

Combined Incubation of Cadmium, Docosahexaenoic and Eicosapentaenoic Acid Affecting the Oxidative Stress and Antioxidant Response in Human Hepatocytes In Vitro

P. LINHARTOVA^{1,2}, I. GAZO^{1,3}, S. SAMPELS^{1,2}

¹South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, České Budějovice, ²Institute of Aquaculture and Protection of Waters, České Budějovice, ³Research Institute of Fish Culture and Hydrobiology, Vodňany, Czech Republic

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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 docosahexaenoic 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

Corresponding author

P. Linhartova, University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Institute of Aquaculture and Protection of Waters, Husova tř. 458/102, 370 05 České Budějovice, Czech Republic. E-mail: linhap01@frov.jcu.cz

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 bioaccumulated 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 $\mu\text{g}/\text{l}$.

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-1 β , 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 eicosapentaenoic 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.

Methods

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.

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).

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 % CO₂ 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 Cd²⁺.

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 50 µl and 20 µl 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 0.1 M 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.

Cd²⁺ post-incubations

Fresh stock solutions of Cd²⁺ diluted in distilled sterile water (ddH₂O) were prepared before the transfer experiments. Hep G2 cells were post-incubated for 24 h with Cd²⁺ stock solution with changing the cell culture medium (MEM). The range of Cd²⁺ concentrations were used from min. 1 µM to max. 5 µM.

Confirmation of uptake of cadmium and FA into the cells

The content of cadmium was earlier analyzed 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 Sampels (2015).

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 Cd²⁺ 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.

Oxidative stress and antioxidant response from pelleted cells

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 0.5 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF).

Thiobarbituric acid reactive substances (TBARS)

For evaluation of TBARS (Lushchak *et al* 2005, Lushchak 2011) each pellet (with FA/without FA and with/without Cd²⁺, 1 and 5 µM Cd²⁺) 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 H₃PO₄ 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 (Invitro 200Pro, Tecan Group Ltd., Mannedorf, Switzerland).

Carbonyl proteins (CP)

CP as markers for protein oxidation were analyzed according to a method by (Lenz *et al.* 1989). 10 % TCA in 0.2 M H₃PO₄ 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 2 M 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 6 M 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 (Infinite 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:

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

Superoxide dismutase (SOD)

Inhibition activity of 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:

$$\text{Inhibition rate\%} = [(\text{average blank 1} - \text{average blank 3}) - (\text{average sample} - \text{average blank 2})] / (\text{average blank 1} - \text{average blank 3}).$$

Total antioxidant capacity (TAC)

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 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 standard (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 (Infinite 200Pro,

Tecan Group Ltd., Mannedorf, Switzerland) and the Trolox standard index was counted.

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 \pm SD (n=3). All analyses were performed at a significance level of $p < 0.05$ using STATISTICA 9.0 for Windows.

Results

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 \pm 75 and 138.45 \pm 8.27 μM $\text{Cd}^{2+}/10^6$ 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 \pm 88.03 and 365.84 \pm 76.93 $\mu\text{M}/10^6$ cells, 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).

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 $\mu\text{M}/\mu\text{g}$) and 5 μM Cd^{2+} (4.25 $\mu\text{M}/\mu\text{g}$). However, cells exposed to BSA reached the level of CP to 2.12 $\mu\text{M}/\mu\text{g}$, which was an increase compare to control and FA groups.

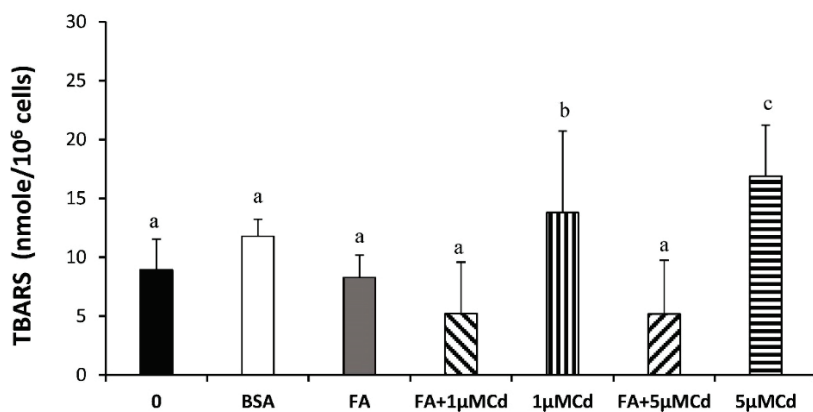


Fig. 1. Effects of BSA, FA and Cd²⁺ on TBARS in Hep G2 cells. Data represent mean values \pm SD, n=3. Different letters indicate significant differences among samples (two factorial, ANOVA, FA and Cd²⁺, $p < 0.05$).

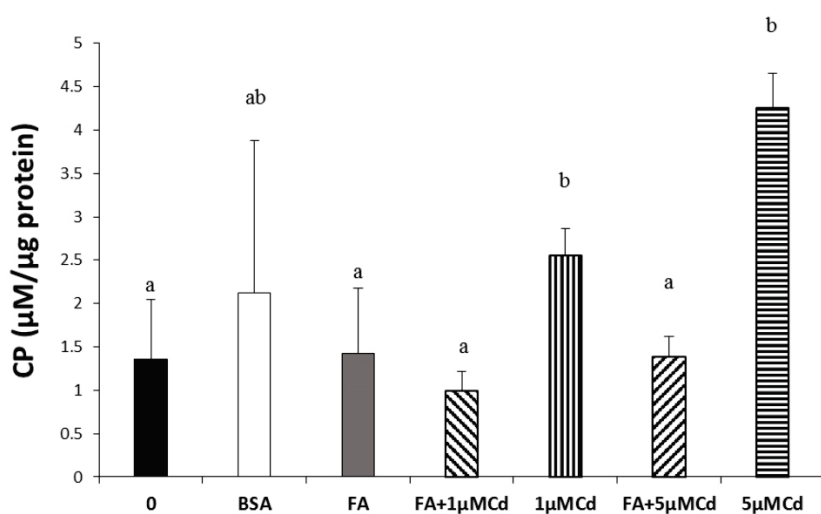


Fig. 2. Effects of BSA, FA and Cd²⁺ on CP in Hep G2 cells. Data are presented as means \pm SD, n=3. Different letters indicate significant differences among samples (two factorial, ANOVA, FA and Cd²⁺, $p < 0.05$).

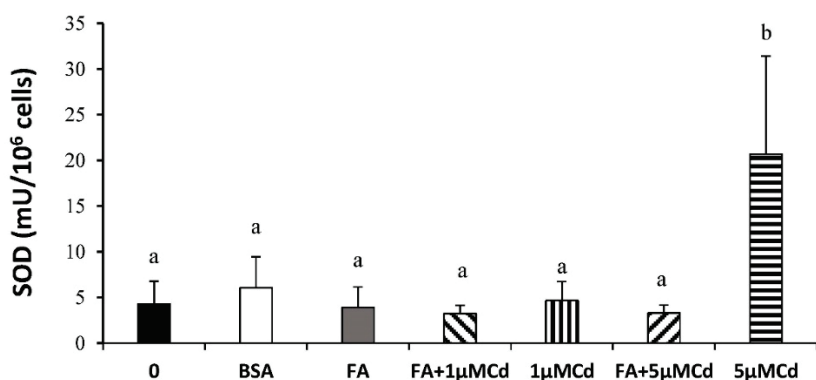


Fig. 3. Effects of BSA, FA and Cd²⁺ on SOD activity in Hep G2 cells. Data represent mean values \pm SD, n=3. Different letters indicate significant differences among samples (two factorial, ANOVA, FA and Cd²⁺, $p < 0.05$).

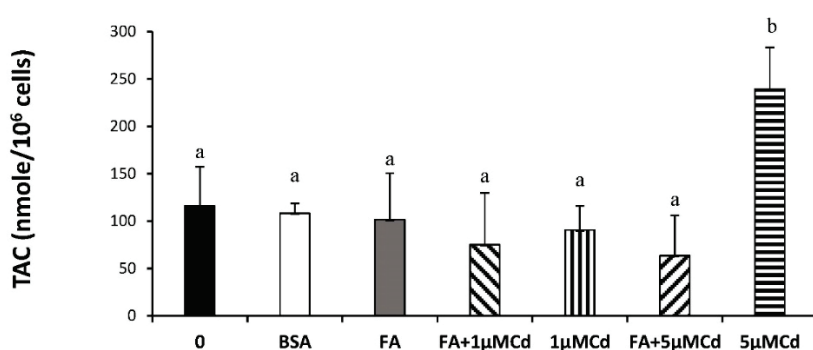


Fig. 4. Effects of BSA, FA and Cd²⁺ on TAC activity in Hep G2 cells. Data are presented as means \pm SD, n=3. Different letters indicate significant differences among samples (two factorial, ANOVA, FA and Cd²⁺, $p < 0.05$).

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+} .

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 (between 10-50 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 antioxidant 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.

Conclusions

Our results showed a positive effect of FA

(5 μ M EPA and 10 μ M 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 Cd²⁺ in concentrations from 1 μ M in hepatocytes. Further research is needed to investigate the mechanism and the relationship between oxidative stress and FA uptake by liver cells.

Conflict of Interest

There is no conflict of interest.

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Abbreviations

BSA: bovine serum albumin; BSA-FA: bovine serum albumin complex with fatty acids; Cd²⁺: cadmium chloride; DHA: docosahexaenoic 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

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