Marine Pollution Bulletin 108 (2016) 70–76

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Absence of selenium protection against methylmercury toxicity in harbour seal leucocytes *in vitro*

Krishna Das [a,*], Aurélie Dupont [a], Marie- Claire De Pauw-Gillet [b], Cathy Debier [c], Ursula Siebert [d]

[a] Laboratory of Oceanology, MARE Center, University of Liège, B6c, Allée du 6 Août 15, 4000 Liège, Belgium
[b] Laboratory of Mammalian Cell Culture (GIGA-R), University of Liège, B6c, Allée du 6 Août 15, 4000 Liège, Belgium
[c] Institute of Life Sciences, Université catholique de Louvain, Croix du Sud 2/L7.05.08, B-1348 Louvain-la-Neuve, Belgium
[d] Institute for Terrestrial and Aquatic Wildlife Research, University of Veterinary Medicine Hannover, Foundation, Werftstrasse 6, 25761 Buesum, Germany

ARTICLE INFO

Article history:
Received 15 September 2015
Received in revised form 23 April 2016
Accepted 29 April 2016
Available online 16 May 2016

Keywords:
Marine mammals
Harbour seal
Phoca vitulina
Methylmercury
Selenium
Lymphocytes
In vitro approach

ABSTRACT

Previous studies described high concentrations of mercury (Hg) and selenium (Se) in the blood of harbour seals, *Phoca vitulina* from the North Sea. In the present study, we evaluated the *in vitro* potential protective effects of sodium selenite (Na_2SeO_3) and selenomethionine (SeMet) on cell proliferation of harbour seal lymphocytes exposed to MeHgCl 0.75 μM. *In vitro* exposure of ConA-stimulated T lymphocytes resulted in severe inhibition of DNA synthesis, likely linked to severe loss of mitochondrial membrane potential at 0.75 μM. Neither selenite nor SeMet showed a protective effect against MeHg toxicity expressed at the T lymphocyte proliferation level for harbour seals. Selenite and SeMet did not show negative effects regarding lymphocyte proliferation and mitochondrial membrane potential.

To conclude, our results clearly demonstrated that MeHg affected *in vitro* immune cells exposure with no protective effects of selenium at a molar ratio Hg:Se of 1:10 in harbour seals from the North Sea.

1. Introduction

High concentrations of mercury (Hg) have been documented previously in the tissues of marine mammals from the North Sea (Bennet et al., 2001; Das et al., 2004; Siebert et al., 1999), with severe immunotoxic effects *in vitro* (Das et al., 2008; Dupont et al., 2016; Kakuschke et al., 2009). Mercury is mainly absorbed under its organic form methylmercury (MeHg), predominant in fish (Baeyens et al., 2003). In mammals, more than 90% of ingested MeHg can be absorbed into blood (Basu and Head, 2010; Scheuhammer et al., 2012). MeHg distribution is systemic, reaching all vital organs and accumulating in several tissues and cell types including muscle, hair and erythrocytes (Clarckson et al., 2007; Habran et al., 2011). The high mobility of MeHg in the body is due to carrier-mediated transport *via* the formation of small molecular weight thiol complexes that are readily transported across cell membranes (Clarckson et al., 2007; Rooney, 2007). Exposure to Hg has been linked to *in vitro* and *in vivo* toxicological effects in wildlife including impairment of specific immune defences in many species including marine mammals (Cámara Pellissó et al., 2008; Das et al., 2008; De Guise et al., 1996; Dufresne et al., 2010; Frouin et al., 2012, 2010; Lalancette et al., 2003; Pillet et al., 2000; Schaefer et al., 2011; Wolfe et al., 1998). Ultimately, MeHg initiates multiple additive or synergistic disruptive mechanisms that lead to cellular dysfunction and cell death (Dupont et al., 2016; Yin et al., 2007). At the intracellular level, addition of Hg^{2+} to mitochondria of rat kidney induced efflux of intramitochondrial Ca^{2+}, accompanied by a decrease of the intracellular nucleotides NAD(P)H/NAD(P) ratio (nicotinamide adenine dinucleotide phosphate) and a decrease of the internal negative membrane potential (Chávez and Holguín, 1988).

Toxicity of Hg can to be partly mitigated by the presence of Se (Cuvin-Aralar and Furness, 1991; Thompson, 1990). The likely protective effect of Se in marine vertebrates are complex and not fully understood at the cellular level, and is species- and cell-dependent (Anan et al., 2010; Cuvin-Aralar and Furness, 1991; Frisk, 2001; Frouin et al., 2012; Ikemoto et al., 2004; Watanabe, 2002; Yoneda and Suzuki, 1997). Possible mechanisms to explain antagonistic effects of Se towards Hg toxicity include *e.g.* i) a diminution of the oxidative stress caused by MeHg, ii) the formation of a complex $(CH_3Hg)_2Se$ and iii) the decomposition and demethylation of MeHg to form inorganic and inert HgSe (tiemannite) generally in the liver of marine vertebrates (Das et al., 2004; Frodello et al., 2000; Koeman et al., 1973; Lailson-Brito et al., 2012; Martoja and Berry, 1980; Nigro and Leonzio, 1996; Nigro, 1994; Rawson et al., 1995; Yang et al., 2008). However, the formation of tiemannite in liver is not the only way Se protects towards Hg toxicity. Se influences the distribution, kinetics and toxic effects of Hg before liver deposition (Cuvin-Aralar and Furness, 1991; Frouin et al., 2012). While first noted for its toxicity and carcinogenicity,

* Corresponding author.
E-mail address: krishna.das@ulg.ac.be (K. Das).

http://dx.doi.org/10.1016/j.marpolbul.2016.04.060
0025-326X/

Se is an essential element for animal nutrition and health (Chapman et al., 2010; Rayman, 2012, 2000; Zwolak and Zaporowska, 2012). Its biological activities are strongly dependent on its chemical form (species, oxidation state) and concentration (Ip et al., 1991; Shibata et al., 1992). The great variety of chemical forms of Se in food and biological systems includes inorganic species such as selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}), and organic species including the aminoacids selenocysteine and selenomethionine (Pedrero and Madrid, 2009). As such, Se is a constituent of numerous proteins, including the selenoenzymes thioredoxin reductases (TrxR) and glutathione peroxidase (GPx). The glutathione peroxidase system quenches free radicals and reactive intermediates (reactive oxygen species) and, in turn, affects demethylation of Hg (Branco et al., 2012).

Se concentrations are generally higher in fish-eating marine mammals than in terrestrial mammals likely due to a greater intake linked to the marine fish diet (Correa et al., 2014; Dietz et al., 2000). Se in fish occurs under both inorganic and organic forms, including selenomethionine (SeMeth) and selenite (Chapman et al., 2010). Once absorbed through the diet, Hg and Se compounds pass into the blood stream, and then funnelled through the hepatic portal; they can interact with blood cells before being processed and distributed in different body organs such as the liver and kidney (Bridges and Zalups, 2010; Chen et al., 2006; Clarckson et al., 2007; Clarkson and Clarckson, 2002). Selenite (Na_2SeO_3) at relatively low concentrations was shown to counteract *in vitro* MeHg toxic effect on outgrowth of rat nerve fibres (Kasuya, 1976), but not on growth inhibition in Chang's liver cells and 3T3 mouse fibroblasts (Potter and Matrone, 1977). These *in vitro* protective effects of selenite were dependent on the concentrations of Na_2SeO_3 and Hg as well as on the chemical form of Hg. More recently Se was shown to provide some limited *in vitro* protection against high concentrations of inorganic and organic Hg in T-lymphocytes of beluga whale, *Delphinapterus leucas* (Frouin et al., 2012). T-lymphocytes are an important component of the cell-mediated arm of acquired immunity, and the proliferation of T cells in response to mitogens is frequently evaluated because of its correlation with *in vivo* host resistance (Luster et al., 1988).

High concentrations of Hg and Se in blood were previously reported in the blood of free-ranging harbour seals, *Phoca vitulina* from the North Sea and these concentrations were highly variable (from 43 to 611 μg total $Hg.L^{-1}$ and 518 to 2261 μg $Se.L^{-1}$) (Das et al., 2008; Dupont et al., 2013; Griesel et al., 2008). However, to our current knowledge, no data are available for the potential interaction between selenium and methylmercury at the lymphocyte level in the harbour seal. Lymphocytes are the active immune cells of adaptive immunity and functional assays have been developed to assess their ability to proliferate and thus mount a proper immune response (Desforges et al., 2016).

The present study focuses on *in vitro* immunotoxic effects of organic Hg and Se on the harbour seal lymphocytes and aims 1) to evaluate the toxic effects of mercuric compounds on lymphocyte proliferation and 2) to characterize the degree of selenium protection against Hg toxicity in lymphoblastic proliferation. Two biogically relevant species of Se were tested, sodium selenite and seleno-L-methionine (SeMet) (Reilly, 2006). Molar ratios of Hg:Se in blood of free ranging harbour seals have been reported at approximately 1:10, therefore lymphocytes were exposed to Hg and Se in this molar ratio in order to reflect natural exposure scenarios.

2. Material and methods

2.1. Blood sample collection and conservation

The field studies were carried out under the relevant permits of the National Park Office Schleswig-Holstein and the animal experiment permit (AZ 312-72241.121-19).

Blood samples were collected from 8 free-ranging harbour seals caught on two sandbanks (Lorenznplate and Kolumbusloch) as previously described (Lehnert et al., 2016) (Fig. 1). Seals were captured in nets and restrained manually for clinical examination. Sex, length and weight were determined before blood sampling (Table 1). Judging by their weight and length, all sampled animals adults. Harbour seals were in good nutritional status and revealed no sign of disease after veterinary check.

Blood was drawn from the extradural venous sinus into sterile evacuated blood collection tubes using a 20 ml syringe and a 1.2 mm × 100 mm needle (Carromco, Hamburg-Norderstedt, Germany). Blood was transferred into Cell Preparation Tubes with Sodium Heparin (CPT™, BD Vacutainer®, Plymouth, UK) for peripheral blood mononuclear cell isolation (PBLs).

Tubes were gently inverted 10 times and stored upright at room temperature until further processing, within maximum 5 h of blood collection for CPTubes™.

2.2. PBL isolation and conservation

PBLs were isolated on Ficoll (Ficoll Hypaque™ contained in CPT™, BD Vacutainer®; same density: 1.077 g/ml) as described previously (Dupont et al., 2016). Blood samples were centrifuged at 1800 ×*g* during 30 min at 20 °C, and cells were resuspended into the plasma as advised by the manufacturer (BD Benelux N.V., Erembodegem, Belgium). After transportation to the cell culture laboratory within 18 h, the PBLs were pipetted into separated vessels and washed 3 times in phosphate buffered saline (PBS, sterile filtered, BioWhittaker®, Lonza, Verviers, Belgium). Cells were suspended in RPMI 1640 medium (BioWhittaker®, Lonza, Verviers, Belgium) supplemented with 10% foetal calf serum (Gibco®, Invitrogen, Paisley, UK), 0.33% L-alanyl-L-glutamine (GlutaMAX™, Gibco®, Invitrogen, Paisley, UK), 1% non-essential amino-acids (BioWhittaker, Lonza, Verviers, Belgium), 1% Na pyruvate (BioWhittaker, Lonza, Verviers, Belgium) and 1% penicillin–streptomycin (100 IU and 100 μg/ml, BioWhittaker, Lonza, Verviers, Belgium). This complete culture medium was further referred to as 'culture medium'. The viability and number of cells were determined with a Nucleocounter® NC-100™ (Chemometec, Allerød, Denmark) according to manufacturer's instruction. After counting, cells were centrifuged, and the pellet was suspended in a determined volume of culture medium added with 10% DMSO (dimethyl sulfoxide, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) at 4 °C to adjust their concentration to 10×10^6 viable cells/ml. The cell suspension was transferred into cryovials (1 ml/vial) and placed into a freezing container (Mr Frosty, Nalgene) at −80 °C during 24 h, before being transferred in liquid nitrogen.

2.3. PBL in vitro cultures

PBL cryovials were quickly thawed and transferred into 50-ml Falcon tubes containing 40 ml of complete culture medium pre-warmed at 37 °C. PBLs were centrifuged at 300 g during 10 min, the supernatant was discarded and the cell pellet was resuspended in warm culture medium. Monocytes were partially depleted by plastic adherence in tissue culture flasks (Vented Cap, Sarstedt, Newton, NC, USA) incubated during 1 h at 37 °C in a humidified atmosphere enriched with 5% CO_2. The cell suspension was harvested, the viable cells were counted with a nucleocounter© (Chemometec, Allerød, Denmark) and the concentration was adjusted to 1×10^6 cells/ml (final concentration).

Cells were also co-stained with propidium iodide (PI) and Annexin V-fluorescein isothiocyanate (AV-FITC) conjugate according to the manufacturer's instructions (Annexin V: FITC Apoptosis Detection Kit I, BD, Erembodegem, Belgium) and analysed by flow cytometry in order to determine the proportions of living, early apoptotic and dead cells (in late apoptosis and necrosis). The Annexin V assay was used to detect phospatidylserine translocation on the membrane surface of cells undergoing apoptosis (Vermes et al., 1995). Cells were displayed as scatter plots based on their sizes, estimated by forward scatter