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

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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,

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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

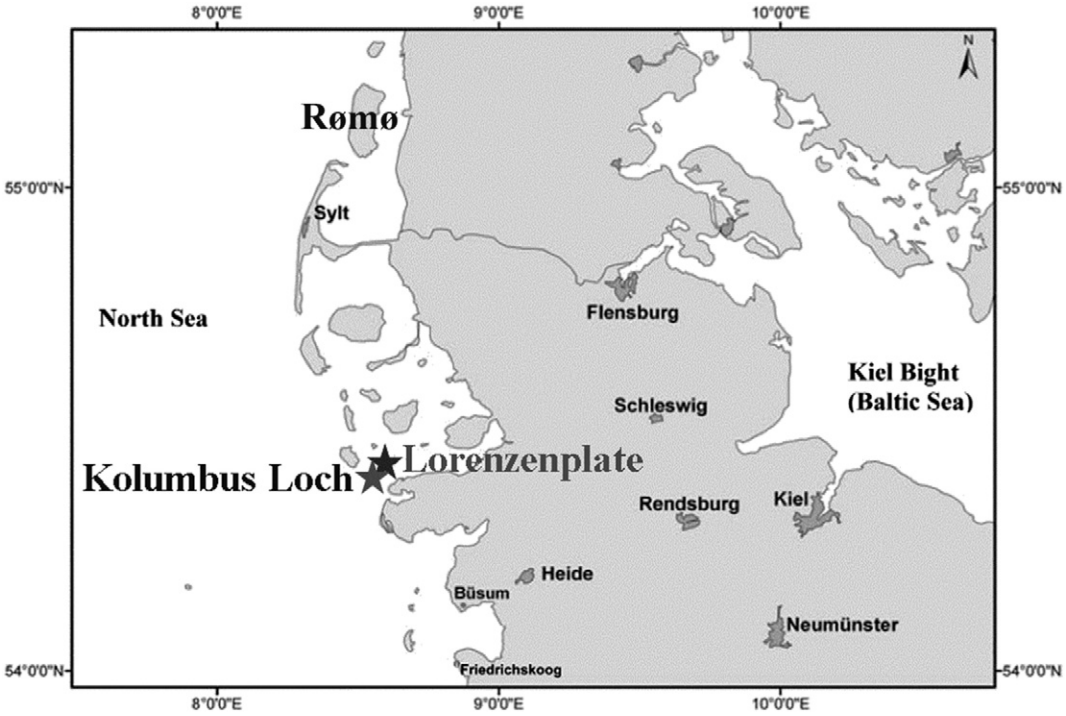

Fig. 1. Sampling location of the 8 harbour seals, *Phoca vitulina* in the North Sea.

(FSC), and internal complexity (granulosity), estimated by side scatter (SSC). The analysis was conducted with a FACSCanto™ II (BD, Erembodegem, Belgium) flow cytometer. Scatter plots obtained by flow cytometry showed that all leucocyte subpopulations (*i.e.*, lymphocytes, monocytes and granulocytes) were present, and that cell suspensions were composed of lymphocyte-enriched leucocytes (Dupont et al., 2013). All cells were analysed for AV-FITC and PI labelling. AV-PI negative cells were further referred to as 'living cells', and PI negative cells (counted with the nucleocounter©, comprising living and early apoptotic cells) were further referred to as 'viable cells'.

2.4. BrdU cell proliferation assay

Proliferation induced by Concanavalin A (ConA, Sigma-Aldrich Chemie GmbH, Steinheim, Germany), a T-cell-specific mitogen in harbour seals (de Swart et al., 1993), was assessed by culturing PBLs in a flat bottom 96-well plate (transparent, Sarstedt, Newton, NC, USA) at 2×10^5 cells in 200 μl culture medium per well with an optimal concentration of 5 μg/ml ConA in an incubator at 37 °C under humidified atmosphere enriched with 5% CO_2. Control PBLs were cultured in medium alone and stimulated controls, with ConA alone. Stimulated PBLs were cultured with 5 μg/ml ConA and 0.75 μM (final concentration) MeHgCl (CH_3HgCl — methyl mercuric chloride, K&K Laboratories, Inc., Plainview N.Y., California, USA) either alone or in presence of 7.5 μM (final concentration) sodium selenite (Na_2SeO_3, Sigma-Aldrich, Bornem, Belgium; further referred to as selenite) or of 7.5 μM (final concentration) seleno-L-methionine ($C_5H_{11}NO_2Se$, Sigma-Aldrich, Bornem, Belgium; further referred to as SeMet). Each culture condition was in triplicate. The actively proliferating lymphocytes were assayed with bromodeoxyuridine (BrdU) incorporation into newly synthesized DNA (BrdU Proliferation Assay, Calbiochem®, Overijse, Belgium). Two types of controls were used for the BrdU tests: blanks (culture medium alone) and background controls (cells without BrdU). BrdU label was added to wells of the microtiter plate for the last 24 h of culture. After 72 h of incubation, cells were fixed, permeabilised and the DNA denatured to enable antibody (Ab) binding to the incorporated BrdU. Detector anti-BrdU monoclonal Ab was pipetted into the wells and allowed to incubate for 1 h, during which time it binds to any incorporated BrdU. Unbound Ab was then washed away and horseradish peroxidase-conjugated goat anti-mouse was added to bind to the detector Ab.

The intensity of the coloured reaction product, which is proportional to the amount of incorporated BrdU in the cells, was quantified by measuring the absorbance (optical density, OD) in each well using a spectrophotometric plate reader (PowerWave X, Bio-Tek Instruments, Inc., Winooski, VT, USA) at dual wavelength of 450–540 nm. The incorporation of BrdU, representing the lymphocyte proliferation, was expressed as mean percentages of absorbance of the stimulated controls (cells cultured with ConA alone).

2.5. Mitochondrial membrane potential test

The mitochondrial membrane potential was measured with a cationic dye that fluoresces either green or orange depending upon mitochondrial membrane potential status (Mito-ID® Membrane Potential Cytotoxicity Kit for microplates, Enzo Life Sciences, Zandhoven, Belgium). PBLs were seeded in 96-well plates with black wall and clear bottom (black/clear Imaging Plate, BD Falcon™, Erembodegem, Belgium) as described above (2×10^5 cells in 200 μl culture medium per well with an optimal concentration of 5 μg/ml ConA). Positive controls were produced by adding 10 μM CCCP (carbonyl cyanide 3-chlorophenylhydrazone), which causes mitochondrial membrane potential loss, to cells for 30 min before 72 h of incubation. Control PBLs

Table 1
Sampling description for harbour seal *Phoca vitulina* in the North Sea.

Sampling period	Harbour seal number	Gender	Length (cm)	Body mass (kg)
April 2010	5618	F	163	73.5
	5615	F	168	92
September 2010	5944	F	150	54.5
	5942	F	158	61
	5940	M	160	66.5
	5948	M	166	67.5
	5952	F	167	79
	5951	M	170	63.5

were cultured in medium alone and stimulated controls, with ConA alone. Each culture condition was in triplicate. After the incubation time, microplates were centrifuged at 400 ×*g* during 8 min at room temperature (Beckman Allegra R21), the culture medium was discarded and 100 μl of prepared Mito-ID® Membrane Potential (MP) Dye Loading Solution was added to each well, following manufacturer instructions (Mito-ID® Membrane Potential Cytotoxicity Kit for microplates, Enzo Life Sciences, Zandhoven, Belgium).

Microplates were incubated during 30 min in the dark, at room temperature before being read in a fluorescent microplate reader (Victor³ 1420 Multilabel Counter, serial number 299811812, Perkin-Elmer) using an excitation setting of 480 nm and an emission setting of 590 nm. In living and healthy cells, the mitochondria had an orange fluorescence following aggregation of the Mito-ID® MP dye, emitting at 590 nm. The microplate reader ran with the Wallac 1420 Workstation Software.

2.6. Flow cytometric analysis

In addition, to confirm the presence of lymphoblasts in stimulated conditions and to evaluate the cytotoxicity in the different cell cultures, the cell suspensions were stained with PI and Annexin V (Annexin V: FITC Apoptosis Detection Kit I, BD, Erembodegem, Belgium) and analysed by flow cytometry as described above.

2.7. Data analysis

Statistical analysis was performed using Statistica 9.1 and Wizard Pro. Each culture condition was in triplicate for each individual. Optical density values (BrdU test) and cell counts were averaged for each individual, giving one average value per culture condition. Comparisons of the OD means of control and stimulated PBLs after 72 h of incubation was assessed by the Wilcoxon test for paired samples. The normality of all data distribution was assessed through the Shapiro–Wilk test.

Comparisons of the means of number of viable cells, proliferative values and mitochondrial membrane potentials between culture conditions, sexes, locations, seasons and years of sampling were performed using the Student's t test (two-tailed), respecting the normality of distribution and equality of variances (Bartlett, $p < 0.05$;). When variances were unequal, the Satterthwaite's approximate t test was used.

When normality of distribution could not be achieved despite the log-transformation, the non-parametric Mann–Whitney U test was used. The level of statistical significance was defined at $p <$ t 0.05.

3. Results

3.1. PBL in vitro cultures

The Ficoll density fluid is originally used to separate peripheral blood mononuclear cells (PBMCs, including lymphocytes and monocytes) and plasma from granulocytes and erythrocytes. However, flow cytometry scatter plots of cell suspensions indicated that all leucocyte subpopulations (*i.e.*, lymphocytes, monocytes and granulocytes) were present. The lymphocyte proliferation responses to *in vitro* ConA stimulation were characterized for the 8 harbour seals from the North Sea after 72 h of incubation. The viability of cells isolated by density gradient centrifugation ranged between 89 and 96% before and after thawing. After 72 h of incubation, lymphocyte proliferation in presence of ConA was demonstrated by the BrdU test analysis. The flow cytometry AV-PI labelling tests showed that the majority of the lymphocytes were out of the apoptotic and necrotic pathways in stimulated controls.

3.2. Lymphocyte proliferation test

Cell proliferation differed significantly between control conditions and Con-A stimulated cells (Mann–Whitney U test, $p < 0.01$). In presence of ConA and 0.75 μM MeHgCl, the lymphocyte proliferation decreased by an average of 33% compared to the stimulated control (with ConA alone) (Fig. 2). No significant difference was observed when selenite or SeMet was added simultaneously to MeHgCl. Cells in presence of MeHgCl and SeMet displayed a lower BrdU incorporation in newly synthetized DNA than those in presence of MeHgCl alone or with MeHgCl and selenite, but this was however not statistically significant ($p < 0.05$).

Cells cultured in presence of ConA and SeMet did not display significantly lower proliferation compared to the stimulated control ($p = < 0.05$). According to the Student t test, cells cultured with ConA alone presented a proliferation significantly different to that in all other culture conditions. Cells cultured with Se, either organic or inorganic, displayed highly variable interindividual proliferative responses. Three individuals showed clear decrease of their lymphocyte proliferative response, down to 30% of the stimulated control. Nevertheless, proliferative responses of cells cultured with Se alone were never lower than those of cells cultured with MeHg, with or without Se.

3.3. Mitochondrial membrane potential test

Exposure to 0.75 μM MeHg, concomitantly to Se or not, resulted in a significant decrease of mitochondrial membrane potential (Fig. 3), as demonstrated by decreased Mito-ID MP dye fluorescence (Mann–Whitney U test, $p < 0.05$). On the contrary, there was no significant difference between the stimulated control and values obtained for cells cultured with selenite or SeMet.

4. Discussion

Seal lymphocytes were affected by *in vitro* exposure to MeHg at concentrations currently observed in wild populations from the North Sea (Das et al., 2008; Dupont et al., 2016; Habran et al., 2013). A lingering question remains the degree to which harbour seals are able to cope with these elevated Hg levels in their tissues. Previous studies suggested that Hg levels may already be sufficient to elicit an adverse effect on immune function in wild populations of marine mammals (Bennet et al., 2001; Desforges et al., 2016; Dietz et al., 2013; Siebert et al., 1999).

Present results clearly demonstrated that MeHg affected *in vitro* immune cells function with no protective effects of selenium at a molar ratio Hg:Se of 1:10. This molar ratio reflects Hg and Se concentrations measured in the blood of free-ranging harbour seals from the North

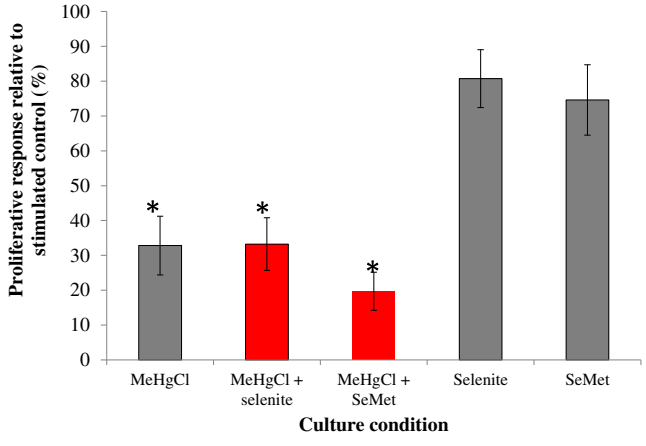

Fig. 2. Proliferation of stimulated lymphocytes (ConA) in presence of MeHgCl, MeHgCl and sodium selenite (selenite), MeHgCl and seleno-L-methionine (SeMet), sodium selenite and SeMet. Data expressed as a percentage (%) of the control response (unexposed cells activated with ConA) ($n = 8$ for each test; mean ± standard error). The T lymphocyte proliferative response to ConA was evaluated according to the incorporation of BrdU in newly synthetized DNA, and absorbance was measured at 450 nm. * significantly different to the control cells, $p < 0.05$.

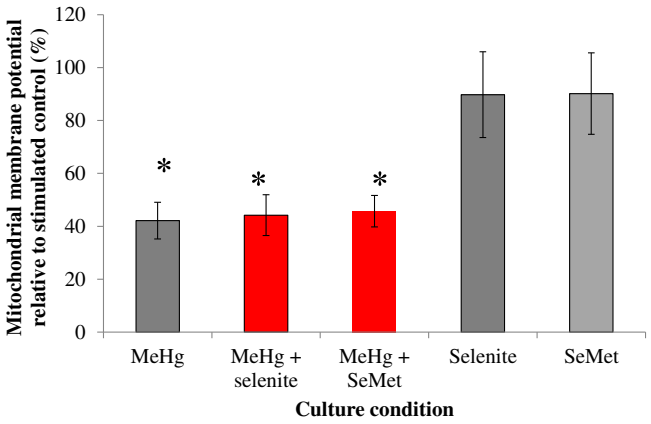

Fig. 3. Mitochondrial membrane potentials of stimulated lymphocytes (ConA) in presence of MeHgCl, MeHgCl and sodium selenite (selenite), MeHgCl and seleno-L-methionine (SeMet), sodium selenite and SeMet. Data expressed as a percentage (%) of the control response (unexposed cells activated with ConA) (n = 8 for each test; mean ± standard error). The mitochondrial membrane potential was evaluated by measurement of the fluorescence of Mito-ID® Membrane Potential dye measured at 590 nm. * significantly different to the control cells, $p < 0.05$.

Sea (Dupont et al., 2013; Kakuschke and Griesel, 2015). We observed a reduced proliferative response at 0.75 μM MeHg suggesting that the immunotoxic effects observed was not linked to cytotoxic effects. *In vitro* exposure of ConA-stimulated T lymphocytes resulted in severe inhibition of DNA synthesis, likely linked to severe loss of mitochondrial membrane potential. The decrease of mitochondrial membrane potential observed in our study is in accordance with previous studies (Yin et al., 2007). Inner mitochondrial membrane potential is a very sensitive endpoint for MeHg toxicity. MeHg exposure at 1 μM was shown to be associated with increased mitochondrial membrane permeability, alterations in glutamine/glutamate cycling, increased ROS formation and consequent oxidative injury in rat primary astrocyte cultures (Yin et al., 2007).

Se is a natural methylmercury (MeHg) antagonist that potently counteracts or eliminates symptoms linked to MeHg exposures (Cuvin-Aralar and Furness, 1991; Ralston and Raymond, 2010). Demethylation of MeHg and protection by Se (both absorbed from the diet), have been suggested as possible Hg detoxification mechanisms in marine mammals. Tiemannite (HgSe), a crystalline mercuric selenide has been observed in several tissues of marine mammals (Lailson-Brito et al., 2012; Martoja and Berry, 1980; Nigro and Leonzio, 1996). However, selenium may reduce the toxicity of Hg other than through the formation of tiemannite. Mechanisms for the antagonistic effect between Hg and Se were summarized in five pathways (other than HgSe formation) as detailed previously (Khan and Wang, 2009): (1) formation of MeHg–Se compounds, (2) Se-aided demethylation of MeHg, (3) redistribution of inorganic Hg in the presence of Se, (4) Se inhibition of methyl radicals from MeHg, and Hg-induced Se deficiency. The three first pathways are based on the assumption that the toxicity of Hg and MeHg is caused by the Hg^{2+} or $MeHg^{+}$, whereas the last two pathways attribute the observed Hg toxicity, at least in part, to the methyl group and Se deficiency, respectively (Khan and Wang, 2009).

Sodium selenite was previously shown to have a protective effect towards Hg toxicity in beluga whale ConA-stimulated PBLs *in vitro* when the Hg:Se molar ratio was similar to or higher than 1:1 (Frouin et al., 2012). When the Hg:Se molar ratio was similar to or higher than 1:2, Se provided protection following exposure to highest concentrations of MeHgCl (10 μM). A selenium-dependent protection was evidenced by an increase in T-lymphocyte proliferation (BrdU assay) only in cells exposed to relatively high MeHgCl concentration (10 μM) at Hg:Se ratios of 1:2 to 1:4. Their finding was consistent with reports in Atlantic spotted dolphin (*Stenella plagiodon*) renal cells (Sp1K cells), where concurrent exposure to 80 μM Na_2SeO_3 provided full protection against the decrease in cell proliferation induced by 20 μM $HgCl_2$ (Wang et al., 2001). Selenite and SeMet alone did not affect lymphocyte proliferation and mitochondrial membrane potential in cultures lymphocytes (Figs. 2 and 3). However, selenite and seleno-L-methionine did not show any protective effect against MeHg toxicity expressed at the T lymphocyte proliferation level for harbour seals.

The absence of protection of Se against the decrease of both cell proliferation and mitochondrial membrane potential remains unclear. Although Hg–Se antagonism is generally observed, additive or even synergistic effects of Hg and Se have also been reported in the literature (reviewed by Khan and Wang, 2009). This is not surprising, as Se, though an essential element for animals and humans, will cause toxic effects when the concentration is elevated above what is considered optimal. The net effect is dependent on the relative concentrations of Hg and Se, their bioavailabilities, and the sensitivity of the species. Previous study suggested that selenium's 'protective effect' against mercury toxicity may simply reflect the importance of maintaining sufficient free selenium to support normal selenium-dependent enzyme synthesis and activity (Raymond and Ralston, 2004). MeHg is a highly specific, irreversible inhibitor of Se-dependent enzymes (selenoenzymes). Selenoenzymes are required to prevent and reverse oxidative damage throughout the body (Ralston and Raymond, 2010). As intracellular concentrations of MeHg approach, and especially as they exceed 1:1 M stoichiometries with Se in these vulnerable tissues, vital selenoenzyme activities are increasingly inhibited and formation of insoluble Hg selenide (HgSe) deplete availability of Se for subsequent cycles of selenoprotein synthesis.

To conclude, our observations suggest that low Hg levels may already be sufficient to elicit an adverse effect on immune function in harbour seal populations from the North Sea. The present *in vitro* approach suggests no protective effect of sodium selenite or seleno-L-methionine towards methylmercury immunotoxicity in the 8 tested harbour seals. Caution is clearly needed because of the limited sample size but these preliminary data reflect the complexity of Hg–Se interactions in marine mammals and deserve further investigations.

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