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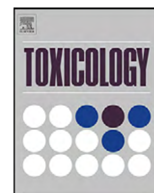

The *in vitro* effects of Trolox on methylmercury-induced neurotoxicity

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ABSTRACT

Methylmercury (MeHg), an environmental toxicant primarily found in fish and seafood poses a dilemma to both consumers and regulatory authorities given the nutritional benefits of fish consumption vs. possible adverse neurological damage caused by MeHg. The present study addresses whether supplementation with 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), alters the neuro-oxidative effects of MeHg in C6-glioma and B35-neuronal cell lines. As indicators of cytotoxicity, reduced glutathione (GSH), reactive oxygen species (ROS) and mitochondrial activity (MTT) were measured. The cellular mercury (Hg) content was measured with high resolution-inductively coupled plasma mass spectrometry (HR-ICPMS). The amount of MeHg-induced ROS was significantly reduced ($p < 0.05$) after treatment with 50 μM Trolox in C6 glial cell line. However, treatment with Trolox did not induce any significant increase in GSH levels or MTT activity in either of the cell lines. In addition, treatment with Trolox did not induce any significant changes in intracellular MeHg levels. The MeHg and Trolox treated C6 glial cell line differed significantly ($p < 0.05$) from the B35 cell line for MTT, ROS and GSH activity. These findings provide experimental evidence that preincubation with Trolox prevents MeHg-induced ROS generation in C6 glial cell line by quenching of free radicals and not by changes in intracellular GSH or MeHg content.

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1. Introduction

Methylmercury (MeHg) is a well-known contaminant in fish. Its consumption from adulterated fish elicits a public health issue as the risks associated with MeHg intake vs. the benefits of fish consumption per se remain elusive. For example, various regulatory agencies, such as the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 1978), National Institute of Environmental Health Sciences (NIEHS, 1999) and National Academy of Sciences (National Research Council, 2000) have stressed uncertainties regarding the risks associated with intake of MeHg from seafood diet. In addition, the discrepancies reported in health outcomes in fish eating populations from New Zealand (Kjellstrom et al., 1986, 1989) and Faroe Islands (Grandjean et al., 1997) as opposed to Canada (Keown-Eyssen et al., 1983), the Seychelles (Davidson et al., 1998; Myers et al., 1997), Peru (Marsh et al., 1995) and the US (Mozaffarian and Rimm, 2006; Oken et al., 2005) indicate that there is a possible disparity between the estimated toxic effect of MeHg and its risk assessment. These differences indicate that besides intrinsic genetic factors, the phenotypic responses to MeHg exposure may ultimately depend on complex interactions between mercury and dietary factors. Hence there is a need to assess dietary nutrients as well as neurotoxic exposures in determining and refining the risks and benefits of fish consumption (Myers et al., 2007).

Vitamin E is the most significant physiologic membrane-associated antioxidant available from seafood. However, there are limitations to testing the role of this nutrient in the aqueous medium of cell culture. On the other hand, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water soluble analog of vitamin E, can be added directly to the test system and serves as a convenient tool for studies on natural biological systems. The absence of the phytyl side chain renders the Trolox molecule soluble in water (Frankel, 2005). The present study investigates the effect of Trolox as an essential seafood component in modulat-

Abbreviations: MeHg, methylmercury; Trolox, 6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid; GSH, reduced glutathione; ROS, reactive oxygen species; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide]; Hg, mercury; HR-ICPMS, high resolution-inductively coupled plasma mass spectrometry; DMEM, Dulbecco's minimum essential medium; MCB, monochlorobimane; CMH_2DCFDA, chloro methyl derivative of di-chloro di-hydro fluoresceindiacetate; HEPES, N-2-hydroxy-ethylpiperazine N′-2-ethansulfonic acid.

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ing the effect of MeHg. We hypothesize that Trolox can attenuate neurotoxic effects of MeHg. To test the hypothesis, we have determined whether Trolox is able to prevent neuro-oxidative effects of MeHg. This may provide an improved risk assessment of MeHg contaminated fish diet.

MeHg-induced oxidative stress plays a key role in the *in vivo* pathological process of MeHg intoxication (Ali et al., 1992; Sanfeliu et al., 2001; Sarafian, 1999; Yee and Choi, 1996). Both Trolox and vitamin E can act as scavengers of radicals via the H-donating groups (Frankel, 2005; Lee et al., 2005). The radical scavenging properties of Trolox and vitamin E have been demonstrated in a variety of experimental studies (Massey and Burton, 1990; Persoon-Rothert et al., 1990; Suttorp et al., 1986; Wu et al., 1990). However, some studies have pointed out that Trolox serves as a better antioxidant than vitamin E (Raspor et al., 2005; Sagach et al., 2002). This has been attributed to its improved access to the hydrophilic compartments of the cells (Garcia et al., 2000), as well as stochiometric properties, i.e., the ability to trap two membrane lipid peroxyl radicals per molecule of Trolox (Barclay and Vinqvist, 1994). Treatment with Trolox has been reported to protect against MeHg-induced decrease in mitochondrial electron transport system enzyme activities (Usuki et al., 2001). However, the ability of Trolox to modify MeHg-induced oxidative stress in neural cells has not been investigated before. In particular, little is known regarding the effect of Trolox on MeHg sensitive neural cells from cerebellum and visual cortex. The innovative aspect of this study resides in the simultaneous measurement of oxidative stress and intracellular MeHg content in different neural cells after MeHg and Trolox exposure. The recognition of the protective effects of Trolox and identification of its mechanisms via *in vitro* models establishes that vitamin-dependent antioxidant defences are important factors in specific cells for attenuating the neurotoxic effects of MeHg-containing fish diet.

2. Materials and methods

2.1. Materials

24-well plastic tissue culture plates were purchased from VWR Norway (USA). Fetal bovine serum (Cat. No. A15-151) was purchased from Fischer Scientific (Norway). The medium for culturing C6-gliomas (F12 Kaighn's nutrient mixture, Cat. No. 21127) was purchased from Invitrogen (Norway). The DMEM media (Cat. No. E15-810) for culturing B35 cells was purchased from Fischer Scientific (Norway). Trolox (Cat. No. 23,881-3), MCB (Cat. No. 69899), MTT (Cat. No. M2128) and poly-D-lysine (Cat. No. P1024) were purchased from Sigma–Aldrich (Norway). The fluorescent indicator CMH_2DCFDA (Cat. No. C6827) was obtained from Molecular Probes, Inc. (Eugene, OR, USA). MeHgCl (Cat. No. 23308) was purchased from K&K Laboratories (Plainview, NY, USA). All other chemicals were of analytical grade.

2.2. Cell lines

The C6-glioma and B35-neuronal cell lines were obtained from the ATCC-LGC Promochem (Sweden) and stock samples were kept in liquid nitrogen. Both cell lines were selected due to their suitability for live cell fluorescence measurements. Freshly thawed cells were used after three passages. On day one, 60,000 cells per well for the C6 cell line and 160,000 cells per well for the B35 cell line were seeded in 24-well plates. The C6 cell line was cultured with F-12 media containing penicillin (10,000 units/ml), streptomysin (10,000 μg/ml) and 15% of heat inactivated fetal bovine serum. For the B35 cells, DMEM media with penicillin (10,000 units/ml), streptomycin (10,000 μg/ml) and 10% of non-heat inactivated fetal bovine serum was used. Poly-D-lysine coating was used for plating the B35 cells. The different seeding concentrations were used due to the difference in cell division (C6 cells dividing at a much higher rate compared to B35 cells). The amount of protein present on the day of experimentation was between 128 ± 7 and 110 ± 17 μg protein per well for the C6 and B35 cell lines, respectively. The seeding density was chosen to optimize the fluorescence measurements.

2.3. Treatments

Trolox was used to modify the vitamin E content in the cell cultures. A stock solution of 525 mM Trolox was freshly prepared by dissolving in 1N NaOH and respective media without serum. From this stock solution, a range of concentrations

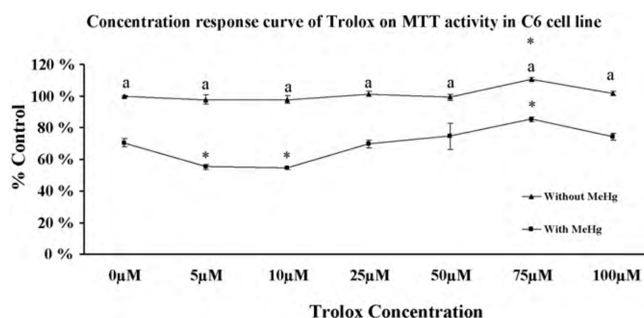

Fig. 1. Concentration response curve of Trolox on MTT activity in C6 cell line. Results are expressed as mean ± standard deviation ($n = 4$ replicates for each cell type in two independent experiments). Superscript (a) indicates $p < 0.001$ when without MeHg vs. with MeHg for each type of treatment; $^*p < 0.001$ when 0 μM MeHg as compared to Trolox (5–100 μM) treated group; $^*p < 0.001$ when with MeHg as compared to Trolox + MeHg-treated group. Values represented the percentage of activity relative to control cells.

(0–100 μM) were prepared in media without serum (Figs. 1 and 2). Similar to previously reported studies (Malcolm et al., 2000), a concentration response curve for MTT activity (Fig. 1) and cellular ROS (Fig. 2) was obtained with Trolox, and based on these results 25 and 50 μM Trolox were selected for further study. For the treatment group, Trolox and for the control group, media was introduced directly to the wells on day 2 post-seeding. From a stock solution of 1 mM MeHgCl in 5 mM Na_2CO_3, a working solution of 10 μM MeHg was prepared in HEPES buffer [122 mM NaCl, 3.3 mM KCl, 0.4 mM $MgSO_4 \cdot 7H_2O$, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 10 mM glucose and 25 mM HEPES adjusted to pH 7.4 with 10 N NaOH]. On day 3, i.e., after additional 24 h, the cells were washed and incubated in the presence of 10 μM MeHg for 50 min. For the last 20 min of the MeHg exposure, the cells were incubated with either fluorescent probes CMH_2DCFDA (7 μM), MCB (40 μM) or MTT (2.4 mM). The MeHg concentration and exposure time were selected on the basis of a previous study (Kaur et al., 2007).

2.4. Determination of MeHg content in cells

MeHg content in the cells was analyzed by HR-ICPMS using a Thermo (Finnigan) model Element 2 instrument (Bremen, Germany). The samples were digested in an UltraClave (Milstone) by adding 1.0 ml 65% HNO_3 (ChemScan, Scanpure) in TMF (Teflon) vessels. After the digestion, the samples were diluted to 24 or 960 ml with ultrapure water (Ultra Analytic, Elga) to achieve a final acid concentration of 0.6 mol/l. The Radio Frequency power was 1400 W and approximately 0.1% methane was added to the plasma. The samples were introduced using a SC-E2 with SC-FAST option auto sampler (ESI) with a peristaltic pump (pump speed 0.25 ml/min). The samples were then passed over a PFA concentric Mainhard nebuliser and a PFA Scott spray chamber. The instrument was calibrated with 0.6 mol/l HNO_3 solutions of multi-element standards at appropriate concentrations. To check for possible drift in the instrument, a standard solution with known elemental concentrations was analyzed for every 16 samples. In addition, blank samples (0.6 mol/l HNO_3, Suprapur) were analyzed after approximately every 16 samples. The samples were analyzed in random order, and the analyst was blinded to the samples. MeHg was determined as the Hg^{200} isotope in the low-resolution mode.

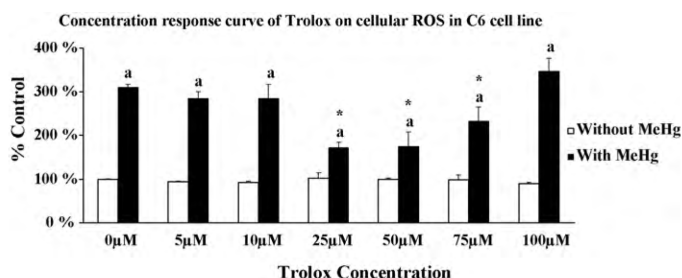

Fig. 2. Concentration response curve of Trolox on cellular ROS in C6 cell line. Results are expressed as mean ± standard deviation ($n = 4$ replicates for each cell type in two independent experiments). Superscript (a) indicates $p < 0.001$ when with MeHg vs. without MeHg for each type of treatment; $^*p < 0.001$ when with MeHg as compared to Trolox + MeHg-treated group. Values represented the percentage of activity relative to control cells.

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