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Cytotoxicity evaluation and antioxidant enzyme expression related to heavy metals found in tuna by-products meal: An in vitro study in human and rat liver cell lines

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ABSTRACT

Heavy metals can accumulate in organisms via various pathways, including respiration, adsorption and ingestion. They are known to generate free radicals and induce oxidative and/or nitrosative stress with depletion of anti-oxidants. Tuna by-product meal (TBM) is rich in proteins and can, therefore, offer an attractive protein source for animals. This study was undertaken to assess the effects of metals present in TBM, namely cadmium (Cd), lead (Pb), and mercury (Hg), separately or in combination with oxidative stress, on cell viability. Three cell models: rat liver FTO2B, human hepatoma HepG2, and human hepatic WRL-68, were used. Cell viability was determined following exposure to various concentrations of the metals. Two antioxidant genes, catalase (CAT) and superoxide dismutase (SOD), were measured to obtain a better understanding of oxidative stress-associated gene expression. Among the metals present in TBM, only Cd at a concentration of 30 μM was noted to exhibit cytotoxic effects. This cytotoxicity was even more pronounced after co-stimulation with H_2O_2, used to mimic systemic oxidative stress. At non-toxic concentrations, Hg and Pb were noted to aggravate oxidative stress toxicity. The results further revealed that exposure to Cd, Pb, and a co-stimulation of H_2O_2 with Hg resulted in the increased expression of antioxidant gene SOD. A risk assessment of toxic contaminants in TBM indicated that food safety objectives should consider the human health impacts of foods derived from animals fed on contaminated meal and that much care should be taken when TBM is used in animal diet.

1. Introduction

Industrialization has resulted in increased concentrations of persistent toxic substances with adverse effects on human health, natural ecosystems, and the environment. Fisheries and fish processing industries worldwide generate large amounts of fish by-products that are often discarded indiscriminately into the environment. In Tunisia, the tuna-processing industry alone generates huge quantities of tuna by-products. Tuna by-product meal (TBM) is rich in crude protein (35–45%) and lipid (4–7%) (M.S. Azaza and A. Abdelmouleh, unpublished data; Saïdi et al., 2010) and can, therefore, offer a promising source for supplementary protein. When used for these purposes TBM should be handled with special care particularly because it comes from an aquatic environment wherein several chemical pollutants are also omnipresent. These pollutants include a number of heavy metals (lead, cadmium, mercury, zinc, and copper) that have deleterious cytotoxic effects on the biota that exist there and, consequently, on human health (Tan et al., 2008). Moreover, predator fish, such as Tuna, can have high concentrations of these metals in their tissues due a bioaccumulation process from their diet along the aquatic food chain (Besada et al., 2006).

In a previous study we have demonstrated in an in vivo study that the use of TBM could be considered as a strong candidate to substitute expensive protein sources (fish meal) for Nile tilapia (Saïdi et al., 2010). However, some indications were found that, due to co-existing chemical pollutants, a number of TBM constituents

Abbreviations: TBM, tuna by-product meal; FM, fish meal; Cd, cadmium; Pb, lead; Hg, mercury; Cu, copper; Mn, manganese; FTO2B, rat liver cell line; HepG2, human liver cell line; WRL-68, human embryonic liver cell line; H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; CAT, catalase; GAPDH, glyceraldehydes-3 phosphate dehydrogenase; ROS, reactive oxygen species; GPx, glutathione peroxidase; OD, optical density; mRNA, messenger RNA.

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are likely to pose toxic effects on animal and human health. At present, fish-consumption advisories related to human health protection do not consider fish by-products fed to farmed animals (Dorea, 2006). Animals (especially farmed fish) that are fed tuna by-products can, however, bioaccumulate heavy metals, which, in turn, can pass through animal-derived foods into components of human diet. Accordingly, the evaluation of TBM composition, particularly in terms of heavy metal contents, is extremely important for the safety of animal and human health.

Several studies have been carried out in the past few decades to investigate the mechanisms by which heavy metals induce toxic effects. Various mechanisms of action, including oxidative stress, interference with essential metals and enzymes, and interactions with cellular macromolecules, have been described (Ercal et al., 2000; Kitchin, 2001; Snow, 1992; Valko et al., 2005). The formation of large amounts of reactive oxygen species (ROS), such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}HO$), and singlet oxygen ($^1O_2$), has been reported to promote the induction of oxidative stress (Stohs et al., 2000). Hydrogen peroxide is also produced by a wide variety of enzymes, including oxidases. Reactive oxygen species play important roles in cell signaling and are used by the immune system to attack and kill pathogens (Segal, 2005). In humans, oxidative stress is involved in the development and subsequent aggravation of several diseases, including cancer (Halliwell, 2007), Parkinson's disease (Jenner, 2003), Alzheimer's disease (Valko et al., 2007), atherosclerosis (Singh and Jialal, 2006), heart failure (Singh et al., 1995), and myocardial infarction (Ramond et al., 2011).

The liver plays a central role in the metabolism of biological substances as well as in detoxification. Most of the substances absorbed by the intestine pass first through the liver where toxins and heavy metals may accumulate. The liver is, therefore, an important organ to consider when investigating the effects of pollution. Moreover, the antioxidant defense system is known to consist of enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) (Livingstone et al., 1990) that play key roles in maintaining cellular homeostasis by removing ROS (Rudneva, 1999). The induction of these enzymatic antioxidant genes as possible biomarkers for oxidative stress has attracted increasing attention in recent research.

In brief, the evaluation of the effects of heavy metals present in TBM on animal and human liver cell lines might contribute to the understanding of the toxic potential of TBM and can help to determine the levels at which TBM can be used with no harm to animal and human health. Accordingly, the present study was undertaken to investigate the effects of heavy metals from TBM (cadmium, lead, and mercury) in terms of oxidative stress, gene expression and induction of cell death using three in vitro cell models, namely rat liver FTO2B, human liver HepG2, and embryonic human liver WRL-68 cells. To evaluate the potential aggravation of the toxicity of these metals by oxidative stress, the cytotoxic effects involved in the co-treatment of liver cells by hydrogen peroxide and metals were analyzed as a model for the systemic production of ROS-molecules by the organism. The mRNA expression level of two important antioxidant enzymes (CAT, SOD) was also determined in an FTO2B cell model.

2. Materials and methods

2.1. Heavy metals present in tuna by-product meal

Three metals were investigated in the cytotoxicity assays namely cadmium (Cd), mercury (Hg), and lead (Pb). These metals were selected because they were also present in the TBM previously used in the diets of juvenile Nile tilapia (*Oreochromis niloticus*). Tuna by-product meal was provided by a local private tuna processing and manufacturing factory (Medi thon, the Sultan, Sfax, Tunisia). In brief, edible parts of tuna muscles were cut and shaped into meat blocks for human consumption. Unused parts were collected as by-products and stored at −20 °C until further processing (i.e., drying in an oven and powdering).

The total mercury (Hg) concentration in TBM was determined using a slightly modified version of the method described by Shinyashiki et al. (1998). The samples were heated with 7 ml of nitric acid and perchloric acid (2 v/1 v) in a hot plate (Gerhardt, Germany) until clear solutions were obtained. After digestion, mercury concentrations were measured by the reducing vaporization-spectrometry atomic absorption method, using Nippon Instruments Mercury Analyzer, Model RA-2 (Tokyo, Japan). The residual concentrations of lead, copper, cadmium, and manganese were determined by atomic absorption spectrometry (Zeenit 700 spectrophotometer, AnalytikJena).

2.2. Cell culture

2.2.1. Cell culture medium

The liver plays a central role in detoxification, most toxins and heavy metals accumulate here and therefore the liver is the prime organ to investigate the effects of pollution. Human cells were used for cytotoxicity evaluation since toxic substances (metals) passing from animal food to animals may end up in the human body. Accordingly, human liver HepG2 cell line (HB-8065, ATCC, Rockville, MD) was cultured in William's Medium E (WEM, InVitrogen) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 20 mU/ml insulin, 50 nM dexamethasone, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, 50 μg/ml gentamycin, and 100 μg/ml vancomycin. Rat liver cell line FTO2B (ECACC nr 89121403) was cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin, streptomycin, and fungizone. Human embryonic liver cell line WRL-68 (CL-48, ATCC) was cultured in MEM (Minimal Essential Medium) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/L penicillin, 100 μg/ml streptomycin, and fungizone.

2.2.2. Cytotoxicity assays

Stock solutions of Cd $(CH_3COO)_2$ (15 mM), Hg_2 $(NO)_3$ (250 μM), and $Pb(NO_3)_2$ (10 mM) were prepared in sterile H_2O. They were prepared with reference to the concentration found in tuna waste but approximately 500 times concentrated (Table 2). The cells were grown in 75 cm^2 tissue culture flasks and, when the cultures were nearly confluent, were trypsinized and counted. They were then plated in 24-well plates (100,000 cells/well in 1 ml medium). Twenty-four hours after cell seeding, the medium was removed and replaced by a fresh medium containing different concentrations of metals and H_2O_2 (9.79 M stock solution). After an additional 72 h, the medium was removed and substituted by a fresh medium containing XTT, and the cell number was determined.

For the cytotoxicity assays, five serial concentrations of the stock solution were used for Pb (500, 200, 40, 20*, and 10 μM), Hg (12.5, 5, 1, 0.5*, and 0.25 μM), and Cd (30*, 6, 1.5, 0.3, and 0.06 μM) (* concentration used for co-treatments with H_2O_2). The actual in vivo concentration of these metals was a function of the concentration found in TBM, and not in the concentration previously used in the diet of *O. niloticus*, which was particularly because it could serve as an appropriate terrestrial biomonitor of metal contamination for different animals fed on contaminated tuna by-product meal.

2.2.3. XTT assay

The cells were cultured for 72 h in the presence of heavy metals after which the medium was replaced by 500 μl of an XTT