter 3). From the data presented on Hep G2 cells, we concluded that FA have a a possible protective effect against Cd^{2+} toxicity, whereas higher doses of Cd^{2+} had increased oxidation parameters, indicating oxidative stress and the production of ROS. Also the the antioxidant response suggests a protective effect of the FA, even if though a significant increase of the antioxidant response was only visible at the highest Cd^{2+} concentration (5 μM), and significant changes between cells exposed to FA were found (Chapter 3). We measured a significant decrease in phospholipid CL after 24h incubation with 5 μM Cd^{2+} independent from incubation with or without FA. At this level of cadmium, only 12.1% CL was detected in cells compared to 14.9% at 1 μM Cd^{2+} and 15.4% without any Cd^{2+} treatment (control group). However, we need to further investigate the exact mechanism behind the significant decrease of CL related to 5 μM Cd^{2+}. In summary, we could see that EPA and DHA had a slight protective effect against the increased oxidative stress caused by Cd^{2+}. Cd^{2+}, but on the other hand, seemed to affect the lipid metabolism as the combined incubation of FA and Cd^{2+} at the highest concentration resulted in increased DPA.

In addition to direct effects on human and animal health, many environmental contaminants have been shown to induce reproductive abnormalities in both wildlife and humans (Chitra et al., 2003; Hulak et al., 2008). Our data suggests that environmentally realistic levels of xenobiotics, i.e. those that can be found in nature, such as DQ, TBBPA or BPA (Zhou et al., 2006; Chen et al., 1997; Aitken et al., 1998; Lahnsteiner et al., 2005) may have an impact on the reproduction of fish via the production of ROS (Chapters 4–6). The reaction of spermatozoa to oxidative-stress causing compounds can be considered as a measure of offspring quality and fitness, therefore, sperm DNA damage assessment is currently gaining special attention

(Peknicova et al., 2002; Meeker et al., 2010; Li et al., 2010). We evaluated sperm quality characteristics (motility and velocity), changes in ATP metabolism or antioxidant response in the sperm as the consequence of exposure to three xenobiotics that cause oxidative damage and stress to cells (Chapters 4–6).

DNA fragmentation in sperm was evaluated by the comet assay (Li et al., 2008). This simple method for assessing DNA strand breaks in different mammalian spermatozoa, was reviewed by Cortés-Gutiérrez et al. (2014). In our study, the comet assay analysis indicated an increase in DNA fragmentation in sperm cells after only 2h exposure to DQ, TBBPA and BPA (Linhartova et al., 2013, Chapter 4; Linhartova et al., 2015, Chapter 5; Hulak et al., 2013, Chapter 6). Changes in DNA fragmentation were represented by an increasing amount of DNA in the comet tail, especially for the highest concentrations of the used xenobiotics, where DNA disappeared from the nuclei of sperm cell into the external environment (water). Our data indicated that the highest used BPA and TBBPA concentrations (10 $\mu g.L^{-1}$), were associated with 6% and 3.8% of DNA fragmentation for BPA, TBBPA respectively and in 8% DNA fragmentation for DQ used in a higher concentrations of 150 μM. We demonstrated that the short-term exposure of sterlet spermatozoa to different types of xenobiotics caused a dramatic increase in DNA damage expressed by two different parameters (tail DNA % and Olive tail moment) (Chapters 4, 5 and 6).

Additionally the sperm quality parameters (motility and velocity) were assessed after a 2h *in vitro* exposure of sterlet sperm to DQ, TBBPA and BPA. We observed reduced spermatozoa motility and velocity compared to the control group at 10, 30, 60, 90, and 120s post-activation, changes in flagellar movement and the C-shaped flagella (showing dead sperm cells), as well as sperm with very low velocity (mostly in 90 and 120 s post sperm activation). Spermatozoa motility was lowered significantly in all concentration ranges of DQ, TBBPA and BPA (10s and 30s post-activation) (Linhartova et al., 2013, Chapter 4; Linhartova et al., 2015, Chapter 5; Hulak et al., 2013, Chapter 6). We observed a decreasing trend in sperm motility (%) and velocity ($\mu m.s^{-1}$) with increasing concentrations of xenobiotics. The exact mechanism could be related to oxidative changes in the plamsa membranes. Li et al. (2012b) reported that plasma membrane plays an integral role in the initiation of sperm motility and its modification may significantly alter spermatozoan movement. Related to this, Thomas et al. (1998) demonstrated that non-estrogenic, as well as estrogenic organic compounds, such as TBBPA, BPA or estradiol, may bind to membrane receptors of sperm and upregulate their motility. Singh et al. (2016) found that 5 $mg.kg^{-1}$ bw of BPA significantly reduced sperm concentration in chicken; however, overall fertility and testis histology were not affected.Their results indicate that BPA adversely affects sperm characteristics in adult kadaknath chicken (*Gallus gallus domesticus*) without affecting fertilization potential (Singh et al., 2016). Our results (Chapter 6) agree with this study and with several other studies performed with different species of fish like brown trout or goldfish, demonstrating lower motility and velocity of sperm cells exposed to relative concentrations of BPA occurring in nature (Hatef et al., 2012; Lahnsteiner et al., 2005).

In addition to spermatozoan quality, we also evaluated changes in ATP content because ATP metabolism is another potential target of harmful xenobiotics. The ATP molecule is an integral component of spermatozoan movement; decreased ATP results in lower motility and velocity (Perchec et al., 1995). Many studies have pointed out that aquatic pollution might result in intracellular ATP depletion (Perchec et al., 1995; Rurangwa et al., 2002). The toxic effects of ROS on fish sperm has been reported for several species (Li et al., 2010; Dietrich et al., 2005). Not only fish, but mammalian cells as well are accumulate products from peroxidation in the mitochondria leading to a decrease in ATP production and threaten

the cell homeostasis (Chance et al., 1979). In our study, the ATP levels significantly declined after 2h of spermatozoa exposure to BPA or TBBPA starting at concentrations 2.5 $\mu g.L^{-1}$, while in case of TBBPA the lowest level of ATP (3.1 nmol ATP/10^8 spermatozoa) was found for the highest concentration (10 $\mu g.L^{-1}$). Above that significant correlation between ATP content and the level of xenobiotics (DQ, TBBPA and BPA) and protein oxidations (CP) were shown (Chapters 4–6).

Storey (1997) indicated that the major contributor to cell function damage under oxidative stress is lipid oxidation (LO), which is mostly indicated by TBARS in fish (Oakes and Van der Kraak, 2003). A significantly increased level of LO (measured by TBARS method) was observed in spermatozoa exposed to TBBPA compared to non-exposed spermatozoa (Chapter 5) (5 $\mu g.L^{-1}$ and higher) and for BPA (Chapter 6) beginning at concentration levels of 2.5 $\mu g.L^{-1}$. A significantly increasing ROS production indicated by the CP level (protein oxidation) was observed in sperm samples exposed to 10 $\mu g.L^{-1}$ TBBPA and the maximum detected level of CP was detected for BPA (level). Additionally, *in vitro* exposure of fish spermatozoa to 0–150 μM DQ for only 2h showed a significant increase in both ROS parameters, the LO and CP levels (Chapter 4). Not only ROS, but also the antioxidant response activity was assed by the evaluation of activity of SOD in fish spermatozoa exposed to DQ, TBBPA and BPA *in vitro*. SOD is considered as a primary defence against effects of harmful oxyradicals in the cells (Li et al., 2012a). In the present study a significant increment of SOD activity was observed beginning at concentrations 0.5 $\mu g.L^{-1}$ of TBBPA, 2.5 $\mu g.L^{-1}$ of BPA. SOD activity increased significantly in all DQ treatments (25–150 μM) as compared to the control spermatozoa group. We concluded that the antioxidant system is an adaptive response to toxic stress reacting on the impact of a changed ROS generation with neutralization actions. The antioxidant system apparently responds to enhanced lipid peroxidation and formation of carbonyl proteins. However, it seems that the antioxidant capacity of sterlet sperm is not sufficient to prevent cell damage. Therefore spermatozoa antioxidant response can be suggested as weak in case of protection against certain environmental xenobiotics.

CONCLUSIONS

The present work includes five publications which describe new insight into the effect of xenobiotics on cell metabolism at different levels, with a focus on oxidative stress. In addition also effects of fish nutrients in relation to xenobiotics were determined.

To summarize, the environmentally relevant concentrations of tested xenobiotics caused oxidative stress to spermatozoa, and subsequently reducing both spermatozoa movement parameters (motility + velocity), DNA integrity, and ATP content. Hence, the antioxidant system apparently responds to lipid and protein oxidation but the results also indicate that the antioxidative capacity of sperm is low; for example, exhibited by the enhancement of SOD activity in fish sperm after short-term exposure to various xenobiotics. Similarly, in the Hep G2 cells, the antioxidant response system is not sufficient to prevent cell damage caused by certain amounts of Cd^{2+}, whereas the addition of FA seems to have a protective effect against production of ROS. Our results showed a positive effect of FA (5 μM EPA and 10 M DHA) against oxidative stress in the Hep G2 cells. Therefore, we can hypothesize that the accumulation of free radicals in different cell species (like spermatozoa or liver cells) could indirectly modify energy metabolism. Moreover the antioxidant defense system, lipid peroxidation and oxidative damages are positively affected by the FA pre-incubations with the chosen ratio of FA in the determined doses in Hep G2 cells. Furthermore, it seems to be possible to load the cells with increased amounts of EPA and DHA by subsequent incubations

of lower doses of FA, without the initiation of the toxic effects caused by higher incubation doses of these FA. On the metabolic level, an increased substrate also seems to result in an increased metabolism from EPA towards DPA and possibly DHA.

Additionally, we can deduct from our data that the different pollutants caused oxidative stress and viability changes in the cells (Hep G2 and fish spermatozoa). Our results also showed that the effects were dose dependant (has been reported in Chapters 4–6), but that there are opposing effects from other substances, in our case FA (Chapters 2 and 3), showing that it the results of single substance studies should be viewed with caution.

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ENGLISH SUMMARY

Effects of xenobiotics on oxidative stress, lipid metabolism, DNA integrity and cell viability in human cells and fish spermatozoa *in vitro*

Pavla Linhartová

Pollution of the aquatic environment by inorganic and organic chemicals is a factor posing a serious threat to the survival of aquatic organisms including fish. Balancing risks and benefits of fish consumption is an intensively discussed public health topic. Spermatozoa of almost all fish species are released into water environment where they can be directly exposed to various compounds prior to fertilization; these include various xenobiotics such as toxic metals. In addition, exposure of parental adults to various xenobiotics may affect gamete quality, which can subsequently reduce fertilization success. On the other hand the advantages of eating fish are well-known, not only in the point that fish is a healthy source of protein and other nutrients, but eating fish may also confer various health benefits. Research over the past few decades has shown that the nutrients and particularly the n-3 fatty acids (FA) found in fish and seafood, are protective against cardiac diseases and have a positive impact on brain development. Sufficient fish consumption has been shown to decrease several modern life-style diseases. On the other hand, consumption of contaminated fish may cause health problems to humans and especially to pregnant women, for whom ingestion of essential n-3 FA is a necessity during fetal development and after birth of the baby.

The thesis provides a focus on two different cell model types. Firstly, human hepatocellular cells (Hep G2, ATCC) were used as *in vitro* tool for studying the effect of the intake of cadmium (Cd^{2+}) contaminated fish on cytotoxicity, oxidative stress and fatty acid and phospholipid class compositions. Uptake of contaminated fish was mimicked by incubating the cells with a combination of Cd^{2+} (0.25–20 μM) as possible contaminant and eicosapentaenoic acid (5 μM EPA) and docosahexaenioc acid (10 μM DHA) as metabolic important FA specific for fish. The main aim was to determine in which level of FA concentration the co-incubation with EPA and DHA could influence the viability and toxic effect that Cd^{2+} has on cells. Therefore, oxidative stress indices (TBARS and CP) and antioxidant response (TAC and SOD) caused by heavy metal were evaluated (Chapters 2 and 3).

Secondly, spermatozoa of a threatened species of fish, sterlet (*Acipenser ruthenus*) were used as an *in vitro* model for studying effects of potentially hazardous xenobiotic compounds' occurring in open waters. These compounds can attack a variety of biological macromolecules such as DNA, proteins and lipids, leading to oxidative damages inside of the cells. Sperm from sterlet were exposed for 2h to environmentally relevant concentrations of DQ (0–150 μM, Chapter 4), TBBPA (0–10 $\mu g.L^{-1}$, Chapter 5), and BPA (0–10 $\mu g.L^{-1}$, Chapter 6) at 4 °C. Sperm functional parameters (motility and velocity), assesment of DNA damage (comet assay), oxidative stress indices (TBARS and CP), antioxidant response (SOD activity) and ATP content were analyzed (Chapters 4–6).

In Hep G2 cells, we first established the appropriate non-toxic FA concentration. A significant increased proportion of EPA and DHA were measured in the treated cells compared to the control group, confirming the uptake. Since doses of 25 μM DHA were toxic to the cells, lower FA concentrations: 5 μM EPA in combination with 10 μM DHA (to keep ratio 1:2 of FA to simulate fish FA content) were established. We found no differences in uptake of FA in relation to the time of incubation (2h versus 48h), but we found significantly higher contents of EPA and DHA after repetitive incubations (3 times 2h) were measured. The pre-incubation

with FA (EPA + DHA) and post-incubation with heavy metal resulted in significantly increased cell viability in comparison to the group affected with Cd^{2+} only. On the metabolic level, increased concentrations of 24h EPA and DHA combined incubation resulted in an increased proportion of docosapentaenoic acid (DPA), indicating an increased metabolism. In addition, incubation with 5 μM Cd^{2+} for 24h decreased the total cardiolipin (CL) fraction from the identified phospholipids. We did not find any significant changes in ROS levels for group of cells exposed to FA (24h) and Cd^{2+} (24h), whereas increased lipid (TBARS) and protein (CP) oxidation levels were indicated in the cells exposed to only Cd^{2+} (1 and 5 μM). These results indicate that the pre-incubation with the chosen FA significantly reduced the oxidative stress caused by the Cd^{2+}. Moreover, at the antioxidant level activity no significant difference in SOD and TAC levels were observed between control and groups incubated with FA or with 1 μM Cd^{2+}. However, the antioxidant response was significantly enhanced in cells exposed to 5 μM Cd^{2+} (SOD: 20.68 mU/10^6 cells and TAC: 239.35 nmole/10^6 cells).

The measured spermatozoa velocity ($\mu m.s^{-1}$) and percentage of motile spermatozoa (%) were significantly decreased for each xenobiotic at each time post-activation in comparison with control. The level of DNA damage expressed as % DNA in Tail and Olive Tail moment increased when spermatozoa were exposed to a concentration of 2.5 $\mu g.L^{-1}$ of TBBPA and BPA and above. The 8% DNA fragmentation for DQ was measured for the highest concentration (150 μM). The level of oxidative stress indices such as lipid oxidation (LO, measured by TBARS method), carbonyl derivatives of proteins (CP) and antioxidant activity (SOD) increased significantly with increased concentrations of DQ, TBBPA and BPA. Additionally significant differences ($df = 4$, $p < 0.05$) in intracellular ATP levels were observed with exposures at 100 and 150 μM DQ, with the lowest ATP content at 45 nmol ATP/10^8 spermatozoa. In case of TBBPA and BPA a significant decrease of ATP appeared in at concentrations 2.5 $\mu g.L^{-1}$ of and above.

The observations in the present thesis gave us new insights into the effects of fish nutrients and xenobiotics on cell metabolism at different levels with a focus on changes in lipid classes and oxidative stress in human hepatoma cell line and fish spermatozoa *in vitro*.

CZECH SUMMARY

Účinky xenobiotik na oxidační stres, metabolizmus lipidů, integritu DNA a životaschopnost lidských buněk a rybích spermií *in vitro*

Pavla Linhartová

Znečišťování vodního prostředí anorganickými a organickými chemikáliemi je jedním z faktorů představujících vážnou hrozbu pro přežití vodních organizmů včetně ryb a jejich konzumaci. Proto je hodnocení rizik a přínosů týkajících se konzumace ryb intenzivně diskutované téma veřejného zdraví. Spermie většiny hospodářsky významných druhů ryb jsou před oplodněním uvolňovány do vodního prostředí, kde mohou být přímo vystaveny různým sloučeninám, jako jsou například cizorodé látky zahrnující též toxické kovy. Kromě toho i samotná expozice dospělých jedinců různým škodlivinám může ovlivnit kvalitu rybích gamet, jejímž následkem bude snížená oplozovací schopnost a oplodnění samotné. Výhody konzumace ryb jsou dobře známé, a to nejen kvůli bohatému zdroji bílkovin ale i dalších látek s příznivým dopadem na lidské zdraví. Věda a výzkum v posledních několika desetiletích ukázaly, že živiny, zejména n-3 mastné kyseliny (MK), vyskytující se u ryb a mořských plodů, jsou prospěšné pro funkci srdce a nezbytné pro vývoj mozku. Spotřeba ryb je také úzce spojena se snižováním rizika vzniku různorodých chorob plynoucích z našeho moderního životního stylu. Z opačného hlediska by ale konzumace ryb žijících ve znečištěném prostředí mohla způsobit zdravotní problémy lidem, např. těhotným ženám, konzumujících ryby kvůli příjmu esenciálních n-3 MK nezbytných pro vývoje plodu a v období krátce po porodu dítěte.

Celá práce byla založena na hodnocení dvou rozdílných typů modelových buněk. Lidské jaterní buňky (Hep G2, ATCC) byly použity jako nástroj pro studium cytotoxicity kadmia (Cd^{2+}) *in vitro* formou simulace požití kontaminované ryby a následných změn ve složení mastných kyselin a fosfolipidových tříd. Příjem kontaminovaných ryb byl napodoben inkubací buněk v kombinaci s Cd^{2+} (0,25–20 μM), jako možné škodliviny, a kyselinou eikosapentaenovou (5 μM EPA) a dokosahexaenovou (10 μM DHA) představujících u ryb specifické, metabolicky důležité MK. Hlavním cílem bylo zjistit, v jaké výši by samotná zvolená koncentrace obou MK (EPA a DHA) a kadmia byla toxická nebo mohla ovlivnit životaschopnost buněk. Z tohoto důvodu byl také měřen oxidační stres a antioxidační reakce způsobené těžkým kovem (kapitoly 2 a 3). Druhým typem modelových buněk pro hodnocení účinků potenciálně nebezpečných xenobiotik vyskytujících se ve volných vodách byly spermie jednoho ohroženého druhu ryby, jesetera malého (*Acipenser ruthenus*) *in vitro*. Xenobiotika mají schopnost napadat řadu biologických makromolekul, jako jsou DNA, proteiny a lipidy, a vedou k oxidačnímu poškození uvnitř buněk. Spermie jesetera malého byly vystaveny po dobu 2 h při 4 °C koncentracím xenobiotik vyskytujících se v životním prostředí. Jmenovitě u DQ (0–150 μM, kapitola 4), TBBPA (0–10 $\mu g.L^{-1}$, kapitola 5) a BPA (0–10 $\mu g.L^{-1}$, kapitola 6) byly analyzovány funkční parametry spermií (pohyblivost a rychlost), hodnocena míra poškození jejich DNA (comet test), indexy oxidačního stresu (TBARS a CP), antioxidační odezvy (SOD aktivita) a v neposlední řadě i úbytek ATP (kapitoly 4–6).

U Hep G2 buněk byla nejprve hledána vhodná netoxická koncentrace MK. U ošetřených buněk byl naměřen jak příjem MK, tak i významně zvýšený podíl EPA a DHA v porovnání s kontrolní skupinou. Vzhledem k tomu, že dávky 25 μM DHA se ukázaly být pro buňky toxické, byly stanoveny nižší koncentrace MK, a to 5 μM EPA v kombinaci s 10 μM DHA (kde byl udržen poměr 1 : 2 MK, simulující obsah MK v rybím mase). Nenašli jsme odlišnost v hladinách MK u buněk při odlišných délkách inkubace v médiu (2 h versus 48 h), ale byla naměřena významně vyšší hladina EPA a DHA po opakované inkubaci (3 x 2 h). Pre-inkubace

MK (EPA + DHA) a jejich následná post-inkubace těžkým kovem způsobila významně vyšší životaschopnosti buněk v porovnání se skupinou postiženou pouze Cd^{2+}. Na metabolické úrovni vedla zvýšená koncentrace EPA a DHA po 24 h působení na buňky ke zvýšení podílu kyseliny dokosapentaenové (DPA), což naznačilo jejich zvýšený metabolizmus. Proto byly ověřeny i fosfolipidové třídy, kde se v důsledku přítomnosti 5 μM Cd^{2+} významně snížil cardiolipin (CL). Na hladině reaktivních částic kyslíku (ROS) nebyly evidovány významné změny mezi skupinami buněk vystavených MK (24 h) a Cd^{2+} (24 h), i když zvýšená lipidová (TBARS) a proteinová (CP) oxidace byla zaznamenána u skupin buněk vystavených pouze dávkám Cd^{2+} (1 a 5 μM). Tyto výsledky naznačují, že pre-inkubace námi zvolenými MK významně snížila oxidační stres způsobený účinkem Cd^{2+}. Navíc na antioxidační úrovni nebyl pozorován žádný významný rozdíl v množství SOD a TAC mezi kontrolní skupinou a skupinami buněk nainkubovaných s MK nebo 1 μM Cd^{2+}. Nicméně antioxidační odpověď byla významně vyšší u buněk vystavených působení 5 μM Cd^{2+} (SOD: 20,68 mU/106 buněk a TAC: 239,35 nmol/106 buněk).

U rybích spermií byly účinkem každého zvoleného xenobiotika významně sníženy rychlost spermií ($\mu m.s^{-1}$) a procento pohyblivosti spermií (%) po jeho aktivaci v porovnání s kontrolou. Míra poškození DNA byla průkazně vyšší, pokud byly spermie vystaveny koncentracím 2,5 $\mu g.L^{-1}$ TBBPA nebo BPA a vyššími. Osmiprocentní fragmentace DNA byla naměřena u nejvyšší koncentrace DQ (150 μM). Indexy oxidačního stresu, jako je oxidace lipidů (LO, měřena metodou TBARS), karbonylové deriváty proteinů (CP) a antioxidační aktivita (SOD), byly podstatně zvýšeny se stoupající koncentrací DQ, TBBPA a BPA. Významné rozdíly (df = 4, $p < 0,05$) v množství vnitrobuněčného ATP byly pozorovány po expozici spermatu 100 a 150 μM DQ, kdy byla zjištěna nejnižším množství ATP, a to pouze 45 nmol ATP/10^8 spermií. V případě TBBPA a BPA byl významný pokles ATP zjištěn při koncentracích 2,5 $\mu g.L^{-1}$ a vyšších.

Zjištěné výsledky při řešení této práce nám poskytly nový pohled na vliv některých živin a xenobiotik na různých úrovních buněčného metabolizmu, a to zejména se zaměřením na změny lipidových tříd a oxidačního stresu u dvou výše zmíněných typů buněk *in vitro*.

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Picture Nr.2 (Hep G2 cells):
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Picture Nr.3 (capsule of omega-3 FA):
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http://images-of-elements.com/cadmium-4.jpg

LIST OF PUBLICATIONS

PEER-REVIEWED JOURNALS WITH IF

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ABSTRACTS AND CONFERENCE PROCEEDINGS

Linhartova, P., Sampels, S., 2015. Effects of combined EPA, DHA and Cd^{2+} on Fatty acids and phospholipid classes in human hep G_2 cells. 13th Euro Fed Lipid Congress, Florence, Italy, September 27–30, 2015 (lecture).

Linhartova, P., Sampels, S., 2014. Effects of Cd^{2+} and fish lipids on human cell viability *in vitro*, 4th Lipidomic meeting, Graz, Austria, September 21–24, 2014 (poster).

Linhartova, P., Gazo, I., Shaliutina, A., Hulak, M., Kaspar, V., 2013. Effects of tetrabrombisphenol A on DNA integrity, oxidative stress and quality of sterlet (*Acipenser ruthenus*) spermatozoa. In the IVth International Workshop on the Biology of Fish Gametes 2013, Portugal, September 15–19, 2013 (lecture).

Linhartova, P., Gazo, I., Shaliutina, A., Hulak, M., 2012. The *in vitro* effect of duroquinone on functional competence, genomic integrity, and oxidative stress indices of sterlet. In The International Conference and Exposition AQUA 2012, Prague, September 1–5, 2012 (poster).

TRAINING AND SUPERVISION PLAN DURING STUDY

Name:	Pavla Linhartová
Research department:	Laboratory of Lipid Metabolism, FFPW, IAWP
Supervisor:	Assoc. Prof. Sabine Samples
Period:	30th September 2013 until September 2016

Ph.D. courses	**Year**
Fish nutrition	2014
Basics of scientific communication	2014
Fish as food	2015
English language (FCE certificate)	2015
Biostatistics	2015
Scientific seminars	**Year**
Seminar days of Ph.D. students in FFPW	2014 2015 2016
International conferences	**Year**
Linhartova, P., Gazo, I., Shaliutina, A., Hulak, M., 2012. The *in vitro* effect of duroquinone on functional competence, genomic integrity, and oxidative stress indices of sterlet. In The International Conference and Exposition AQUA 2012, Prague, September 1–5, 2012 (poster).	2012
Linhartova, P., Gazo, I., Shaliutina, A., Hulak, M., Kaspar, V., 2013. Effects of tetrabrombisphenol A on DNA integrity, oxidative stress and quality of sterlet (*Acipenser ruthenus*) spermatozoa. In the IVth International Workshop on the Biology of Fish Gametes 2013, Portugal, September 15–19, 2013 (lecture).	2013
Lihartova, P., Sampels S., 2014. Effects of Cd^{2+} and fish lipids on human cell viability *in vitro*, 4th Lipidomic meeting, Graz, Austria, September 21–24, 2014 (poster).	2014
Linhartova, P., Sampels S., 2015. Effects of combined EPA, DHA and Cd^{2+} on Fatty acids and phospholipid classes in human hep G_2 cells. 13th Euro Fed Lipid Congress, Florence, Italy, September 27–30, 2015 (lecture).	2015
Foreign stays during Ph.D. study	**Year**
University of Münster, Germany, Laboratory for food and chemistry, under supervision of Prof. Dr.Tanja Schwerdtle and Dr. Franziska Ebert, 4 months stay (September–December).	2013
University of Potsdam, Germany, Institut für Ernährungswissenschaft, under supervision of Prof. Dr.Tanja Schwerdtle and Dr. Franziska Ebert, 4 months stay (March-June 2014).	2014
Teaching	**Year**
Professional seminar – How to write bachelor thesis (3rd Bachelor students), guarantor of subject: Jan Mráz, Ph.D., 2014–2015.	2014 2015 and 2016
Quality of fish products (2nd Master students) guarantor: Jan Mráz, Ph.D., 2014–2015.	2014 and 2015

Summer school students supervision		**Year**
Laura Mims (USA)	Combined incubation of Cadmium, docosahexaenoic and eicosaentaenoic acid affecting the oxidative stress, antioxidant response and lipid metabolism in human Hepatocytes *in vitro*.	2015
Gakuto Oishi (Japan)	Oxidative stress related to omega-3 fatty acids found in fish: An *in vitro* study on human Hep G2 cells.	2015

CURRICULUM VITAE

PERSONAL INFORMATION

Name: Pavla
Surname: Linhartová
Title: M.Sc.
Born: October 15th 1985, Strakonice, Czech Republic
Nationality: Czech
Language: Czech (mother tongue), English (B2 level – FCE certificate), French (active speaker), German (active speaker)

EDUCATION

September 2013 – present	Ph.D. student in Fishery, Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Czech Republic
2011–2013	M.Sc. student at University of South Bohemia, Faculty of Fisheries and Protection of Waters, České Budějovice, Czech Republic
2007–2011	B.Sc. student at VŠCHT (University of Chemistry and Technology) in Prague, Faculty of Biochemistry and Microbiology, Czech Republic
1998–2006	Eight-year general secondary school (grammar school), Vodňany.

RESEARCH STAY AND COLLABORATIONS

September–December 2013	University of Münster, Germany, Laboratory for Food and Chemistry, under supervision of Prof. Dr.Tanja Schwerdtle and Dr. Franziska Ebert.
March–June 2014	University of Potsdam, Germany, Institut für Ernährungswissenschaft, under supervision of Prof. Dr. Tanja Schwerdtle and Dr. Franziska Ebert.

PROFESSIONAL EXPERIENCE

Date from–to	Place	Institution	Post	Characterisation of activity
From 2013	České Budějovice, Czech Republic	FFPW USB	Ph.D.	Work in Laboratory of Fish Nutrition and Lipid Metabolism
2011–2013	Vodňany, Czech Republic	FFPW USB	student	Work in Laboratory of Molecular, Cellular and Quantitative Genetics
2007–2013	Prague, Czech Republic	VŠCHT	student	Work in Laboratory of Biochemistry and Microbiology

PART OF PROJECT

2013–2016	Grant Agency of Czech Republic (GACR No. 13-01543S) and the Grant Agency of the University of South Bohemia in Ceske Budejovice (GAJU No. 134/2013/Z and GAJU No. 060/2016/Z).

COMPLETED COURSES

2015	LGC Cell Biology Seminar Czech Republic, Prague