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Cellular and Molecular Mechanisms Behind Methylmercury-Induced Neurotoxicity

Thesis for the degree philosophiae doctor

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Cellulære og molekylære mekanismer ved metylkvikksølvindusert nevrotoksisitet

Kvikksølv er et metall kjent og brukt til mange forskjellige formål i flere hundre år. Kvikksølv inngår i mange kjemiske forbindelser og på 1950-tallet ble det vist at en organisk form for kvikksølv, metylkvikksølv (MeHg), er nevrotoksisk. Til tross for omfattende forskning kjenner vi fortsatt ikke den molekylære mekanismen som ligger bak MeHg's giftighet. MeHg reagerer med thiol-grupper og disse er vesentlige for enzymers normale funksjon. På denne måten kan MeHg skade de fleste enzymer i det cellulære maskineriet. MeHg's giftvirkningen skyldes trolig et multippel av effekter på flere cellulære prosesser.

En av de viktige mekanismer bak MeHg-indusert giftighet er dannelse av reaktive oksygen radikaler (ROS) og reduksjon av mengden av glutation (GSH) i celler. Balansen mellom de antioksidante og reduktive prosesser er viktig for MeHg-indusert nevrotoksisitet. Cellekultursystemer er mindre komplekse enn hjernen og ble brukt som modell for å studere forandringene i slike cellulære prosesser etter MeHg-eksponering. MeHg-indusert oksidativt stress ble studert i primære nevroner og astrocytter fra stor- og lillehjernen, samt cellelinjer. Cellekulturene ble behandlet med N-acetyl cystein (NAC) som øker cellulært GSH eller med dietylmaleat (DEM) som reduserer cellulært GSH-innhold. Fluorescens-mikroskopi ble benyttet til identifisering og kvantifisering av cellulær ROS og GSH i levende celler. Reduksjon av GSH førte til økt cellulært opptak av MeHg og forsterket MeHg-indusert oksidativ stress. Redusert GSH-nivå i nevroner i forhold til astrocytter viser betydningen av nevron-glia interaksjon med hensyn til MeHg nevrotoksisitet. Økningen av MeHg i cerebellare nevroner kan forklare økt følsomhet av cerebellare nevroner i forhold til cerebellare astrocytter. Modulasjonen av GSH forklarer i noen grad forskjellen i ROS-endringer i cerebellare og kortikale kulturer. Behandling med NAC eller DEM førte til økt cellulært opptak av MeHg i cerebellare kulturer i forhold til kortikale kulturer.

Epidemiologiske studier av fiskespisende populasjoner har gitt noe forskjellig resultat med hensyn til hvordan MeHg kan påvirke hjernens utvikling. Mengden av ernæringsfaktorer som dokosaheksaensyre (DHA) kan påvirke MeHg-giftighet og bidra til å forklare uoverensstemmelsene i de ulike studiene. I denne avhandlingen har vi sett på hvordan DHA kan påvirke MeHg-indusert nevrotoksisitet i cellelinjer og primær-cellekulturer. I primær cellekulturer ble det vist hvordan DHA kan redusere mengden av MeHg i cellene samt redusere den oksidative effekt. Dette støtter hypotesen om at næringsstoffer fra fisk kan bidra til å beskytte hjernen mot MeHg.

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I. Acknowledgements

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II. Abbreviations

Hg	mercury
MeHg	methylmercury
PCB's	poly chlorinated biphenyls
SH	sulfhydryl
CNS	central nervous system
NAC	N-acetyl cysteine
DMPS	2,3-dimercapto-1-propane sulfonate
GABA	gamma amino butyric acid
ROS	reactive oxygen species
GPx	glutathione peroxidase
GSH	glutathione
PUFA's	poly unsaturated fatty acids
DHA	docosahexaenoic acid
GFAP	glial fibrillary acidic protein
CMH_2DCFDA	chloro methyl derivative of di-chloro di-hydro fluoresceindiacetate
MCB	monochlorobimane
DCF	dichlorofluorescein
FID	flame ionization detector
MTT	[3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide]
DEM	di-ethyl maleate
FAF-BSA	fatty acid free bovine serum albumin
AA	arachidonic acid
EPA	eicosapentaenoic acid
LA	linoleic acid.

III. Summary

Methyl mercury (MeHg), a metal known and used for various purposes over many years became recognized as a neurotoxicant during the 1950's. In spite of decades of research, the toxic mechanisms of MeHg still remain an enigma. It can react rapidly with any thiol group and since this binding is reversible, it can jump freely from one protein thiol group to another. Therefore, it can damage any protein present in the cellular machinery. There is no single specific mechanism of action ascribed to MeHg and its toxicity likely involves multiple coordinated effects on several parallel processes in the cell.

One of the major mechanisms behind MeHg-induced toxicity is via generation of reactive oxygen species (ROS) and depletion of glutathione (GSH). The balance between the oxidative and reductive cellular processes is critical for MeHg-induced neurotoxicity. Tissue culture systems being less complex than the heterogenous brain were selected as a model for studying the changes in these cellular processes after MeHg exposure. Moreover, cell cultures can provide new insights into the mechanisms of neurotoxic compounds. The role of MeHg-induced oxidative stress was studied in primary neurons and astrocytes from cerebrum and cerebellum and in neuronal and glial cell lines. The cell cultures were treated with N-acetyl cysteine (NAC) which is known to increase the cellular GSH or with Di-ethyl maleate (DEM) which decreases the cellular GSH status. The technique of fluorescence microscopy allowed the identification as well as quantification of cellular ROS and GSH in live cells. The relationship between GSH and MeHg concentrations was also estimated. The depletion of GSH increased the MeHg accumulation and enhanced MeHg-induced oxidative stress. Conversely, supplementation with GSH precursor protected against MeHg exposure *in vitro*. The presence of increased GSH in neurons as compared to astrocytes indicated the importance of neuronal-glial interactions with respect to MeHg neurotoxicity. In addition, the increased cell-associated MeHg in cerebellar neurons provided an explanation for the increased susceptibility of cerebellar neurons as compared to cerebellar astrocytes. To a certain extent, the modulation of GSH also explained the differential sensitivity of MeHg towards ROS generation in cerebellar and cortical cultures. The increased cell-associated MeHg was observed in cerebellar cultures after treatment with NAC or DEM as compared to cortical cultures. Since *in vitro* cultures could be influenced by changes in cell density and concentration of

neurotoxic compounds; the importance of using an optimum concentration, time and cell density in cell cultures for assessing MeHg toxicity was also addressed.

There have been discrepancies in the outcomes of epidemiological studies estimating the effect of MeHg from fish diet. The availability of nutritional factors such as docosahexaenoic acid (DHA) might influence MeHg toxicity and may explain the discrepancies from the different studies. Therefore, the effect of DHA on modulating MeHg-induced neurotoxicity was studied in cell lines as well as primary cell cultures. In the cell lines, DHA augmented the response of MeHg-induced oxidative effects. However, in primary cell cultures the importance of DHA to reduce the cell-associated MeHg and prooxidant response from MeHg was addressed. This novel finding supported the hypothesis that fish-derived nutrients can offer possible neuroprotection from MeHg as well as highlighted the importance of using an appropriate model for investigating DHA and MeHg-induced effects.

IV. List of papers

1. Kaur, P., Schulz, K., Heggland, I., Aschner, M., and Syversen, T.
   The use of fluorescence for detecting MeHg-induced ROS in cell cultures.
   Toxicology in Vitro 2008; Accepted for publication and in press.

2. Kaur, P., Aschner, M., and Syversen, T.
   Glutathione modulation influences methyl mercury induced neurotoxicity in primary cell cultures of neurons and astrocytes.
   Neurotoxicology 2006; 27: 492-500.

3. Kaur, P., Aschner, M., and Syversen, T.
   Role of glutathione in determining the differential sensitivity between the cortical and cerebellar regions towards mercury-induced oxidative stress.
   Toxicology 2007; 230: 164-177.

4. Kaur, P., Schulz, K., Aschner, M., and Syversen, T.
   Role of docosahexaenoic acid in modulating methylmercury induced neurotoxicity.
   Toxicological Sciences 2007; 100: 423-432.

5. Kaur, P., Heggland, I., Aschner, M., and Syversen, T.
   Docosahexaenoic acid may act as a neuroprotector for methylmercury-induced neurotoxicity in primary neural cell cultures.
   (Submitted to Neurotoxicology)

V. List of all my publications

1. Kaur, P., Yousuf, S., Ansari, M.A., Almas Siddiqui, A., Ahmad, A.S., and Islam, F. Tellurium-induced dose dependent impairment on antioxidant status: differential effects in cerebrum, cerebellum and brain stem of mice. Biological Trace Element Research 2003, 94: 247-258.

2. Kaur, P., Yousuf, S., Ansari, M.A., Ahmad, A.S., and Islam, F. Dose and duration dependent alterations by tellurium on lipid levels: differential effects in cerebrum, cerebellum and brain stem of mice. Biological Trace Element Research 2003, 94: 259-271.

3. Kaur, P., and Islam, F. Nutritional Aspects of mustard oil. Book Chapter in Health, Nutrition and Value Addition of Indian Mustard. Eds. D. Mathur and N. Bharti, 2003, 62-70.

4. Islam, F., Zia, S., Sayeed, I., Kaur, P., and Ahmad, A.S. Effect of selenium on lipids, lipid peroxidation, and sulfhydryl group in neuroendocrine centers of rats. Biological Trace Element Research 2004, 97: 71-82.

5. Kaur, P., Schulz, K., Heggland, I., Aschner, M., and Syversen, T. The use of fluorescence for detecting MeHg-induced ROS in cell cultures. Toxicology in Vitro 2008; in press.

6. Kaur, P., Aschner, M., and Syversen, T. Glutathione modulation influences methyl mercury induced neurotoxicity in primary cell cultures of neurons and astrocytes. Neurotoxicology 2006; 27: 492-500.

7. Kaur, P., Aschner, M., and Syversen, T. Role of glutathione in determining the differential sensitivity between the cortical and cerebellar regions towards mercury-induced oxidative stress. Toxicology 2007; 230: 164-177.

8. Kaur, P., Schulz, K., Aschner, M., and Syversen, T. Role of docosahexaenoic acid in modulating methylmercury induced neurotoxicity. Toxicological Sciences 2007; 100: 423-432.

9. Kaur, P., Heggland, I., Aschner, M., and Syversen, T. Docosahexaenoic acid may act as a neuroprotector for methylmercury-induced neurotoxicity in primary neural cell cultures. (Submitted to Neurotoxicology).

10. Kaur, P., Evje, L., Aschner, M., and Syversen, T. The *in vitro* effects of selenomethionine on methylmercury-induced neurotoxicity. (In preparation).

VI. Introduction

a) Background

Mercury (Hg) is a metal of many uses that has been known for thousands of years. Hg occupies the atomic number 80 in the periodic table and has an atomic weight of 200.6. The physical properties of Hg include a density of 13.6 and its melting and boiling points at normal pressure are -38.9 and 356.6^{o}C, respectively. Thus, it is the only metal which is liquid at room temperature. Hg exists in nature mainly as three different molecular species; elemental (Hg^{o}), inorganic (e.g., Hg^{+}, Hg^{2+}) and organic (e.g., methylmercury, ethylmercury, phenylmercuric acetate). One of the organic forms of Hg: methylmercury (MeHg) became a prime environmental heath issue during the 1950's.

The first case of fatal occupational poising caused by MeHg was recorded in 1863 where the researchers working on the synthesis of organic mercurials were gravely affected (Edwards, 1865, 1866). Later in 1940, the first report on MeHg neurotoxicity by occupational exposure in four adults was reported (Hunter *et al.,* 1940). Several catastrophic epidemics resulting from environmental contamination at the two cities in Japan - Minamata from 1953-1956 (Igata, 1993) and Niigata from 1964-1965 (Tsubaki *et al.,* 1967) highlighted the potentially disastrous effects of MeHg. In mid-1960's in Sweden (Westöö, 1966) MeHg was recognized as a widespread environmental issue. Later in the late 1960's and early 1970's exposure to MeHg via consumption of bread baked with Hg treated seed grains in Iraq (Bakir *et al.,* 1973) caused a large outbreak of human intoxication from organic Hg. Similar incidents in Pakistan, Guatemala and Ghana led to the recognition of MeHg as a ubiquitous environmental toxicant (Clarkson, 2002). It is a hazardous trace metal that is still released into the environment from both natural and anthropogenic sources (ATSDR, 1999; US EPA, 1997). Hg released from such sources is sustained in the marine ecosphere (Stokes and Wren, 1987; Veiga *et al.,* 1994) and becomes methylated to MeHg in the upper sedimentary layers of sea or lake beds by the action of microorganisms (Jensen and Jernelöv, 1969). The MeHg formed is rapidly taken up by the living organisms in the aquatic environment. MeHg is then biomagnified through the food chain from 10,000-100,000 times (US EPA, 1997; Wiener *et al.,* 2003). After bioaccumulation it reaches humans through fish consumption (Clarkson, 1997; Kamps *et al.,* 1972; Spry and Wiener, 1991). A recent study by Boudou *et al.,* (2005) reported higher concentrations of Hg in

a gold mining river at French Guiana resulting in high MeHg content in the fish. In general, nearly all fish contain detectable amounts of MeHg which when consumed in large amounts by humans can exert neurotoxic effects due to MeHg overload (Clarkson *et al.,* 1988). However, the enrichment of MeHg in the food chain is not uniform. It is dependent upon the Hg content in the water and bottom sediments, pH of the water, redox potential of the water, species, age and size of the fish. In addition Boudou *et al.,* (2005) also reported that environmental conditions such as anoxia favor the growth of microorganisms which increase the methylation of Hg and might account for high concentrations in fish. They observed that fish collected in rivers downstream of gold mining sites in French Guiana had 8-fold higher concentrations of MeHg than fish collected upstream.

The current recommended maximal dose for MeHg is 0.4μg/kg body weight/day by the World Health Organization and US Food and Drug Administartion and more recently 0.1μg/kg/day by the US Environmental Protection Agency (US EPA, 1997, 2001). Unfortunately, these levels can easily be attained with only a few meals of fish per week, depending on the source of the fish and its position in the food chain. The Hg intake via fish consumption in different epidemiological studies is shown in Table 1. In populations which consume large amounts of fish as for example on the Faroe Islands, an increase in hair-Hg levels up to 4.27 ppm during pregnancy were associated with impaired psychomotor test performance of the child at 7 years of age (Grandjean *et al.,* 1997). This population consumed mainly pilot whale which contained both MeHg and PCB's (poly chlorinated biphenyls). This type of diet led to average cord blood MeHg level of 22.9 μg/l and was correlated with deficits in neurophysiological and neuropsychological tests (eg finger tapping speed, reaction time on a continued performance task, cued naming, deficits in motor, attention and verbal tests) performed in children. In a follow up study performed on 14 year-old children at Faroe Islands it was reported that correlation between MeHg exposure via fish and deficits in scores of neurological tests were still persistent (Debes *et al.,* 2006). Moreover, Budtz-Jorgensen *et al.,* (1999), reported that PCB's were not responsible for these effects. In Japan, strong association between the prevalence of mental retardation and Hg concentration in the umbilical cord were reported at Minamata (Harada, 1978) and Niigata (Tsubaki and Irukayma, 1977). In another study from New Zealand (Kjellstrom and Kennedy, 1985) intake of MeHg via fish increased the maternal hair Hg levels to 5-20 ppm. In

this study, maternal hair Hg levels exceeding 6 ppm correlated with the deficit in the Denver developmental screening test and neurological screening test in children at 4 years of age. However, studies carried out on the Seychelles (Davidson *et al.,* 1998; Huang *et al.,* 2005; Myers *et al.,* 2003) showed no adverse effects at maternal hair Hg level of 6.8 ppm. Another epidemiological study in the province of Quebec, Canada (Keown-Eyssen *et al.,* 1983) also indicated no consistent relation between hair MeHg levels of 24 ppm or lower in the mother during pregnancy and developmental outcomes in the female offspring. Although, the objective of both the Faroe Island and Seychelles studies was to evaluate the offspring of mothers exposed to MeHg during pregnancy, the apparent differences in outcomes between the Faroe Islands and Seychelles studies could be due to many reasons. For example, the effect of some of the confounding factors, such as socioenvironmental factors, differences in genetic disposition, nutritional status as well as pattern of exposure can be difficult to control and adjust for in the statistical analysis. In addition in the Faroe Islands study, umbilical cord blood was the preferred biomarker of exposure, although maternal hair was also collected and analyzed. In addition in the Seychelles study, maternal hair was used as the measure of fetal exposure. Moreover, the assessment of neurodevelopmental outcomes depends greatly upon the appropriate uses of the test for the skills being assessed and the age of the subjects being evaluated. At the Faroe Islands the tests were performed on 7 year olds but on the Seychelles it was performed on 5½ year (66 months) olds. Furthermore, the first testing in the Seychelles study was performed on 6-month-olds (Myers *et al.,* 1995) using the Denver Developmental Screening Test. This test is specific but insensitive, especially when administered to 6-month-olds. The co-exposure with confounders such as selenium, and omega-3 fatty acids might also influence the outcome of these studies. Conversely, it is also possible that the different populations simply produced different results due to differences in genetic dispositions.

The MeHg content in food products excluding fish varies from few µg to 50µg/kg (Bouquiaux, 1974). Consumption of MeHg-contaminated bread in Iraq led to MeHg concentrations in hair exceeding 50 ppm and was correlated with severe psychomotor retardation (Marsh *et al.,* 1980). In the United States in 1970, a case of MeHg exposure was reported in a family that consumed the meat of a pig fed treated grain (Likosky *et al.,* 1970). Even recent contamination of rice with MeHg has also been reported from Jiangsu province in China (Shi *et al.,* 2005). Therefore, the risk of exposure to MeHg

via contaminated food still persists and requires constant surveillance. When neurological impairments from MeHg consumption appear, the duration of exposure is of importance for recovery and rehabilitation. From the epidemiological studies, the duration of exposure can be divided into three categories (Table 1): a) chronic exposure at high levels as in Minamata or b) acute exposure at high levels as in Iraq or c) chronic exposure at low levels such as in Sweden, the Faroe Islands and the Seychelles. The outlook for recovery and rehabilitation seems to be better in the case of acute exposure compared with prolonged exposure (Amin-Zaki *et al.,* 1978). However, exposure levels seem to be a critical factor in determining the extent of recovery.

Table 1. Compilation of some epidemiological studies of MeHg exposure

Epidemiological study	Hg intake	Source
Prolonged low level		
Swedish Population (Swedish Expert Group, 1971)	1-20 µg/day	Fish
American Samoan Population (Marsh *et al.,* 1974)	200-300 µg/day	Fish
Seychelles (Davidson *et al.,* 1998)	12 fish meals/week leading to maternal hair level of 6.8 ppm (median)	Fish
Faroes (Grandjean *et al.,* 1997)	1-3 fish meals/week leading to maternal hair level of 4.27 ppm (geometric mean)	Fish and pilot whale
New Zealand (Kjellstrom and Kennedy, 1985)	fish consumption leading to maternal hair level of 5-20 ppm	Fish
High level exposure		
Minamata and Niigata (Eto, 1997)	5 mg/day	Fish
Iraq (Bakir *et al.,* 1973)	hair levels above 50 ppm blood levels above 500 µg Hg/l	Wheat

b) Pharmacokinetics

MeHg can be absorbed via skin (Friberg *et al.,* 1961) or after inhalation where it readily penetrates the membranes of the lung at absorption level of 80% of the exposure. MeHg ingested via food is likely to be bound to proteins in the intestinal tract (Aberg *et al.,* 1969). The hazards involved in the intake of MeHg via food or occupational exposure is due to efficient absorption (90%) and long retention time (half life of 70 days) in man. After ingestion, the distribution to the blood compartment is complete within 30 hrs and the blood levels account for about 7% of the ingested dose (Kershaw *et al.,* 1980). In blood, MeHg is accumulated to a larger extent in the red cells bound to cysteinyl residues on the beta-chain of the hemoglobin molecule (Doi, 1991). From the blood MeHg is distributed slowly to the organism and the equilibrium between blood and body is not reached until 4 days (Kershaw *et al.,* 1980). It was shown by using radiolabeled MeHg that equilibrium between blood and brain requires about 3 days (Aberg *et al.,* 1969). In humans about 10% of body content of MeHg is retained in the brain. MeHg is incorporated into the hair during its formation. The MeHg concentration in blood and hair reflects the body burden. The ratio of blood/hair concentration in man is 1/250 under steady state conditions (Skerfving, 1974).

Demethylation of MeHg into inorganic Hg is the key step in the excretion process of MeHg. This process occurs mainly through microbial activity within the intestine (Rowland *et al.,* 1984) or *in vitro* in rat liver microsomes (Suda and Hirayama, 1992) and has also been reported to occur in brain but at a slower rate (Charleston *et al.,* 1995). MeHg is predominantely excreted (about 90%) via the fecal route. The net excretion rate in humans is approximately 1% of the body content at non-symptomatic body burden (Swedish Expert Group, 1971). Most of the MeHg is eliminated through liver into the bile and through the kidney into the urine. From the bile most of the MeHg gets absorbed in the gut leading to enterohepatic circulation of MeHg. Slower excretion of MeHg via urine has been reported to result in more toxicity in female rats as compared to male rats (Hirayama and Yasutake, 1986). MeHg is also excreted in the breast milk which is about 5% of the MeHg in the maternal blood (Bakir *et al.,* 1973). An influence of diet on the excretion rate of MeHg has also been shown (Landry *et al.,* 1979) since the diet interferes with the reabsorption of MeHg in the lower part of the intestines.

MeHg has a remarkable affinity for the anionic form of sulfhydryl (-SH) groups (log K, where K the affinity constant is in the order of 15-23) (Hughes, 1957). Despite the high thermodynamic stability of the MeHg-SH bond, very rapid exchange of MeHg between -SH groups is known to occur (Rabenstein and Fairhurst, 1975). In cells, MeHg can form a complex with the -SH containing amino acid cysteine (Bridges and Zalups, 2004). The MeHg-S-Cys complex behaves as a mimic of the neutral amino acid, methionine, which is a substrate of the neutral amino acid transporter system L (Landner, 1971). This mimicry has been reported to be responsible for MeHg uptake into the cells. For MeHg, the uptake into the cells is both an active, energy dependent (e.g. MeHg-cysteine) as well as passive uptake (e.g. MeHgCl) depending on the Hg species (Aschner *et al.,* 1990)

c) Neurological disturbances

The brain and the central nervous system (CNS) are the primary target sites where the adverse effects of MeHg are observed (ATSDR, 2003; WHO, 2000). MeHg is a potent neurotoxicant that affects both the developing and mature CNS (Atchison, 2005; Clarkson *et al.,* 1988). There is usually a latent period of weeks to months between exposure and the onset of symptoms (Clarkson *et al.,* 2003). The pathological changes found in adult brain are different when compared to fetal brain (Lapham *et al.,* 1995). The pathology of the Minamata disease is shown in Fig. 1 where MeHg poisoning results in focal damage in adults as compared to the widespread and diffuse damage in the fetal brain. MeHg from mother's blood is transported through the placenta to the fetus (Reynolds and Pitkin, 1975). The brain levels of MeHg in fetus can be higher than in the mother (Berlin and Ullberg, 1963). In infants, MeHg poisoning results in an unspecific infantile cerebral palsy (Swedish Export Group, 1971) involving ataxic motor disturbances and mental symptoms. The brain is found to be hypoplastic upon autopsy with a symmetrical atrophy of cerebrum and cerebellum. The histological features involve decreased number of neurons and distortion of cytoarchitecture in the cortical areas (Choi *et al.,* 1978; Takeuchi, 1977). In less severe cases, psychomotor retardation and increased incidence of seizures has been reported (Marsh *et al.,* 1980). These generalized symptoms in infants with neuronal loss throughout the brain are mainly irreversible. A body burden of 0.5 mg/kg body weight in pregnant women may result in inhibited brain development of the fetus with psychomotor retardation of the child.

In adults, chronic MeHg poisoning results in degeneration of the sensory cerebral cortex and severe neurological disturbances, such as paresthesia in the distal extremities, ataxia, sensory and speech impairment, and constriction of the visual field (Bakir *et al.,* 1973; Elhassani, 1982; Harada, 1995). In severe cases clonic seizures have been observed. The pathological changes involve general neuronal degeneration with gliosis in the calcarine, precentral and postcentral areas of the cerebral cortex. In the cerebellar cortex, loss of granular cells in the neocerebellum is observed (Hunter and Russell, 1954). The signs and symptoms associated with the changes in the specific regions of the adult brain can be reversible at a very slow rate. Specific therapies against MeHg poisoning are aimed at lowering the MeHg body content and thus the concentration at the site of action. In severe cases the first choice is hemodialysis combined with extracorporeal infusion of chelating agents such as N-acetylcysteine (NAC) or cysteine and oral administration of DMPS- 2,3-dimercapto-1-propane sulfonate (Aposhian, 1998).

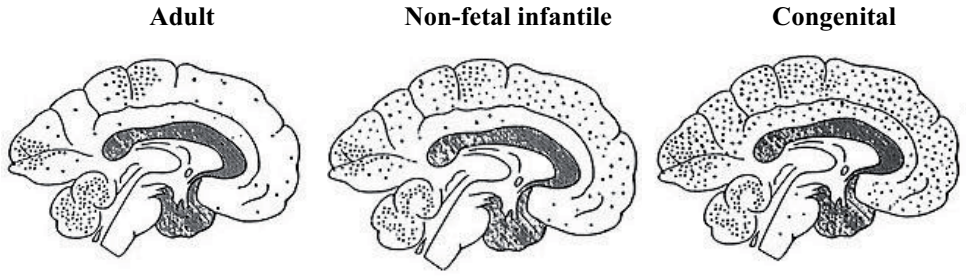

Fig.1. Pathology of Minamata disease: Comparison of the distribution of lesions among the adult, non-fetal infantile and congenital Minamata disease (Takeuchi, 1968).

d) Biochemical mechanisms behind MeHg toxicity

Hg is covalently bound to the carbon moiety in MeHg (CH_3-Hg^+). The carbon-Hg bond is chemically stable because of the low affinity of Hg for oxygen. MeHg exists only at a very low concentration as a free, unbound cation in biological systems (Hughes, 1957) and the chloride form is highly soluble in organic solvents and lipids. MeHg is found to bind to protein -SH groups of amino acids such as cysteine which is also present in glutathione (Clarkson, 1993). This affinity of Hg for sulphur and sulfhydryl groups is a major factor underlying the biochemical properties of MeHg. The binding of MeHg to -

SH groups of proteins in membranes and enzymes may interfere with the membrane structure and function. This in turn, results in interference with the enzyme activity of several cellular targets. The main mechanisms involved in MeHg toxicity include:

- inhibition of macromolecule synthesis (DNA, RNA and protein),
- microtubule disruption,
- increase in intracellular Ca^{2+} with disturbances of neurotransmitter function,
- oxidative stress,
- excitotoxicity, secondary to altered glutamate homeostasis.

MeHg reacts with DNA and RNA resulting in changes in secondary structure of these molecules (Gruenwedel and Lu, 1970). This could give rise to disturbances in the synthesis of protein, DNA and RNA and has been reported by several authors using cells (Choi *et al.,* 1980; Gruenwedel and Cruikshank, 1979), mice (Chang *et al.,* 1972) and rats (Farris and Smith, 1975) as a model. Inhibition of protein synthesis (Verity *et al.,* 1975), as well as increased protein synthesis due to reactive astrogliosis has been reported after *in vivo* exposure to MeHg (Brubaker *et al.,* 1973). Using neuron enriched fractions from rat brain it has been shown that *in vivo* exposure to MeHg may induce temporary changes in protein and RNA synthesis (Syversen, 1977, 1982), indicating that some types of neurons may be able to repair the initial reduction in protein syntheis. It is still open for speculation as to whether such repair-mechanisms may play a role in the cellular selectivity of MeHg's action in the nervous system. Due to the ability of MeHg to react with DNA and RNA, it has been proved to be mutagenic (Ramel, 1972). It can also cause chromosomal aberrations in human lymphocytes (Skerfving *et al.,* 1974) and leukocytes and in bone-marrow cells from MeHg exposed cat (Miller *et al.,* 1979).

Inhibition of polymerization of tubulin by MeHg (Fig. 2.) is among major mechanisms behind MeHg toxicity (Sager *et al.,* 1982). Microtubular fragmentation has been reported in cultured primary rat cerebellar granular neurons at a MeHg concentration of 0.5-1μM (Castoldi *et al.,* 2000). Since microtubules participate in cell division, their fragmentation by MeHg results in antimitotic effects. Fragmentation of microtubule by MeHg results in inhibition of neuronal migration and causes degeneration of neuritis (Choi *et al.,* 1980).

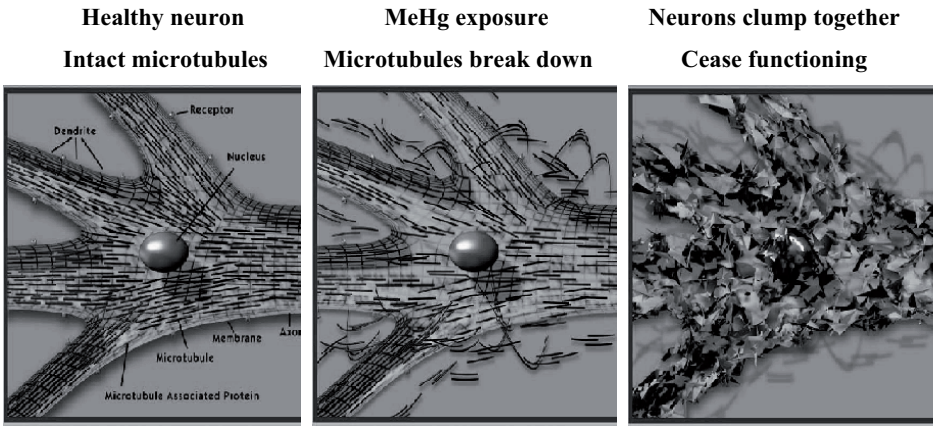

Fig. 2. Methylmercury targets the cell structure and disturbs neuron migration (Mercury and the developing brain, Report of the National Environmental Trust for Clean the Air).

MeHg depolarizes the presynaptic membrane which increases the Na^{2+} and decreases K^{+} ion concentration. This causes disruption of Ca^{2+} homeostasis leading to increased intracellular Ca^{2+} concentration (Komulainen and Bondy, 1987; Oyama *et al.,* 1994). Blockers of voltage dependent Ca^{2+} channels prevent the appearance of neurological signs (Sakamoto *et al.,* 1996). The damaged cell membranes due to increased Ca^{2+} levels are associated with disruption of neurotransmitter signaling. Disturbances in neurotransmitter concentrations (Fig. 3.), such as increased release of dopamine, glutamate, GABA-gamma amino butyric acid, glycine, choline (Bondy *et al.,* 1979) and acetylcholine (Juang, 1976) also occur after MeHg exposure. Inhibition of uptake of excitatory amino acids like glutamate and aspartate by astrocytes has been one of the major mechanisms behind MeHg-induced neurotoxicity (Aschner *et al.,* 1993, 2000). Antagonists of the N-methyl-D-aspartic acid receptor have been reported to inhibit the toxic effects of MeHg (Park *et al.,* 1996).

MeHg also alters the cellular energy metabolism. It affects respiratory control in synaptosomes both *in vitro* and *in vivo* (Fox *et al.,* 1975; Verity *et al.,* 1975). It causes decrease in state 3 and increase in state 4 respirations. Effects on mitochondrial respiration in the brain have been reported to occur at 10-100µM MeHg (Verity *et al.,* 1975; Von Burg *et al.,* 1979). This causes inhibition of glycolysis and tri-carboxylic-acid cycle activity and decrease in adenosine triphosphate utilization. Since nervous

tissue is strictly dependent on glucose, high oxygen utilization and excitability renders it especially susceptible.

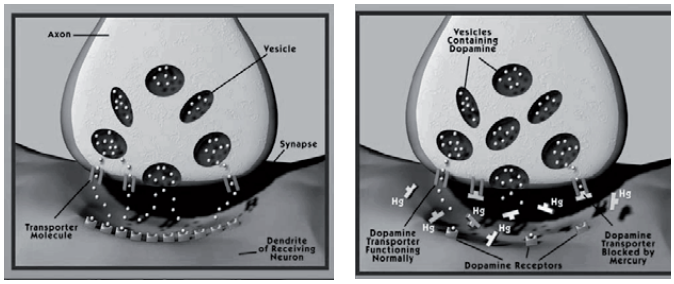

Fig. 3. Methylmercury blocks the release of neurotransmitters such as dopamine (Mercury and the Developing Brain, Report of the National Environmental Trust for Clean the Air).

Alterations in the heme biosynthetic pathway and porphyrinurias have also been reported after MeHg exposure (Woods and Fowler, 1977; Woods *et al.,* 1991). These porphyrins have been reported to catalyze the formation of reactive oxygen species (ROS) (Woods and Sommer, 1991). Since the adult human brain consumes >20% of the oxygen utilized by the body and comprises only 2% of the body weight, the ROS are generated at high rates during oxidative metabolism of the brain (Clarke and Sokoloff, 1999). Disruption of redox cellular homeostasis by an excess of ROS formation leading to cumulative oxidative stress appears to be an important contributor to MeHg neurotoxicity (Fig. 4. and 5.). MeHg is known to induce oxidative stress (Sarafian, 1999) both *in vitro* and *in vivo* which is evidenced by membrane peroxidation (Ali *et al.,* 1992; Fujimoto *et al.,* 1985; LeBel *et al.,* 1990; Sarafian and Verity, 1990; Shanker and Aschner, 2003; Taylor *et al.,* 1973; Yee and Choi, 1994; Yonaha *et al.,* 1983). The production of ROS by MeHg exacerbates the toxicity by facilitating cell death through apoptotic pathways. Inhibition of glutathione peroxidase (GPx) by MeHg further potentiates lipid peroxidation. Conversely, several studies have demonstrated partial amelioration of MeHg toxicity in the presence of antioxidants by inhibition of ROS (Gasso *et al.,* 2001; Sanfeliu *et al.,* 2001; Shanker and Aschner, 2003). A major source of MeHg-increased ROS generation may be the mitochondrial electron transport chain. The damaged mitochondrion increases oxidative stress, leading to decrease in defense mechanisms such as reduced glutathione (GSH) content and excitotoxic damage. Both these triggered chains of events interact leading to

amplification of toxicity. In addition, inhibition of protein synthesis and microtubule assembly adds to the serious consequences on neurotransmission and neural cell development. Interaction of MeHg with respiratory enzyme complexes and its ability to cause oxidative damage in the mitochondria has been reported by Verity *et al.,* (1975) and Yee and Choi, (1996). It has also been reported that MeHg causes increased ROS generation after stimulation of the ubiquinol: cytochrome c oxidoreductase complex in isolated mitochondria (Yee and Choi, 1996). Furthermore, blockage of the mitochondrial transition pore by cyclosporin A in brain synaptosomes has been reported to lower MeHg-induced ROS production (Myhre and Fonnum, 2001). MeHg binds to GSH which is one of the principal endogenous antioxidants and this binding is reported to be responsible for the excretion of MeHg. Decreased GSH levels usually parallel increased oxidative stress by MeHg (Sarafian and Verity, 1990; Sarafian *et al.,* 1994; Vijayalakshmi and Sood, 1994). The upregulation (Li *et al.,* 1996) or the induction of an increased synthesis of GSH (Choi *et al.,* 1996) has been reported to be neuroprotective against MeHg-induced neurotoxicity.

Oxidative stress and antioxdant defences **Mechanisms of oxidative stress**

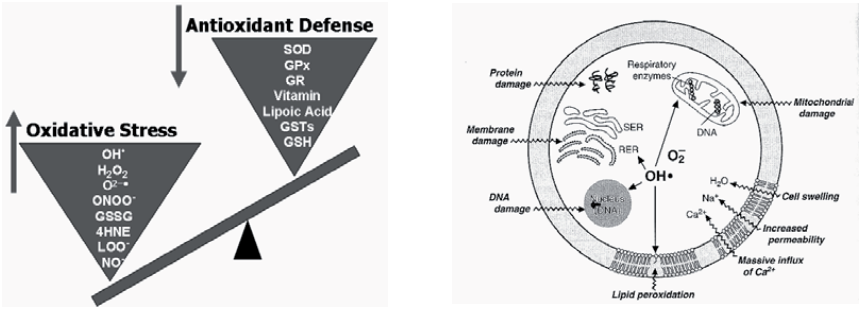

Fig. 4. Methylmercury causes increase in oxidants and decrease in antioxidants (James, 2005).

Other effects of MeHg include inhibition of spermatogenesis (Homma-Takeda *et al.,* 2001), immune responses (Koller, 1980), changes in the axonal flow in the sciatic nerve (Wakabayashi *et al.,* 1976) and decreased activity of lysosomal enzymes (Vinay and Sood, 1991).

e) Advantages and disadvantages of brain cells and cell cultures: implications for neurotoxicity

Brain tissue is a heterogeneous system comprising of two distinct compartments known as neurons and glia (Van den Berg *et al.,* 1969). The neuron is the functional unit responsible for transmitting and processing information in the nervous system (Augustine, 2004). The glial cells play a significant role by supplying neurons with a number of metabolites and precursors. In the cerebrum, the neurons are greatly outnumbered by glial cells (Nedergaard *et al.,* 2003). In contrast to cerebrum, the cerebellum is one of the most evolutionary primitive brain regions where neurons outnumber the glial cells (Andersen *et al.,* 1992). Of three main types of glial cells- microglia, oligodendrocytes and astrocytes, the astrocytes regulate the chemical environment of the brain. Due to the heterogeneity and complexity of the brain, the molecular mechanisms leading to neurological abnormalities following exposure to neurotoxic substances are difficult to study in the CNS *in vivo*. In a heterogeneous system numerous factors such as neural, hormonal, and hemodynamic are not under experimental control. Hence, a simplified model, such as tissue culture, is indispensable as a tool for studying the cellular and molecular mechanisms of neurotoxicity produced by a variety of compounds. Tissue cultures allow direct evaluation of the effects of toxic agents on the CNS as toxins can be easily added and withdrawn from the cultures, and long term effects may be studied. In addition, tissue culture systems also could be very useful in studying the modification of the effects of neurotoxic substances since they can provide clues to reduce the effectiveness of neurotoxic agents and new insights into the mechanisms of their action.

Primary cultures are prepared by taking cells directly from an organism in contrast to cell lines which originate from transformed cells (e.g. tumor cells). The primary cells are obtained after an initial mechanical and/or enzymatic dissociation of the tissue and consist of normal diploid cells. Once the cellular purity, content and degree of maturation have been established, monolayer cultures of astrocytes and neurons afford a host of advantages over *in vivo* techniques. Cell morphology, protein synthesis and release (myelin), energy metabolism, receptor interaction, neurotransmitter uptake and release, electrophysiological studies utilizing patch clamping as well as electrolyte and non-electrolye uptake and release can be easily studied using cell cultures. Direct effects of chemicals on a relatively homogeneous population allows for study of

specific aspects of the growth and differentiation of cells, as well as the kinetics of uptake and metabolism of the parent compound. The culture model also makes it possible to study regional specialization, and can be extended to study astrocytic-neuronal interactions by co-culturing astroglial and neuronal cells as two separate monolayers in the same culture dish (at a distance from each other). The system may provide information on how astrocytes respond to the neuronal environment, and vice versa, and how astrocytic homeostasis affects neuronal development and function. Primary cell cultures are advantageous over the cell lines as their properties and metabolism closely resemble that of corresponding cells *in vivo*. On the other hand, cell lines are economical, easy to grow and handle and possess several features of nerve and glial cells. For example dibutyryl cAMP agents produce morphological differentiation in both glioma and normal embryonic glial cells (Vernadakis and Nidess, 1976).

Although the use of cultured astrocytes and oligodendrocytes in toxicity testing has emerged as a powerful tool to evaluate the responses of target cells at the cellular and molecular level, one must bear in mind some intrinsic pitfalls of culture systems. For example for the use of primary cells, the timing is very crucial to obtain viable cells. In mice, the neurogenesis is almost completed at the time of birth. Only the interneurons in cerebellar cortex such as granule neurons develop from day 2 until 15 after birth. Therefore, the tissue must be at a particular developmental stage which favors the cultivation of a particular cell type. Such developmental stage requirements might be a challenge if cellular maturity or age is an important aspect of the toxicity mechanism. In addition, the cells can undergo varying degrees of differentiation. From the toxicologic viewpoint, the extent of cellular differentiation must be carefully defined, since multiple phenotypic states may exhibit different toxicologic responsiveness and the phenotypic expression of cells in culture may itself be the target of toxic insult. Since glial cells and neurons promote mutual functional differentiation of each other, cell types resulting from purified cultures may result in undifferentiated cells or cells with altered differentiation, making results difficult to interpret. The sensitivity of undifferentiated cells to neurotoxicants has not been assessed.

The effect of MeHg in cell cultures depends on the total biomass present. It was shown by Furukawa *et al.,* (1982) that cytolethal sensitivity of the mitotic cells to MeHg was equal to that of exponentially growing cells. Gülden *et al.,* (2001), reported that for a

variety of toxic compounds including MeHg, the EC_{50} values increased with increasing cell concentration. They concluded that cell binding can significantly affect the availability of compounds *in vitro* and thus their toxic potencies and toxic equivalency factors. In addition, it was also reported that the presence of albumin concentrations in medium greatly influences the toxic potency of MeHg (Seibert *et al.,* 2002). This may be due to the presence of extra binding sites for MeHg, which can dilute the effective concentration. Therefore, caution should be taken when extrapolating results from *in vitro* experiments to the whole brain. Moreover, diverse cell types exhibit different sensitivity towards MeHg, which might be dissimilar from the *in vivo* situation. These differences in sensitivity can also be seen within different species. For example, the monkey brain resembles the human brain with respect to effects of MeHg exposure as the calcarine cortex is highly vulnerable and changes in vision are detected early (Berlin *et al.,* 1975b). However, in rats, peripheral neuropathy is most commonly observed (Cavanagh, 1973). The characteristic effect of MeHg, i.e. loss of granular cells preceding Purkinje cell loss is found in rats (Klein, 1972), mice (MacDonald and Harbison, 1977) and rabbit cerebellum (Jacobs *et al.,* 1977). However in rats, the haemoglobin has an extra chain, so there is a greater retention of MeHg in blood which to some extent limits the utility of rat as a model. Therefore, use of appropriate model is crucial for studying MeHg toxicity.

In addition, it still needs to be determined whether the receptor phenotype observed *in vitro* accurately reflects the *in vivo* situation. The observed phenotype might be a function of culture conditions or inherent astrocyte heterogeneity. While chemicals can be easily added and withdrawn from the cultures, and their effects directly probed in culture systems, caution should be used when correlating effects occurring *in vitro* to those which are observed in the intact animal, where additive interactions are likely to occur. One must remember several concepts:

- A number of different, sometimes competing, processes influence the ability of a toxin to attack and destroy specific cells. Metabolism of the administered agent by a non-target cell or tissue may be responsible for bioactivation and/or detoxification of the compound or its metabolite, affecting the vulnerability of the cells to the neurotoxin.

- A cell culture is many fold more homogeneous and simpler than any tissues, in particular the CNS. Removal of many cell types and barriers can facilitate diffusion or even active transport of the compound or its metabolite, limiting or enhancing toxicity by determining at which sites the toxin can reach sufficiently high concentrations to interfere with vital cellular processes.
- The capacity of the cell to repair or replace damaged organelles or enzymes can also be critical in determining cell survival after toxic insult and may obviously depend on neighboring cells and physical barriers, which may be absent in the culture altogether. Accordingly, characteristics which are described as advantageous in particular circumstances may be described as disadvantageous in others.

f) Neuronal- glial interactions with respect to MeHg neurotoxicity

Neuronal-glial interactions play an important role in MeHg neurotoxicity (Fig. 5.). Neuronal damage in response to MeHg exposure has been suggested to be mediated by astrocytes (Aschner *et al.,* 2007; Morken *et al.,* 2005). Astrocytes support neurons and supply them with various factors which neurons themselves are unable to synthesize. The tripeptide glutathione (γ-glutamyl-L-cysteinylglycine) is the most abundant thiol present in mammalian cells. It is also involved in the disposal of exogenous peroxides by astrocytes and neurons. MeHg toxicity has been reported to be caused by the reduction in the amount of intracellular GSH (Choi *et al.,* 1996; Miura and Clarkson, 1993; Sarafian *et al.,* 1994) which leads to augmentation of ROS formation (Ali *et al.,* 1992; Gasso *et al.,* 2001; Sanfeliu *et al.,* 2001; Sarafian, 1999; Shanker and Aschner, 2003; Sorg *et al.,* 1998; Yee and Choi, 1996). Astrocytes contain higher concentrations of GSH than neurons (Kranich *et al.,* 1996; Sagara *et al.,* 1993). The astrocytes supply the rate limiting precursor molecules, e.g. cysteine, glycine and glutamine to neurons for GSH synthesis (Shanker and Aschner, 2001). Other enzymes which are present only in astrocytes include pyruvate carboxylase (Shank *et al.,* 1985) and glutamine synthetase (Norenberg and Martinez-Hernandez, 1979). Glutamine synthetase is the enzyme required for synthesis of glutamine which is one of the substrates for glutathione synthesis. Glutamine is first converted to glutamate (Sonnewald *et al.,* 1993) by the mitochondrial enzyme phosphate activated glutaminase (Kvamme *et al.,* 1988). Glutamate then binds to cysteine and is converted to γ-glutamylcysteine with the help of the enzyme γ-glutamylcysteine synthetase. The γ-glutamylcysteine is then acted

upon by glutathione synthetase which leads to the formation of glutathione. Glutamine is also an important precursor for amino acids such as glutamate and GABA (Schousboe *et al.,* 1977; Sonnewald *et al.,* 1993). During glutamatergic neurotransmission, glutamate is released from neurons and is mainly taken up by astrocytes (Danbolt, 2001) and this is compensated for by the net flow of glutamine from astrocytes to neurons. The reason for such compensation is that the major anaplerotic enzyme in brain, pyruvate carboxylase, is only present in astrocytes, as pointed out above. The spontaneous release of glutamate and GABA from neurons (Atchison and Hare, 1994) and the inhibition of its uptake in astrocytes (Aschner *et al.,* 2000) are among the major mechanisms of MeHg-induced neurotoxicity. The drain of GABA from neurons to astrocytes is relatively modest (Hertz and Schousboe, 1987) and the glutamine transport is more intense in glutamatergic neurons than cortical neurons and astrocytes (Su *et al.,* 1997). However, in the glutamatergic cerebellar granule cells, glutamate re-uptake might be important for glutamate homeostasis (Olstad *et al.,* 2007).

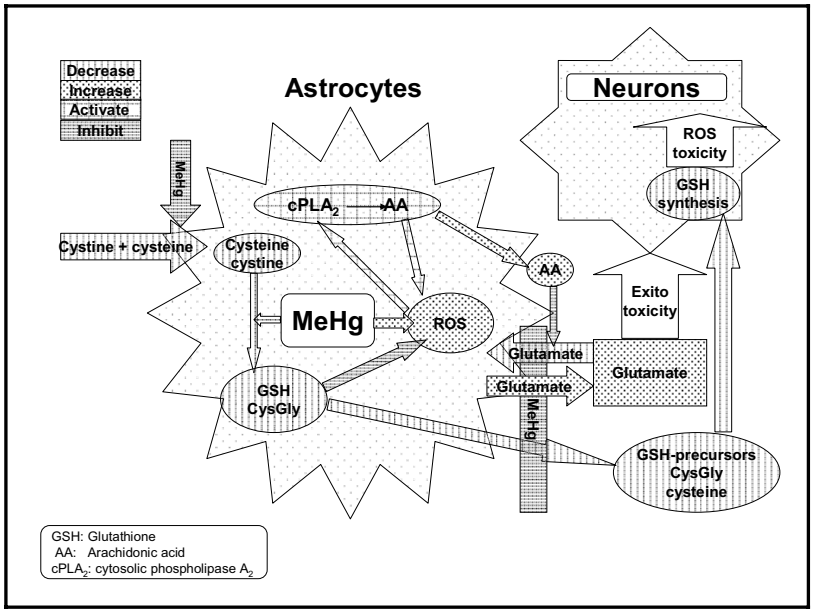

Fig. 5. Neuronal-glial interactions with respect to MeHg neurotoxicity (Modified from Shanker *et al.,* 2005a)

Long chain poly unsaturated fatty acids (PUFA's) such as docosahexaenoic acid (DHA; 22:6*n*-3) are essential for normal brain development (Innis, 1991). Astrocytes can readily release DHA into the extracellular fluid while neuronal DHA is not readily

released (Garcia and Kim, 1997; Kim *et al.,* 1999; Moore, 2001). Neurons cannot produce DHA because they lack desaturase activity (Moore *et al.,* 1991). The transport of DHA from astrocytes to neurons is considered trophic. Astrocytes may protect neurons from low doses of MeHg by upregulation of metallothionein synthesis (Aschner *et al.,* 1997). Upon MeHg exposure, astrocytes also increase local interleukin-6 release that leads to neuroprotection in 3 D brain cell cultures (Eskes *et al.,* 2002). Therefore, the interactions between neurons and glial cells play a critical role in MeHg-induced neurotoxicity.

g) Prevailing conundrums behind MeHg neurotoxicity

Conundrum 1**:** The relationship between GSH and MeHg concentration in brain is unknown. Molecular oxygen is essential for many biological events associated with aerobic metabolism which results in the constant formation of ROS (Powis *et al.,* 1995). Conversely, GSH is the most abundant thiol tripeptide present in mammalian cells for scavenging ROS (Fang *et al.,* 2002; Roberts *et al.,* 1980). Several reports have implicated a critical role of GSH in modulating MeHg neurotoxicity (Choi *et al.,* 1996; Miura and Clarkson, 1993). However, it still remains unclear whether the protection afforded by GSH is due to protection against ROS generated by MeHg or due to the reduction of the intracellular concentration of MeHg. In addition, little is known concerning the relationship between GSH and MeHg concentrations in specific anatomical regions of the brain.

Conundrum 2: The reason for the selective sensitivity of certain brain regions to MeHg remains unknown. MeHg has a high association constant (15<pKa<23) for -SH groups (Carty and Malone, 1979). It can react with any -SH group (Hughes, 1957) leading to conformational changes and thus inhibition of many enzymes. This indicates that more than one mechanism may contribute to the expression of MeHg-induced neurotoxicity. However, the existence of similar enzymes such as acetylcholine esterase in different regions makes it difficult to reconcile this with the specific pattern of neurological damage associated with MeHg, such as in the cerebellar granule cell layer and in the calcarine region of the occipital cortex in humans (Choi *et al.,* 1978; Eto, 1997; Hunter and Russell, 1954; Takeuchi, 1982) and in rodent brains (Nagashima, 1997). Moreover in the cerebellum, which is a major target of MeHg effects, the Purkinje cell layer which tends to accumulate more Hg is spared as compared to the sensitive granule cell

layer (Clarkson and Strain, 2003). Neuronal dysfunction has been proposed to be secondary to disturbances in astrocytes (Allen *et al.,* 2001). However, the sparing of Purkinje cells and the sensitivity of granule cells in the cerebellum cannot be attributed solely to the vulnerability of cerebellar astrocytes towards MeHg, for under these circumstances, both Purkinje and granule cells would be expected to respond in a similar fashion. Therefore, despite considerable scientific efforts, the reason for this selective degeneration of certain areas of the nervous system has not been satisfactorily explained.

Conundrum 3: The effect of DHA on modulation of MeHg-induced neurotoxicity remains unknown. The major dietary route of human exposure to MeHg is via the ingestion of seafood for adults (Clarkson, 1997; Kamps *et al.,* 1972; Spry and Wiener, 1991). The nervous system is also highly enriched in long chain PUFA's which are also provided via consumption of fish and mother's milk (Franco *et al.,* 2006; Manfroi *et al.,* 2004). DHA, in particular, is the most abundant PUFA in the brain which is essential for normal brain function (Kim, 2007; Salem *et al.,* 1999; Uauy *et al.,* 2001). DHA plays a crucial role in diverse cellular functions ranging from controlling the cell body size (Ahmad *et al.,* 2002) and outgrowth of neurites by promoting cell differentiation (Ikemoto *et al.,* 1997) to being antiapoptotic (Akbar and Kim, 2002) and neuroprotective (Martin, 1998). The availability of DHA is dependent upon type and amount of fish consumed and it varies within different epidemiological studies. On the Seychelles (Davidson *et al.,* 1998), fish consumption was reported to be around 12 fish meals/week. On the Faroe Islands (Grandjean *et al.,* 1997) the diet included whale meat and 1-3 fish meals/week. At Minamata and Niigata (Eto, 1997), tuna consumption was around 5mg/day. The discrepancies in the outcomes of the studies on the Seychelles (Davidson *et al.,* 1998; Myers *et al.,* 1997) and the Faroe Islands (Grandjean *et al.,* 1997) have raised the question of whether risks outweigh the benefits arising from the combined exposure to a neurotoxicant and neuroprotectant. Therefore, there is a need to investigate whether DHA supplementation would modulate the susceptibility of neural cells to MeHg exposure. Knowledge of this could assist in assessing the risk/benefit from their exposure.

VII. Aims

The aim of the study was to explore the cellular and molecular mechanisms behind MeHg toxicity. More specifically, we wanted to elucidate the role of MeHg-induced oxidative stress by introduction of agents which are known to influence MeHg neurotoxicity.

1. Evaluate the concentration, time and cell density dependent effects of MeHg on generation of ROS using C6-glial and B35-neuronal cell lines. (Paper 1)

2. To investigate the role of GSH modulation on MeHg-induced neurotoxicity using primary cell cultures of cerebellar neurons and astrocytes. (Paper 2)

3. To determine whether GSH is responsible for the differential sensitivity between the cortical and cerebellar cultures towards MeHg-induced oxidative stress using primary cell cultures. (Paper 3)

4. To investigate the effect of DHA in modulating MeHg-induced neurotoxicity in C6-glial and B35-neuronal cell lines. (Paper 4)

5. To investigate the effect of DHA in modulating MeHg-induced neurotoxicity in primary astrocytes and neurons from the cerebellum. (Paper 5)

VIII. Materials and methods

a) Primary cell cultures and cell lines

Cerebellar granule cells are predominately glutamatergic, whereas cortical neurons are mainly GABAergic (Hertz and Schousboe, 1987; Yu *et al.,* 1984). Glutamatergic neurons were prepared from the cerebella of 7-day-old mice according to the method described by Schousboe *et al.,* (1989). GABAergic neurons were prepared from the cerebra of the pups obtained from pregnant mothers at 15 days of gestation (Schousboe *et al.,* 1989). Addition of cytosine arabinoside to the culture medium between 24 to 48 hrs after preparation of neurons reduced the glial cell concentration to 5% (Messer, 1977). The cytosine arabinoside acts by being cytotoxic to the dividing glial cells whereas neurons which are in the post-mitotic stage are spared (Hertz *et al.,* 1989. The neuronal cultures were used one week post isolation.

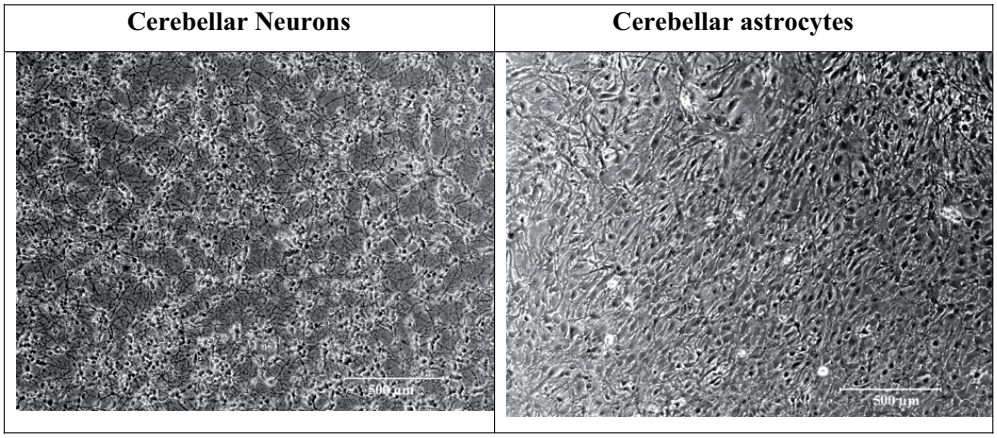

Fig. 6. Primary cell cultures from the cerebellum prepared from 7-day-old mice. Phase contrast images taken with Nikon Eclipse TE 2000-S microscope equipped with SPOT RT Digital Camera.

Primary astrocytes express a large variety of ion channels, neurotransmitter receptors and are glial fibrillary acidic protein (GFAP) specific (Verkhratsky and Steinhauser, 2000). Cerebellar and cortical astrocytes were prepared from the respective brain part according to the method of (Hertz *et al.,* 1989). For the cerebellar astrocytes, 7-day-old mice were used whereas for cortical astrocytes, pups were used within 24 hrs of birth. The method used for the preparation of cerebellar astrocytes has been estimated to yield about 95% astrocytes (Hertz *et al.,* 1989). Minor contaminations in these

cultures are phagocytic cells and oligodendrocytic precursors. The regular medium changes and use of dibutyryl-cAMP reduces the contamination from phagocytic cells. The insignificant amount of oligodendrocytes is due to their formation between three weeks to three months. Astrocytes were used for the experiment 21 days post isolation. Poly-D-lysine coating was used for all the cell cultures to improve the attachment of cells. Cell lines are widely used as a model to study neurotoxicity since they are economical, easy to grow and possess several features of nerve and glial cells. Therefore, both C6-glial (Benda *et al.,* 1968) and B35-neuronal (Schubert *et al.,* 1974) cell lines from rat brain were selected to represent each cell type in the present study.

Cell type

- In Paper 1 and 4, C6-glial and B35-neuronal cell lines have been used.
- In paper 2, 3 and 5, primary neurons and astrocytes from cerebellum were used. In addition, cortical neurons and astrocytes were used in paper 2 where dialyzed serum was used for culturing cortical neurons to reduce the glutamate content in the media.

Cell density

- In Paper 1 and 4: For C6 cell line, 60,000 cells per well and for B35 cell line, 160,000 cells per well were seeded in 24 well plates.
- For Paper 2: Kainic acid was used for culturing cerebellar neurons and the cells were seeded at a density of 0.5 cerebella / 24 well culture plate (astrocytes) and 0.8 x 10^6 cells / ml (neurons).
- For Paper 3 and 5: Kainic acid was not used for culturing cerebellar neurons since it did not significantly inhibit the GABA content in neurons (Sonnewald *et al.,* 2004, 2006). The cells were seeded at a density of 1 cerebella / 24 well culture plate (astrocytes) and 1.5 x 10^6 cells / ml (neurons).

b) Fluorescence microscopy

Fluorescence reflects the property of some atoms, molecules or solids to absorb energy and get excited to a high energy level and then subsequently emit light of longer wavelength while coming back to the lower energy level. The intensity of fluorescence is very weak in comparison with the excitation light. Fluorochromes are stains that attach themselves to visible or sub-visible molecules and have highly specific biological targets. The technique of fluorescence microscopy has become an

essential tool in biology and the biomedical sciences. The application of an array of fluorochromes has made it possible to identify cells and sub-microscopic cellular components with a high degree of specificity amid non-fluorescing material. Through the use of multiple fluorescent labels, several target molecules can be identified simultaneously. In many cases it allows work on live cells where it is important to prevent background staining and photobleaching in order to improve the resolution. In a fluorescent microscope, light from the arc lamps or lasers passes thorough field and aperture diaphragms and then into a cube which contains a set of interference filters, dichoric mirror, barrier filter and excitation filter. After passing through the objective and being focused onto the specimen, the specimen gets excited. The reflected fluorescence is then filtered by the emission filter which is then sent to the eyepieces or detector. The oxidative effects of MeHg were detected with the help of fluorescent probes CMH_2DCFDA- chloro methyl derivative of di-chloro di-hydro fluoresceindiacetate and MCB- monochlorobimane. The fluorescent probes CMH_2DCFDA (485/535) and MCB (360/465) can be used for detecting the ROS and GSH content in live cells. The oxidation of the CMH_2DCFDA dye by ROS yields a fluorescent product, 2′, 7′-dichlorofluorescein (DCF), which is retained inside the cell (Shanker *et al.,* 2004; Shimazawa *et al.,* 2005; Liu *et al.,* 2001). It has been reported that CM-H_2DCFDA dye is more useful than other DCF dyes since the addition of a chloromethyl group gives a better retention and more reliable fluorescent signals in live cells as compared to other DCF dyes (Liu *et al.,* 2001). The content of reduced GSH can be determined by MCB (Bellomo *et al.,* 1997) as it can diffuses passively across the plasma membrane into the cytoplasm where it binds with the reduced form of GSH leading to formation of blue fluorescent adducts (Haugland, 1996). The Fluorescent images of the cell cultures were taken with Nikon Eclipse TE 2000-S microscope which was equipped with SPOT RT Digital Camera. The excitation filters B-2A and UV-2 E/C*(DAPI) were used at the microscope and the emitted fluorescence was detected at Victor$^3{}_{TM}$ 1420 Multilabel counter by scanning the whole well using the desired excitation and emission wavelengths.

c) Gas chromatography-flame ionization detection

Chromatography is a technique for separating chemical substances that relies on differences in partitioning behavior between a flowing mobile phase and a stationary phase to separate the components in a mixture. The sample is carried by a moving gas

stream through a tube packed either with a finely divided solid material or which may be coated with a film of a liquid. The substances having the greater interaction with the stationary phase are retarded to a greater extent and consequently separate from those with smaller interaction. As the components elute from the column they can be quantified by a detector and/or collected for further analysis. The flame ionization detector (FID) uses an air-hydrogen flame to break the components to produce ions. The ions are collected on a biased electrode and produce an electrical signal. The greater the concentrations of the component, the more ions are produced and greater is the current. Because of its simplicity, sensitivity, and effectiveness in separating components of mixtures, gas chromatography is one of the most important tools in chemistry. It is widely used for quantitative and qualitative analysis of mixtures and for the purification of compounds. Since in paper 4 and 5, the cells were supplemented with the fatty acid DHA, it was important to detect the amount and type of intracellular fatty acids after DHA exposure.

d) Other methods

Cytotoxicity: Determined by changes in mitochondrial dehydrogenase activity using colorimetric 2.4 mM MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide] reduction assay (Carmichael *et al.,* 1987; Dahlin *et al.,* 1999).

Cell-associated MeHg: 99% pure ^{14}C-labeled MeHg (20 mCi/mmol) obtained from American Radiolabeled Chemical (St.Louis, Mo, USA) was used and the radioactivity was counted in a 1450 Micro Beta Trilux Liquid scintillation counter (Wallac, Perkin Elmer Life Sciences, Norway).

Estimation of protein: Total cellular protein was used as a measurement of biomass and all the measurements were corrected with respect to the biomass present. Protein concentration was determined by the method of Lowry *et al.,* (1951) using Folin reagent with bovine serum albumin as a standard.

IX. Data analysis

All results are given as mean ± standard deviation. Differences between groups were analyzed statistically with one-way ANOVA followed by the least significant difference or Tukey *post hoc* test for multiple comparisons and $p<0.05$ was considered statistically significant. In addition, a two or three-way ANOVA was carried out to evaluate the interactive effects between different parameters.

X. Summary of papers

a) Paper 1

For evaluating the concentration-, time- and cell density- dependent effects of MeHg, three different MeHg concentrations (5, 10 and 25μM) and time periods (30, 50 and 90 min) and two different cell densities (day3 v/s day4) were selected. For this purpose, C6-glial and B35-neuronal cell lines were selected. The MeHg-induced ROS was measured by the fluorescent probe, CMH_2DCFDA. The cell-associated MeHg was measured with ^{14}C-labelled MeHg. For C6 cells, a significant increase ($p<0.05$) in MeHg-induced ROS was observed at 10 and 25μM MeHg for both 30 and 50 min time intervals. For B35 cells, a significant increase in ROS was observed only at 25μM MeHg. The amount of ROS produced with 25μM MeHg varied significantly ($p<0.001$) at different time periods. For both the cell lines, significant cell density-dependent differences ($p<0.05$) were observed at 10μM MeHg treatment for 50 minutes. A concentration-dependent increase in cell associated-MeHg provided an explanation for increased ROS at 30 and 50min. However, the cell density dependent differences in ROS were not due to differences in cell associated-MeHg. Therefore it was concluded that special attention should be focused upon concentration, exposure time and cell density for assessing MeHg-induced ROS effects by fluorescence.

b) Paper 2

In order to evaluate the effect of GSH on MeHg-induced cytotoxicity, the intracellular GSH content was modified by pretreatment with NAC or di-ethyl maleate (DEM) for 12 hrs. For this purpose, primary cell cultures of cerebellar neurons and astrocytes were used. ROS and GSH were measured using the fluorescent indicators CMH_2DCFDA and MCB. Cell-associated MeHg was measured with ^{14}C-radiolabeled MeHg. Mitochondrial dehydrogenase activity was detected by MTT. The MTT timeline study was also performed to evaluate the effects of both the concentration and duration of MeHg exposure. Treatment with 5μM MeHg for 30 min led to significant ($p<0.05$) increase in ROS and reduction ($p<0.001$) in GSH content. Depletion of intracellular GSH by DEM further increased the generation of MeHg-induced ROS in both cell cultures. Conversely, NAC supplementation increased intracellular GSH and provided protection against MeHg-induced oxidative stress in both cell cultures. MTT studies also confirmed the efficacy of NAC supplementation in attenuating MeHg-induced cytotoxicity. The

cell-associated MeHg was significantly ($p<0.02$) increased after DEM treatment. In summary, depletion of GSH increased MeHg accumulation and enhanced MeHg-induced oxidative stress, and conversely, supplementation with GSH precursor protected against MeHg exposure *in vitro*.

c) Paper 3

The role of GSH behind the differential sensitivity between the cerebrum and cerebellum towards MeHg-induced toxicity was investigated. MeHg-induced oxidative insult was determined in primary neuronal and astroglial cell cultures of both cerebellar and cortical origins. The intracellular GSH content was modified by pretreatment with NAC or DEM. The ROS and GSH were measured with the fluorescent indicators, CMH_2DCFDA and MCB. The cell associated-MeHg was measured with ^{14}C-radiolabeled MeHg. The cerebellar cell cultures were more vulnerable to ROS ($p<0.02$) than the cortical cell cultures after MeHg, NAC or DEM treatment.

A trend towards significant interaction between origin×MeHg×pretreatment was observed only for the dependent variable, ROS (astrocytes $p<0.06$; neurons $p<0.001$). For GSH, a significant interaction between origin×MeHg was observed only in astrocytes ($p<0.05$). The increased content of GSH in cortical astrocytes as compared to cerebellar astrocytes accounted for the increased ROS production in cerebellar astrocytes. However, the similar content of GSH in cortical and cerebellar neurons after MeHg exposure did not provide an explanation for the increased susceptibility of cerebellar neurons. The cell associated-MeHg increased when cells were treated with DEM, and the cerebellar cultures were significantly different ($p<0.05$) from the cortical cultures. In summary, GSH modulation influenced MeHg-induced toxicity, and partially explained the differential sensitivity between the cortical and cerebellar cultures.

d) Paper 4

It is important to identify the biochemical mechanisms involved with fatty acids and MeHg exposure for understanding how fish consumption could modulate MeHg-induced neurotoxicity. For this purpose, the effect of DHA in modulating MeHg-induced neurotoxicity was investigated in C6-glial and B35-neuronal cell lines. Increased DHA content in both the cell lines after 24 hr supplementation was measured by gas chromatography. Decreased mitochondrial activity evaluated by MTT reduction indicated that 10μM MeHg treatment for 50 min led to a significant ($p<0.001$) and similar decrease

in MTT activity in both cell lines. However, DHA pretreatment led to more pronounced depletion ($p<0.05$) in the MTT activity in C6 cells as compared to B35 cells. Upon DHA and MeHg exposure, the C6 cells exhibited a more apparent decrease in GSH and increase in ROS ($p<0.001$) as compared to B35 cells. The cell associated-MeHg measurement using ^{14}C-labelled MeHg indicated a decrease ($p<0.05$) in MeHg accumulation upon DHA exposure in both cell lines. These findings provide experimental evidence that although pretreatment with DHA reduced cell associated-MeHg, it caused increased ROS ($p<0.001$) and GSH depletion ($p<0.05$) in C6 cells.

e) Paper 5

The following reasons prompted us to ascertain the effects of DHA on MeHg-induced neurotoxicity in primary cell cultures. Firstly, the DHA exposure increased the MeHg-induced ROS in tumor cell lines. We wanted to investigate whether primary cell cultures would respond similarly to DHA exposure. Secondly, the B35 neuronal cell line behaved differently from primary neuronal cells with respect to MeHg exposure. Therefore, the identification of these effects in primary cell cultures would help us in understanding the effect of potential natural modulators such as DHA in influencing the toxicity of fish-bound MeHg. This may improve the risk/benefit assessment of a MeHg-containing fish diet. For this purpose, primary neuronal and astroglial cell cultures from cerebellum were selected. After individual or combined treatment with MeHg (10μM) and DHA (30 and 90μM), the neurons differed significantly ($p<0.001$) from astrocytes exhibiting increased ROS production and decreased MTT activity. After MeHg and 30μM DHA treatment there were no changes in MTT or GSH content but significant decrease ($p<0.001$) in ROS was observed in both the cell types when compared to MeHg alone. The cell associated-MeHg measurements indicated reduced MeHg-accumulation in both cell types ($p<0.05$) upon 30μM DHA exposure. Therefore it was established that DHA pretreatment effectively reduces cell-associated MeHg and prooxidant response from MeHg in both cerebellar astrocytes and neurons. This observation supported the hypothesis that fish-derived nutrients can possibly offer neuroprotection against MeHg-induced neurotoxicity.

XI. Discussion

Though considerable headway has been made in elucidating the effects of MeHg on the nervous system, it still remains an element of mystery. The underlying processes behind its mechanism of action are not very well understood. The reason for its selective sensitivity towards certain brain regions remains unknown. In addition, little is known concerning the cellular uptake of MeHg in various brain regions.

The considerable risk to the public from MeHg-contaminated food has raised the concern about the safe dose of ingested MeHg especially for pregnant women. Various regulatory agencies in the past have stressed uncertainities in their documentation regarding the effects of MeHg. The Joint FAO/WHO Expert Committee on Food Additives had reported in 1978 that ''*foetus may be more susceptible to MeHg toxicity than the adult*'' (JECFA, 1978). The workshop organized by Committee on Environmental and Natural Resources, Office of Science and Technology Policy and The White House in 1998 stated ''*These studies have provided valuable new information on the potential health effects of MeHg but significant uncertainties remain because of issues related to exposure, neurobehavioral endpoints, confounders and statistics, and design*'' (NIEHS, 1999). Later in 2000, the National Academy of Sciences committee reported that ''*60,000 children in the United States were at risk as a result of prenatal exposure''* (National Research Council, 2000). However, they failed to provide any justification or explanation for that conclusion (Clarkson *et al.,* 2003). Hence there is a possible discrepancy between the estimated effect of MeHg and its risk assessment. The issue which is a dilemma for the consumers and regulatory authorities is whether fish consumption should be encouraged for its nutritional benefits to the developing brain or should it be discouraged for the possible adverse effects of MeHg on the developing CNS? This suggests that there is a need to assess dietary nutrients as well as neurotoxic exposures in determining the risks and benefits of fish consumption (Myers *et al.,* 2007). Therefore studies directed towards estimating the effect of modifiers such as essential nutrients available from seafood may provide a better assessment of risk from a contaimated fish diet.

The work presented in this thesis attempts to explore the mechanism of action of MeHg. The cell culture models provide excellent tools to investigate the mechanistic pathyways since the effects on individual cell type could be determined. The present

work investigated the effects of MeHg on oxidative stress and identified the role played by GSH in modifying these effects. The differences in the MeHg-induced oxidative stress related effects in cerebellar and cortical cultures were reported. The cellular uptake of MeHg in these cell cultures was determined and correlated with the oxidative stress related effects. In addition, it identified the biochemical mechanisms behind DHA and MeHg exposure where DHA pretreatment effectively reduced cell-associated MeHg and decreased MeHg-induced ROS in primary cell cultures.

It has been known that toxicity of MeHg is dependent upon the amount of biomass (Furukawa *et al.,* 1982; Gülden *et al.,* 2001; Seibert *et al.,* 2002). Therefore, for all the experiments special attention was focused upon the use of consistent cell densities. In addition, for evaluating the effects of MeHg, three different concentrations and exposure intervals were selected to find out the optimum dose and time interval for the experiments. The dose of 5 or 10μM MeHg for 50min was selected for further experiments as consistent increase in ROS and cell associated-MeHg was observed for this exposure. The 10μM dose was used for estimating the toxicity in cell lines and 5μM MeHg was used for primary cell cultures due to increased sensitivity of primary cell cultures as compared to cell lines. However, in paper 5, a dose of 10μM MeHg was used for primary cell cultures treated with DHA. This was attributable to introduction of small amounts of fatty acid free bovine serum albumin (FAF-BSA) along with DHA which diluted the effect of MeHg.

The well defined MeHg-induced effects on GSH depletion (Yee and Choi, 1996) and ROS generation (Ali *et al.,* 1992; Gasso *et al.,* 2001; Sanfeliu *et al.,* 2001; Sarafian, 1999; Shanker and Aschner, 2003; Shanker *et al.,* 2004, 2005b; Sorg *et al.,* 1998; Yee and Choi, 1996) were corroborated by primary cell cultures used in the presented papers. In papers 2 and 3, the effects of GSH modulation by NAC or DEM provided an important insight into the role of GSH in modulating the effect of MeHg-induced neurotoxicity in cerebellar and cortical cell cultures. Changes in intracellular MeHg content with GSH modulation provided an explanation for the increased susceptibility of neurons and cerebellar cultures towards MeHg-induced neurotoxicity as compared to astrocytes and cortical cultures. The papers 4 and 5 addressed the question of public health concern of whether PUFA intake from fish may modulate the MeHg-associated effects. It identified the biochemical mechanisms behind overall effects of fish

consumption (ie, providing DHA and MeHg). These papers may improve the basis for risk/benefit assessment of a MeHg-containing fish diet and our knowledge regarding the maximum permissible dose of MeHg from fish diet.

a) MeHg-induced ROS effects

In paper 1, it was observed that MeHg-induced ROS effects were dependent upon the MeHg concentration and exposure time. At 10µM MeHg in C6 cells, a consistent increase in ROS was observed with increased exposure time. In addition, at 30 and 50min intervals there was an augmentation in ROS with increased MeHg concentration in both cell types. However, at 25µM MeHg there was a consistent decrease in ROS with an increase in exposure period. This decrease in ROS at 25µM MeHg could be due to leakage of dye from the cells. However, since the cell associated-MeHg did not decrease with time, the decreased ROS could be due to transition from the ROS generating state to the apoptic state in cells. Another possibility is the production of reducing agents in cells which could convert the dye to a reduced form. However, the reason for the decreased ROS needs to be determined.

The ROS induction by MeHg was also observed to be dependent upon the cell density. At 10µM MeHg, decrease in fluorescence was associated with increase in cell density and increased cell-associated MeHg. The decreased fluorescence might be explained by the presence of extra binding sites for MeHg which may actually dilute the effective concentration of MeHg inside the cells. Another possibility could be the differences in cell cycle which may lead to changes in the intracellular microenvironment (Zurgil *et al.,* 1996) which affects the ROS generation.

b) Role of GSH in MeHg-induced neurotoxicity

In Paper 2, it was determined that GSH played a major role in the cytotoxic effects of MeHg in cerebellar neurons and astrocytes. In the present study, NAC was effective in preventing ROS after MeHg exposure in both cell cultures. NAC, a relatively simple, nontoxic N-acetyl derivative of cysteine contains a thiol group that is stabilized by acetylation of the amino group (Aremu *et al.,* 2008). NAC induces GSH synthesis (Zafarullah *et al.,* 2003) and has been reported to enhance MeHg excretion in mice (Ballatori *et al.,* 1998) and rats (Aremu *et al.,* 2008). It has been reported that nucleophilic properties of NAC enables it to inactivate free radicals by direct conjugation and reduction (Moldeus *et al.,* 1986). The loss of protective thiol groups

by DEM on the other hand further augmented MeHg-induced ROS. The low GSH content in neurons as compared to astrocytes was associated with increased intracellular MeHg and ROS in this cell type. The increased cell associated-MeHg in neurons as compared to astrocytes was different from what has been previously reported (Berlin *et al.,* 1975a; Hargreaves *et al.,* 1985). However, it provided an explanation for increased susceptibility of neurons as compared to astrocytes. GSH constitutes the most important antioxidant (Cooper, 1997) and the most abundant thiol with concentrations up to 10 mM in mammalian cells (Sies, 1979). Since MeHg is known to bind to GSH, the decrease in GSH is expected to decrease the cell-associated MeHg. However, the binding of MeHg to GSH is among one of the mechanisms for the efflux of MeHg. Therefore, the reported increase in cell-associated MeHg after GSH depletion could be due to inhibition of MeHg efflux from the cells and has been previously observed to occur in endothelial cells (Kerper *et al.,* 1996). Therefore, the limited availability of GSH might be one of the mechanisms responsible for the increased susceptibility of neurons to MeHg toxicity as compared to astrocytes.

c) Role of GSH in the differential sensitivity of MeHg towards cerebellar and cortical cell cultures

In Paper 3, the susceptibility of cells from the cerebrum and the cerebellum towards MeHg-induced toxicity were compared. It was reported that treatment with MeHg was associated with greater depletion of GSH in cerebellar astrocytes as compared to cortical astrocytes (Paper 3). This indicated that cortical astrocytes were more resistant to MeHg-induced depletion of GSH. However, the cortical astrocytes were more vulnerable when their GSH content was modified with NAC or DEM. This result might be attributed to the presence of a higher GSH content in cortical astrocytes possibly indicating that DEM is able to further deplete the available GSH content, whereas NAC is not able to upregulate the already elevated level of GSH in this cell type.

The cortical neurons did not vary significantly from the cerebellar neurons with respect to their GSH content at either of the tested treatments. However, modulation of GSH content with NAC or DEM pretreatment did significantly influence the MeHg-induced GSH loss in neurons. This indicated that an increase in GSH content

with NAC pretreatment prevented MeHg-induced GSH loss in neurons, whereas a decrease in GSH content with DEM pretreatment augmented this loss. We further report that treatment with MeHg was associated with greater production of ROS in cerebellar cultures as compared to cortical cultures. The higher amount of GSH in cortical astrocytes after exposure to MeHg might explain the decreased production of ROS in this cell type. However, the similar content of GSH in cortical and cerebellar neurons after MeHg exposure does not provide an explanation for the increased susceptibility of cerebellar neurons. The increased susceptibility of cerebellar cultures could possibly be explained by the increase in cell associated-MeHg in these cultures as compared to cortical cultures. However, it has been postulated that the differential and selective vulnerability of cells is not simply due to the preferential accumulation of MeHg since Purkinje cells accumulate more Hg than granule cells (Leyshon-Sørland *et al.,* 1994). However, the study by Leyshon-Sørland using the silver nitrate technique detected only Hg^{2+}, while the assessment of ^{14}C labeled-MeHg provided a measurement of the presence of organic Hg only (Koh *et al.,* 2002; Morken *et al.,* 2005; Simmons-Willis *et al.,* 2002). Therefore, estimation of organic Hg in Purkinje and granule cells would provide a better assessment of organic Hg toxicity in these respective regions.

In addition, the present study also points out the importance of using comparable cell densities for the study of MeHg toxicity as an increase in MTT activity was observed in MeHg treated cerebellar astrocytes when seeded from low to high concentrations. In addition, since GSH content only partially explained the differential MeHg-sensitivity of cerebellar and cortical cultures, the role played by factors other than GSH in protecting cortical cultures from MeHg-induced ROS were discussed. Further studies on identifying these potentially selective targets are necessary.

d) Role of DHA in moldulating MeHg-induced neurotoxicity

There have been obvious differences in the dose effect relationships in population studies on the Faroe Islands (Grandjean *et al.,* 1997; Harada, 1995; Keown-Eyssen *et al.,* 1983; Kjellstrom *et al.,* 1986, 1989) and other studies on the Seychelles (Davidson *et al.,* 1998; Myers *et al.,* 1997), Peru (Marsh *et al.,* 1995) and in the US (Mozaffarian and Rimm, 2006; Oken *et al.,* 2005). The availability of nutritional factors such as DHA might influence MeHg toxicity and may explain the

discrepancies from the different studies. Since dietary supplementation with DHA has been reported to enhance problem solving skills at 20 months of age (Willats *et al.,* 1998), boost the normal development of the visual system (Uauy-Dagach and Valenzuela, 1996) and result in better scores on visual and developmental tests (Makrides *et al.,* 1994); there is a need to understand the overall effects of fish consumption (ie, providing DHA and MeHg). The identification of the biochemical mechanisms behind these effects may improve our basis to establish the maximum permissible dose of MeHg from fish diet. For that reason, the susceptibility of neural cells from MeHg and DHA exposure was investigated in paper 4 and 5.

A relatively high concentration of MeHg (10μM) for a short time period (50min) was used in these studies which simulated the high level exposure conditions observed at Minamata (Eto, 1997). The MeHg content observed in these studies is 10-20 times that of the *in vivo* studies (Eto *et al.,* 2001) or the same as in the extended *in vitro* exposure studies (Sakaue *et al.,* 2006).

In paper 4, neural cell lines (C6-glial and B35-neuronal) were used. In the cell lines, the DHA exposure increased the relative DHA fraction of the total fatty acids content from 2-2.5% in control to 13-24% in DHA treated cells which is in agreement with the previous study (Leonardi *et al.,* 2005). The 90μM DHA caused an increase in DHA, arachidonic acid (AA), eicosapentaenoic acid (EPA) and linoleic acid (LA). The LA can be desaturated and elongated to AA (Salem *et al.,* 1999) and this is likely to occur in C6 and B35 cells. In the present study after DHA exposure, the C6-glial cells exhibited a higher content of DHA, AA and LA as compared to the neuronal-B35 cells. This effect may be likely explained by the dependence of neurons on astrocytes for the availibilty of DHA (Moore, 2001).

The DHA exposure led to reduced cell associated-MeHg in both cell types which is in agreement with the previous study in rat brain (Berntssen *et al.,* 2004) where a significant lower degree of MeHg was observed when rats were fed with naturally contaminated fish as compared to feed where MeHg was added chemically to the same matrix.

In cell lines, the combined exposure to MeHg and DHA resulted in a more pronounced reduction in MTT activity and GSH content and augmentation of ROS which was more evident in C6 cells. The B35 cells, on the other hand exhibited an increase in GSH content after MeHg exposure which might indicate an upregulation of protective mechanisms in this cell type. However, this is contrary to the characteristics of primary neurons which are not able to upregulate the synthesis of GSH on their own as they depend on astrocytes for this purpose (Wang and Cynader, 2000). Our present observation of upregulated GSH in B35 cells may explain the previous finding by (Sakaue *et al.,* 2005) of increased viability of B35 cells towards MeHg.

This peculiar behavior of B35 cells prompted us to study the effects of DHA and MeHg exposure in primary cells. This could provide a better understanding of the effects of DHA and MeHg treatment. Hence in paper 5, primary cerebellar cultures (astrocytes and neurons) were used. The amount of total lipid content in primary cells was not modified after exposure to DHA as compared with the cell lines. However, the amount of mg DHA/g test material in primary cells was reported to be similar to the levels previously reported in cell lines. This could be possibly due to the ability of cell lines to accumulate relatively high amounts of lipid droplets as compared to primary cells. In general, the cerebellar astrocytes had higher fatty acid content than neurons in all the tested groups possibly due to the dependence of neurons on the neighboring astrocytes for provision of long-chain PUFA's (Moore *et al.,* 1990; Spector and Moore, 1992).

The primary neurons had higher Hg levels than B35-neuronal cell line in Paper 4 which may provide an explanation for the increased cytotoxicity in primary neurons to MeHg. Moreover, the reduced cell associated-MeHg observed in primary cells after DHA treatment may indicate its protective effect.

In primary cell cultures, the ability of DHA to afford protection against MeHg-induced generation of ROS was established. The combined exposure to DHA and MeHg was effective in attenuating the MeHg effect, reducing ROS levels in cerebellar astrocytes to levels indistinguishable from controls. Similar effects were seen in cerebellar neurons, where treatment with DHA significantly reduced ROS levels in comparison to MeHg treatment alone. These reduced ROS levels after DHA plus MeHg exposure in

cerebellar astrocytes and neurons did not induce any significant increase in MTT or GSH production when compared with MeHg alone. Moreover, significant reduction in MTT and GSH content was not associated with increased ROS production in neurons after 90μM DHA plus MeHg exposure. This effect could be possibly explained by the increased GPx activity by DHA which could lead to disposal of exogenous peroxides by conversion of reduced GSH to the oxidized form, GSSG. In primary cells, the DHA treatment was associated with an increase in EPA and decrease in AA possibly due to retroconversion (Alessandri *et al.,* 2003). The decreased AA levels could also be integrated with an increase in GPx activity (Bryant *et al.,* 1982; Vericel *et al.,* 1992; Weitzel and Wendel, 1993) and be related to protection against MeHg toxicity (Shanker *et al.,* 2002). Therefore, the decrease in AA levels could be the mechanism behind decreased GSH and ROS levels.

Differences between the cell lines and primary cells were observed with respect to the effect of DHA on MeHg toxicity. The cell lines used in paper 4 were derived from cancer tissue and the ability of DHA to augment ROS in these cancer cell lines might be attributed to the anti-tumor property of DHA (Calviello *et al.,* 1998; Igarashi and Miyazawa, 2000; Merendino *et al.,* 2005; Narayanan *et al.,* 2003; Schley *et al.,* 2005). Therefore the choice of cellular model for studying the effects of DHA is critical. The primary cells may be considered as an improved *in vitro* model which provided better insight into the mechanism behind the effect of DHA on MeHg toxicity. However, the primary cell cultures still represent a highly simplified model since these cultures are established using immature cells taken out of their anatomical context. Therefore, caution should be taken when extrapolating results from *in vitro* experiments to the whole brain. Nevertheless, the insight in mechanisms that can be provided from cell cultures may improve our experimental design for *in vivo* studies. As such, the recognition of the protective effects of DHA and identification of its mechanisms via *in vitro* models may improve the basis for a risk/benefit assessment of a MeHg-containing fish diet.

XII. Conclusion

The present findings provide evidence that concentration, time and cell density play a crucial role while assessing MeHg-induced ROS effects (Paper 1).

The confounding factors such as availability of GSH and fatty acids such as DHA effectively influenced MeHg-induced neurotoxicity. The modulation of GSH by NAC or DEM effectively played a major role in the cytotoxic effects of MeHg in primary cell cultures (Paper 2). To a certain extent, it also explained the differential sensitivity of MeHg towards cerebellar and cortical cultures (Paper 3).

In the cell lines, DHA affected the peroxidative machinery and augmented the response of C6 and B35 cells to MeHg-induced oxidative effects (Paper 4). However, in primary cell cultures the novel ability of DHA to protect from the adverse effects of MeHg was highlighted. The reduction in cell-associated MeHg and the prooxidant response from MeHg challenge in primary cells with DHA was not associated with an increase in MTT activity or GSH content (Paper 5). This further highlights the importance of using an appropriate model for investigating DHA and MeHg-induced effects.

XIII. Future experiments

The differential sensitivity of MeHg towards cerebellar and cortical cultures could be further explored. Since GSH content partially explained the differential sensitivity, the role of -SH groups after low and extended exposure to MeHg could be investigated. The capacity of these cultures to modulate GSH and ROS after low and extended MeHg exposure will be studied. In particular, the mechanism behind the differential sensitivity of granule and Purkinje cells will be explored. For this purpose, the authors would also like to expose the cell cultures to low level of MeHg throughout the maturation period. The protein and RNA isolated from these samples will then be tested for gene expression studies using microarrays and electrophoresis. These studies in different cell culture models would be later verified in animal studies.

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Cellular and molecular mechanisms behind methylmercury-induced neurotoxicity
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List of Changes

Thesis (submitted to the committee)	Thesis (printed version)
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List of Errors

Page #	Line #	Present	Corrected
4	25	increased	decreased
18	16	antioxdant defences	antioxidant defenses
27	12	(Hertz *et al.,* 1989	(Hertz *et al.,* 1989)
27	13	Neurons	neurons
28	22	cells / ml	cells / ml each well
28	26	cells / ml	cells / ml each well
30	2	having the greater	having greater
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Paper I

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The use of fluorescence for detecting MeHg-induced ROS in cell cultures

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Abstract

The effect of methylmercury (MeHg) on reactive oxygen species (ROS) induction in neural cell lines was measured by the fluorescent probe, chloro methyl derivative of di-chloro di-hydro fluoresceindiacetate (CMH_2DCFDA). Three different MeHg concentrations (5, 10 and 25 μM) and time periods (30, 50 and 90 min) were studied in C6-glial and B35-neuronal cell lines. In addition, the relationship between MeHg-induced ROS and cell density (day3 vs. day4) was also explored. The ^{14}C-labelled MeHg measurements were done to determine the cell associated-MeHg content. At 30 and 50 min exposure, a significant increase ($p < 0.05$) in MeHg-induced ROS was observed at 10 and 25 μM MeHg for C6 cells and at 25 μM MeHg for B35 cells. However, the amount of ROS produced with 25 μM MeHg varied significantly ($p < 0.001$) at different time periods. For both the cell lines, significant cell density dependent differences ($p < 0.05$) were observed at 10 μM MeHg treatment for 50 min. MeHg treatments were associated with a concentration as well as cell-density dependent increase in cell associated-MeHg. These findings provide experimental evidence that special attention should be focused upon concentration, exposure time and cell density when assessing MeHg-induced ROS via fluorescence.

Keywords: In vitro; Reactive oxygen species; Methylmercury; Fluorescence

1. Introduction

Methylmercury (MeHg) is a well known neurotoxicant that affects both the developing and mature central nervous system (Clarkson, 2002; Aschner and Syversen, 2005). Major human epidemics in Iraq (Bakir et al., 1973) and Japan (Takeuchi, 1982) have established its neurotoxic effects. A major mechanism for MeHg-induced neurotoxicity is through generation of reactive oxygen species (ROS) (Sarafian and Verity, 1991; Ali et al., 1992; Yee and Choi, 1996; Gasso et al., 2001; Shanker and Aschner, 2003). ROS, such as superoxide anion and hydroxyl radical, initiate oxidative cell damage and the brain is exceptionally sensitive to such free radicals. ROS can be detected by the fluorescent dye-CMH_2DCFDA as its oxidation yields a fluorescent product, 2′,7′-dichlorofluorescein (DCF), which is retained inside the cell (Shanker et al., 2004; Kaur et al., 2006, 2007a). The CMH_2DCFDA dye has been extensively used for measuring cellular ROS (Liu et al., 2001; Shanker et al., 2004; O'Donovan et al., 2005; Shimazawa et al., 2005; Madhavan et al., 2006; Bai et al., 2007). It has been reported that the addition of a chloromethyl group to the CM-H_2DCFDA dye gives a better retention and more reliable fluorescent signals in live cells as compared to other DCF dyes (Liu et al., 2001).

Abbreviations: CMH_2DCFDA, chloro methyl derivative of di-chloro di-hydro fluoresceindiacetate; DMEM, Dulbecco's minimum essential medium; HEPES, *N*-2-hydroxy-ethylpiperazine *N*′-2-ethansulfonic acid; MeHg, methylmercury; ROS, reactive oxygen species.

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Both C6 and B35 cells from rat brain which have been extensively used as a glial (Benda et al., 1968; Schubert, 1974) and neuronal model (Schmid et al., 2000; Otey et al., 2003; Diestel et al., 2005), respectively, were selected to represent each cell type. The present study was designed to assess the differences obtained with the DCF signal by measuring the oxidative effects of MeHg, assessing concentration, time and cell density dependence. This comparative approach allowed us to recognize the importance of these factors for determining ROS-induced neurotoxicity by the use of fluorescence.

2. Materials and methods

2.1. Materials

24-well plastic tissue culture plates were purchased from Falcon (Becton Dickinson Labware, USA). Fetal bovine serum was purchased from PAA Laboratories, Pasching (Austria). The medium for culturing C6 (F12 Kaighn's nutrient mixture) was purchased from Invitrogen (Norway). The DMEM media used for culturing B35 cells was purchased from PAA Laboratories, Pasching (Austria). The fluorescent indicator CMH_2DCFDA was purchased from Molecular Probes, Inc. (Eugene, OR, USA). Radiolabeled ^{14}C-MeHg was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). MeHgCl was purchased from K&K Laboratories (Plainview, NY, USA). All other chemicals were of analytical grade.

2.2. Cell lines

The C6-glial and B35-neuronal cell lines were purchased from the ATCC-LGC Promochem (Sweden). After three passages, approximately 60,000 C6 and 160,000 B35 cells per well per ml of media were seeded in 24 well plates and incubated in a humidified 5% CO_2/95% air atmosphere at 37 °C. The different seeding concentrations were used as the C6 cells divided at a much higher rate as compared to B35 cells. To study the MeHg concentration and exposure time dependent effects, the cells were used at day 4 in vitro. In addition, for investigating the cell density dependent effects, cells were used both at day 3 and day 4. The amount of average protein for control C6 cells at day 3 and day 4 of the experiment was 84.6 ± 7.6 and 135.8 ± 7.9 μg protein/well. For control B35 cells, the amount of average protein at day 3 and day 4 of the experiment was 87.2 ± 4.9 and 137.7 ± 7.5 μg protein/well. The seeding density was chosen to facilitate the fluorescence measurements.

2.3. Treatments

A stock solution of 1 mM MeHgCl was prepared in 5 mM Na_2CO_3. From this stock, a working solution 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 the day of the experiment, the cells were washed once with HEPES buffer and incubated at 37 °C with 450 μl of 0, 5, 10 or 25 μM MeHg per well for 30, 50 or 90 min interval.

2.4. Detection of intracellular ROS accumulation

Intracellular ROS accumulation was monitored in the cells by adding CMH_2DCFDA (7 μM) to the wells containing MeHg for the last 20 min of the 30, 50 and 90 min exposure intervals. There were no washing steps involved before the addition of CMH_2DCFDA and were done only after incubation with MeHg and CMH_2DCFDA was complete. The final values were corrected for intracellular protein in each well and expressed as a percent of fluorescence in control wells as described previously (Kaur et al., 2007b).

2.5. Cellular MeHg accumulation

The cell associated-MeHg studies were done with ^{14}C-labeled 10 μM MeHg (82 nCi/μg Hg) for 30, 50 and 90 min intervals. After incubation, the cells were washed with ice cold buffer and treated with 1 N NaOH for 90 min. Samples were neutralized with 10 N HCl and 100 μl aliquots were added to 500 μl of Ultima Gold (Packard) scintillation cocktail. The samples were then counted in a 1450 Micro Beta Trilux Liquid scintillation counter (Wallac, Perkin Elmer Life Sciences, Norway) and radioactivity was corrected for cellular protein in each well as described previously by Kaur et al. (2007b).

2.6. Estimation of protein

Protein concentration was determined by the Folin reagent with BSA as a standard (Lowry et al., 1951). The protein was measured after MeHg and CMH_2DCFDA incubation using the same wells in which the fluorescence readings had been taken.

2.7. Data analysis

All results are given as mean ± standard deviation. Differences between groups were analyzed statistically with one-way ANOVA followed by the Tukey HSD post hoc test for multiple comparisons and $p < 0.05$ was considered statistically significant.

3. Results

3.1. MeHg-induced ROS effects

ROS levels quantified by the fluorescence intensity of the oxidized product, DCF, for different MeHg concentrations and time of exposure are shown for C6-glial and B35-neuronal cells in Figs. 1 and 2, respectively. A significant increase in ROS ($p < 0.05$) was observed with 10 μM MeHg

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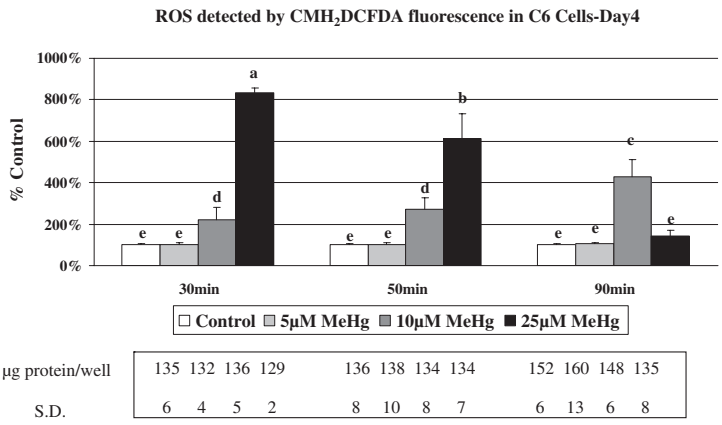

Fig. 1. Cellular ROS detected by CMH_2DCFDA fluorescence in C6-glial cell line. Results are expressed as mean ± standard deviation ($n = 6$ replicates for each cell type). The superscripts (a, b, c, d, and e) not showing common letters are significantly different ($p < 0.05$) from each other. Values were corrected for protein content in each well (provided below) and determined as a percentage of fluorescence with respect to control cells.

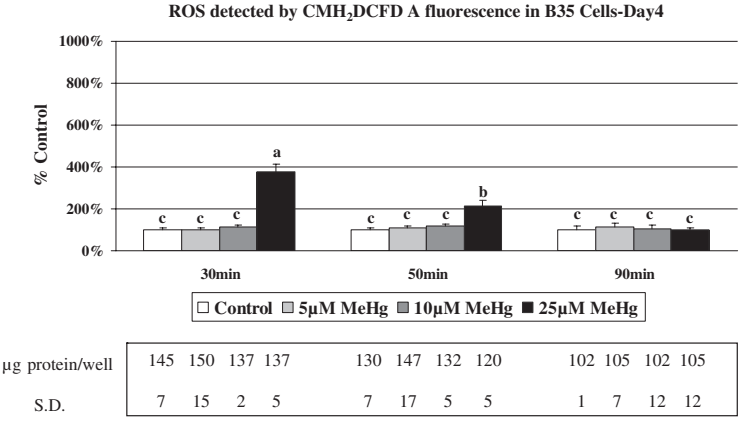

Fig. 2. Cellular ROS detected by CMH_2DCFDA fluorescence in B35-neuronal cell line. Results are expressed as mean ± standard deviation ($n = 6$ replicates for each cell type). The superscripts (a, b, and c) not showing common letters are significantly different ($p < 0.05$) from each other. Values were corrected for protein content in each well (provided below) and determined as a percentage of fluorescence with respect to control cells.

in C6 cells for all the tested time intervals. However, at 25 μM MeHg, a significant increase in ROS ($p < 0.001$) was induced only at 30 and 50 min interval for both the cell lines. In C6 cells, a significant difference was observed between 50 min and 90 min interval upon 10 μM MeHg treatment. Likewise, at 25 μM MeHg, a significant difference was observed between the various time intervals for both the cell lines. The protein values for each type of treatment (provided below) did not vary significantly with any one the above treatments.

3.2. Cell associated-MeHg

Cell associated-MeHg measured with ^{14}C-radiolabeled MeHg for different MeHg concentrations and time of exposure are shown for C6-glial and B35-neuronal cells in Figs. 3 and 4, respectively. A significant increase in cell associated-MeHg ($p < 0.05$) was observed with increasing MeHg concentrations at all the tested time intervals for both the cell lines. The cell associated-MeHg at 50 min increased significantly ($p < 0.05$) at 10 μM MeHg when compared to 30 min in both C6 and B35 cells. In addition, a significant time dependent increase ($p < 0.05$) in cell associated-MeHg was observed for B35 cells at 25 μM MeHg dose.

3.3. Cell density dependent changes in MeHg-induced ROS

The MeHg-induced ROS level at 50 min interval for two different cell densities is shown in Fig. 5. In C6 cells, a

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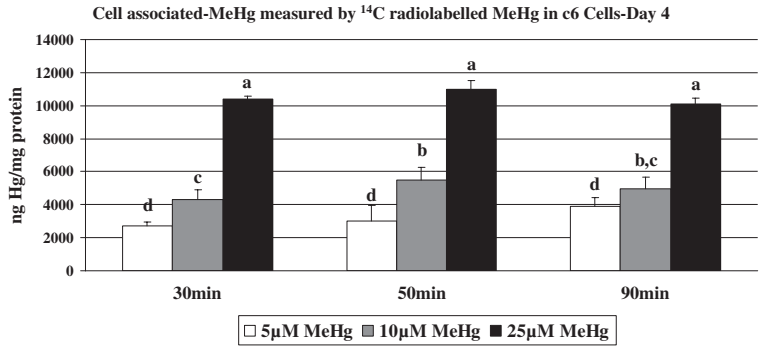

Fig. 3. Cell associated-MeHg in C6-glial cell line as measured by ^{14}C radiolabeled MeHg. Results are expressed as ng Hg/mg protein, mean ± standard deviation ($n = 8$ replicates for each cell type). The superscripts (a, b, c, and d) not showing common letters are significantly different ($p < 0.05$) from each other. Values were corrected for protein content in each well.

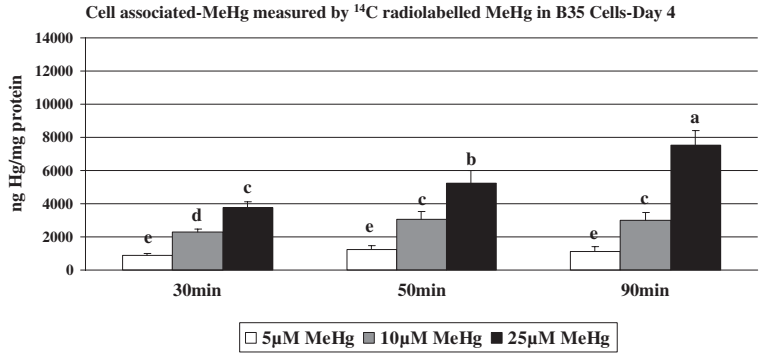

Fig. 4. Cell associated-MeHg in B35-neuronal cell line as measured by ^{14}C radiolabeled MeHg. Results are expressed as ng Hg/mg protein, mean ± standard deviation ($n = 8$ replicates for each cell type). The superscripts (a, b, c, d, and e) not showing common letters are significantly different ($p < 0.05$) from each other. Values were corrected for protein content in each well.

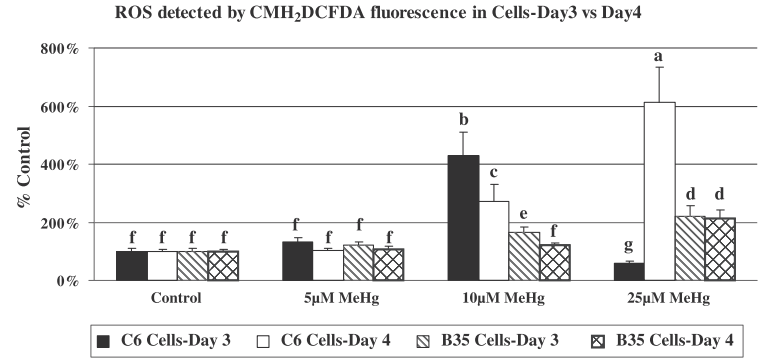

Fig. 5. Cellular ROS detected by CMH_2DCFDA fluorescence in C6-glial and B35-neuronal cell line at day 3 and day 4. Results are expressed as mean ± standard deviation ($n = 6$ replicates for each cell type). The superscripts (a, b, c, d, e, f and g) not showing common letters are significantly different ($p < 0.05$) from each other. Values were corrected for protein content in each well and determined as a percentage of fluorescence with respect to control cells.

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Table 1
Cell density dependent MeHg accumulation in C6-glial and B35-neuronal cell lines as measured by ^{14}C radiolabeled MeHg at 10 μM MeHg for 50 min

Cell type	Day 3	Day 4
C6-glial	2048 ± 231^{c}	5485 ± 755^{a}
B35-neuronal	1499 ± 180^{d}	3076 ± 426^{b}

Values are expressed as ng Hg/mg protein, mean ± standard deviation. Experiments were conducted in eight replications for each cell type. The superscripts (a, b, c, and d) not showing common letters are significantly different ($p < 0.05$) from each other. Values were corrected for protein content in each well.

significant difference ($p < 0.001$) in ROS production was observed for both 10 and 25 μM MeHg dose. In B35 cells, cell density dependent differences in ROS production were observed only for 10 μM MeHg dose.

3.4. Cell density dependent changes in cell associated-MeHg

Cell density-dependent changes in cell associated MeHg (treated with 10 μM for 50 min) are shown in Table 1. For both the cell lines, significant differences in cell associated-MeHg ($p < 0.001$) were observed between day 3 and day 4. For both the cell lines, the cell associated-MeHg was more at day 4 when compared to day 3. In addition, significant differences ($p < 0.05$) between the two cell types were observed with C6 cells exhibiting more cell associated-MeHg when compared to B35 cells.

4. Discussion

Oxidative stress occurs when the normal balance between the oxidative events and antioxidant defenses is disrupted either by loss of reducing agents/antioxidant enzymes or by increased production of oxidizing species. The present study is a part of a larger study aimed at modulation of MeHg toxicity and estimation of intracellular ROS levels (Kaur et al., 2007b). Three different MeHg concentrations used in the present study were selected on the basis of MTT timeline study (Kaur et al., 2007b). The exposure time was selected on the basis of previous uptake studies showing maximum cell associated-MeHg level for 10 μM dose at 50 min of exposure (data not shown). The MeHg content observed in the present study is 10–20 times that of in vivo studies (Eto et al., 2001) and similar to cellular levels after extended in vitro exposure studies (Sakaue et al., 2006). For a range of toxic substances the effective dose during in vitro exposure must be considerable higher than the in vivo situation when the target organ concentration is used as a dose indicator. In order to compare dose in a range of cell lines and primary cultures we prefer to use cell-associated mercury as the dose indicator rather than the nominal concentration in culture media. This becomes vital if the cell density varies between cell culture types or the serum quantity/quality varies in the growth media.

The present study demonstrates increase in MeHg-induced ROS in both C6 and B35 cells; corroborating findings from previous studies (Sarafian and Verity, 1991; Ali et al., 1992; Yee and Choi, 1996; Shanker and Aschner, 2003; Shanker et al., 2004). In addition, it identifies significant differences between different MeHg concentrations and time intervals for the production of ROS. From the MTT Timeline study (Kaur et al., 2007b) it can be seen that 5 μM MeHg dose at 60 min interval resulted in a 15% and 4% reduction in MTT activity in C6 and B35 cell lines, respectively. The primary cells on the other hand are more sensitive (Kaur et al., 2006) exhibiting 40% and 50% reduction in MTT activity in cerebellar astrocytes and neurons. This reduction in MTT activity was associated with significant induction of ROS in primary cells. However, the non-significant reduction in MTT activity observed at 5 μM MeHg in cell lines (Kaur et al., 2007b), indicates that at this dose the cells are not significantly affected to produce significant increase in ROS.

The decrease in the DCF signal at 25 μM MeHg dose with increase in exposure time could have several explanations including signal leakage, quenching and cellular defensive response. Leakage of the DCF signal has been observed in other cell types such as cancer cells (Ubezio and Civoli, 1994) and endothelial cells (Royall and Ischiropoulos, 1993). Quenching of the DCF fluorescence signal may occur as a result of lower intracellular pH which can be induced by extracellular glutamate in primary neurons (Reynolds and Hastings, 1995). Exposure to MeHg may result in increased release of glutamate (Aschner et al., 2000) which in turn may induce a lower intracellular pH. In the present study, the cellular defensive response is shown by B35 cells which counteract the ROS effects more effectively than C6 cells. We have previously reported that this is most likely connected to the ability of B35 cells to upregulate their GSH content (Kaur et al., 2007b). We have made an attempt to address all these considerations in our approach and thus use relatively high MeHg concentration for short exposure times with co-exposure to the DCF probe.

The present study demonstrates decrease in ROS with an increase in cell density at 10 μM MeHg in both the cell lines. The in vitro results can be influenced by the density of cells in culture (Preobrazhensky et al., 2001). Moreover, the effect of MeHg depends on the total biomass present (Furukawa et al., 1982). It was reported by Gulden et al., 2001 that for a variety of toxic compounds including MeHg, the EC_{50} values increased with increasing cell concentration. They concluded that cell binding can significantly affect the availability of compounds in vitro and thus their toxic potencies and toxic equivalency factors. In addition it was also reported that the presence of albumin of variable concentrations in medium greatly influences the toxic potency of MeHg (Seibert et al., 2002). This may be due to the presence of extra binding sites which can reduce the effective dose at the primary toxicity targets. The cell density-dependent production of ROS has also been reported by previous studies using different

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models such as fibroblasts (Pani et al., 2000), granulocytes (Rosenkranz et al., 1992) and neural precursor cells (Limoli et al., 2004). It has been reported by Zurgil et al. (1996) that cell density related differences result in differences in cell cycle which lead to changes in intracellular environment such as changes in membrane fluidity. They reported that in T-lymphocyte Jurkat cell line, at highest cell density the resting phase (G_0/G_1) predominated whereas at low cell density the S or G_2/M phase was predominant. Therefore, the differences in cell cycle or dilution of effective dose at high cell density can be responsible for increased ROS production at day 3 with 10 μM MeHg dose in both the cell lines. The decreased production of ROS in C6 cells at 25 μM MeHg dose at day 3 as compared to day 4 could be due to increased effective dose at low cell density resulting in increased cytotoxicity and loss of the dye. The non-differential production of ROS in B35 cells at 25 μM MeHg dose at day 3 vs. day 4 could be due to the ability of this cell type to upregulate the GSH content resulting in decreased cytotoxicity. Therefore, changes in the intracellular environment may play a crucial role in the generation of ROS detected by the DCF signal.

In cell culture uptake studies of compounds which are sparingly water soluble and strongly bound to proteins, the differentiation between the compounds being attached to the surface of the cells from what is actually inside the cell is difficult. Moreover, the extensive washing procedures may cause the cells to release the compound which has been accumulated inside the cells during the assay. Therefore, for the present study we measure the cell-associated MeHg which represents the MeHg associated to cellular protein structures. For MeHg, the uptake into the cells is both an active, energy dependent (e.g. MeHg-cystein) as well as passive uptake (e.g. MeHgCl) depending on the Hg species (Aschner et al., 1990; Kerper et al., 1992; Wang et al., 2000). Effects of any unspecific binding of ^{14}C-radiolabeled MeHg in the culture well were prevented by washing the cells with cold Hepes buffer prior to solubilization in NaOH. For both C6 and B35 cells, the cell associated-MeHg was saturated after 60 min of exposure at doses ranging from 1 to 10 μM MeHg (data not shown). The increased cell associated-MeHg in C6 cells as compared to B35 cells explains the increased production of ROS in C6 cells. However, the increased cell associated-MeHg at day 4 when compared to day 3 did not correlate with the cell density dependent differences in MeHg-induced ROS. This could be due to the presence of extra binding sites at high cell density resulting in reduction of effective dose. The concentration dependent increase in cell associated-MeHg at 30 and 50 min intervals for both the cell lines correlated with the MeHg-induced ROS effects. However, the changes in cell-associated MeHg at different exposure conditions did not always correlate with the ROS production. The authors do not have an explanation for this phenomenon. This further highlights the importance of using consistent cell densities and exposure conditions when estimating ROS from the DCF signal.

Taken together, our study establishes that concentration, time and cell density effectively contributes to MeHg-induced ROS generation. Therefore, optimum concentration, exposure time and consistent cell densities should be of prime consideration while estimating the fluorescent DCF signal for studying ROS-induced neurotoxicity.

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Conflict of interest statement

The authors confirm that they do not have any financial, personal or institutional interests which may be in conflict with the content or use of the present study.

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Paper II

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Glutathione modulation influences methyl mercury induced neurotoxicity in primary cell cultures of neurons and astrocytes

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Abstract

Methyl mercury (MeHg) is highly neurotoxic and may lead to numerous neurodegenerative disorders. In this study, we investigated the role of glutathione (GSH) and reactive oxygen species (ROS) in MeHg-induced neurotoxicity, using primary cell cultures of cerebellar neurons and astrocytes. To evaluate the effect of GSH on MeHg-induced cytotoxicity, ROS and GSH were measured using the fluorescent indicators chloro methyl derivative of di-chloro di-hydro fluorescein diacetate (CMH_2DCFDA) and monochlorobimane (MCB). Cell-associated MeHg was measured with ^{14}C-radiolabeled MeHg. Mitochondrial dehydrogenase activity was detected by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. MTT timeline study was also performed to evaluate the effects of both the concentration and duration of MeHg exposure. The intracellular GSH content was modified by pretreatment with *N*-acetyl cysteine (NAC) or di-ethyl maleate (DEM) for 12 h. Treatment with 5 μM MeHg for 30 min led to significant ($p < 0.05$) increase in ROS and reduction ($p < 0.001$) in GSH content. Depletion of intracellular GSH by DEM further increased the generation of MeHg-induced ROS in both cell cultures. Conversely, NAC supplementation increased intracellular GSH and provided protection against MeHg-induced oxidative stress in both cell cultures. MTT studies also confirmed the efficacy of NAC supplementation in attenuating MeHg-induced cytotoxicity. The cell-associated MeHg was significantly ($p < 0.02$) increased after DEM treatment. In summary, depletion of GSH increases MeHg accumulation and enhances MeHg-induced oxidative stress, and conversely, supplementation with GSH precursor protects against MeHg exposure in vitro.

Keywords: Neurotoxicity; In vitro; Methyl mercury; Glutathione; Reactive oxygen species

1. Introduction

Methyl mercury (MeHg) is a potent neurotoxicant that affects both the developing and mature central nervous system (CNS). Several catastrophic epidemics resulting from consumption of MeHg-adulterated food in Iraq (Bakir et al., 1973), Pakistan, Guatemala and Ghana, or via environmental contamination in Japan (Harada, 1995) have led to its recognition as a ubiquitous environmental toxicant (for a review, see Clarkson, 2002). The CNS damage caused by MeHg is irreparable and different in adult brain versus fetal brain (Lapham et al., 1995). Severe neurological disturbances, such as paresthesia, ataxia, sensory and speech impairment, and constriction of the visual field are caused by MeHg poisoning (Bakir et al., 1973; Harada, 1995; Elhassani, 1982). Contaminated fish from polluted areas, vaccines and dental amalgams also pose as a potential source of Hg exposure in humans (Clarkson et al., 2003). MeHg has a high association constant ($15 < pK_a < 23$) for sulfhydryl (SH) groups (Carty and Malone, 1979). It can react with any SH-group leading to conformational changes and thus inhibition of many enzymes.

Abbreviations: MeHg, methyl mercury; GSH, glutathione; ROS, reactive oxygen species; CMH_2DCFDA, chloro methyl derivative of di-chloro di-hydro fluoresceindiacetate; MCB, monochlorobimane; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; NAC, *N*-acetyl cysteine; DEM, Di-ethyl maleate; CNS, central nervous system; SH, sulfhydryl; DCF, 2′,7′-dichlorofluorescein; GPx, GSH peroxidase; FCS, Fetal calf serum; DMEM, Dulbecco's minimum essential medium; HEPES, *N*-2-hydroxy-ethylpiperazine *N*′-2-ethansulfonic acid; BSA, bovine serum albumin; LSD, least significant difference

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However, all signs and symptoms of toxicity in adults are confined mostly to the nervous system where it affects primarily the granule layer of the cerebellum and the visual cortex of the cerebrum (Takeuchi, 1982). Despite considerable scientific efforts, the reason for this selective degeneration of the nervous system has not been satisfactorily explained.

The adult human brain consumes >20% of the oxygen utilized by the body although it comprises only 2% of the body weight, indicating that ROS are generated at high rates during oxidative metabolism of the brain (Dringen et al., 2000; Clarke and Sokoloff, 1999). ROS can be detected by the CMH_2DCFDA dye as its oxidation yields a fluorescent product, 2′,7′-dichlorofluorescein (DCF), which is retained inside the cell (Shanker et al., 2004). Antioxidants provide cellular defense against ROS, with GSH constituting the most important and abundant component (Cooper, 1997). Both neurons and astrocytes contain GSH, which is involved in the disposal of exogenous peroxides by acting as a co-substrate in a reaction catalyzed by GSH peroxidase (GPx) (Eklow et al., 1984; Wendel et al., 1980). However, astrocytes contain higher concentrations of GSH than neurons (Kranich et al., 1996; Sagara et al., 1993). The astrocytes provide precursor molecules, e.g., cysteine, to neurons for GSH synthesis (Shanker and Aschner, 2001; Sagara et al., 1993). The availability of cysteine from glial cells becomes rate limiting for synthesis of neuronal GSH (Kranich et al., 1998; Wang and Cynader, 2000). The content of intracellular free thiols and the subcellular compartmentation of GSH could be determined by MCB (Bellomo et al., 1997). The MCB can diffuse passively across the plasma membrane into the cytoplasm where it binds with the reduced form of GSH and other thiol-containing proteins leading to formation of blue fluorescent adducts (Haugland, 1996). Previous studies on the mechanism of MeHg have shown that MeHg toxicity leads to depletion of GSH and generation of ROS (Sanfeliu et al., 2001; Yee and Choi, 1996). Several reports have implicated a critical role of GSH in modulating MeHg neurotoxicity (Choi et al., 1996; Miura and Clarkson, 1993). However, it still remains unclear whether the protection afforded by GSH is due to protection against ROS generated by MeHg or by reduction of intracellular concentration of MeHg. The present investigation addresses this issue by firstly, evaluating the effect of MeHg on the content of GSH and ROS in neurons as well as astrocytes, and secondly, by examining the effect of GSH modulation on the accumulation of MeHg in both cell types. GSH content of primary cultured cells can be modulated by variety of treatments (Dringen, 2000). NAC or DEM were employed for GSH modulation in the present study. DEM depletes cellular GSH by forming adducts with the thiol groups. DEM has been reported to cause high mortality in $HgCl_2$ treated rats (Baggett and Berndt, 1986). NAC is a source of thiol groups and scavenger of free radicals such as H_2O_2 and $OH^{\bullet}$ (Aruoma et al., 1989). Previous studies have indicated the effectiveness of NAC as an antidote against MeHg poisoning (Ballatori et al., 1998; Koh et al., 2002). The present study attempts to resolve the ambiguity concerning the cellular selectivity exhibited by MeHg.

2. Materials and methods

2.1. Materials

Twenty-four-well plastic tissue culture plates were purchased from Falcon (Becton Dickinson Labware). Fetal calf serum (FCS) was acquired from Seralab Ltd. (Sussex, UK) and culture medium was acquired from GIBCO BRL, Life Technologies (Roskilde, Denmark). The fluorescent indicator CMH_2DCFDA was purchased from Molecular Probes Inc. (Eugene, OR). NAC, DEM and MCB were acquired from Sigma Aldrich (Norway). Radiolabeled ^{14}C-MeHg (Cat. no. ARC-1302) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). MeHgCl (Cat. No 23308) was purchased from K&K Laboratories (Plainview, NY, USA). All other chemicals were of analytical grade.

2.2. Animals

NMRI mice were purchased from Møllegaard Breeding Center (Copenhagen, Denmark). Prior to experiments the animals had free access to food and water and were kept under constant conditions of temperature (22 °C), humidity (60%) and 12 h light/dark cycles. The animals were handled in compliance with the NTNU Animal Care and Use Committee.

2.3. Cell cultures

Cerebellar astrocytes were prepared as described earlier (Hertz et al., 1989). Briefly, cerebella obtained from 7-day-old mice were passed through Nitex nylon netting (80 μm pore size) into Dulbecco's minimum essential medium (DMEM) containing 20% (v/v) fetal calf serum (FCS). The cell suspension was then plated at a density of 0.5 cerebella per 24-well culture plate, which was coated previously with poly-D-lysine. Medium was changed 2 days after plating and subsequently twice a week, gradually decreasing the FCS concentration to 10%. At 14 days in culture, dibutyryl-cAMP was added to the medium for 1 week to promote the morphological differentiation of astrocytes. Cells were used for the experiment 21 days post-isolation and the cultures were 80% confluent by this time.

Primary cultures of cerebellar neuronal cells were prepared from cerebella of 7-day-old mice according to the method described by Schousboe et al., 1989. Briefly, mild trypsinized cerebella were triturated in a DNase solution containing a soybean trypsin inhibitor. The cells were suspended in DMEM (19 mM KCl, 31 mM glucose, and 0.2 mM glutamine) supplemented with 50 μM kainic acid, *p*-aminobenzoate, insulin, penicillin, garamycin and 10% (v/v) FCS. Cells were seeded at a density of (0.8×10^6 cells/ml) in 24-multiwell plate, which were previously coated with poly-D-lysine and incubated in a humidified 5% CO_2/95% air atmosphere at 37 °C. Neuronal cultures were used 1 week post-isolation.

notable ($p < 0.001$) at 10 μM concentration and at 60 min exposure for both cell types. A slight increase in MTT activity was observed in astroglial cultures at 1 μM concentration and at 15 min interval, which may indicate inherent protective mechanisms. At any particular concentration and time interval, cerebellar neurons were more susceptible to MeHg-induced reduction in MTT activity than cerebellar astrocytes. At 5 μM MeHg concentration for 30 min there was a 41% ($p < 0.001$) reduction in mitochondrial activity in neurons (Fig. 6) as compared to 25% ($p < 0.05$) reduction in astrocytes (Fig. 6).

4. Discussion

The present study addresses the role of GSH in differential cytotoxic effects of MeHg in cerebellar neurons and astrocytes. It determined that depletion of GSH increases the intracellular concentration of MeHg in both cell types and therefore influences the generation of ROS.

4.1. GSH modification and ROS generation

MCB fluorescence studies indicated that MeHg causes a depletion in GSH content both in neuronal and astroglial cells, which is in accordance with the previous studies (Sarafian and Verity, 1990). It has been reported that NAC is an acetylated analog of cysteine, which easily crosses the cell membrane and is rapidly deacetylated inside the cell to become available for GSH synthesis (Zafarullah et al., 2003). Our present data shows that NAC was effective in preventing MeHg-induced GSH loss in both cell cultures. In the control cells, NAC did not increase the GSH level. However, after MeHg exposure NAC-pretreated cells more readily met the increased demand for reduced GSH. In contrast, loss of protective thiol groups by DEM further augmented MeHg-induced loss of GSH. The average control MCB values indicated that cerebellar astrocytes contained a greater supplement of GSH than cerebellar neurons, which is consistent with previous observation (Raps et al., 1989). The present study demonstrates that neurons were more vulnerable to MeHg-induced GSH depletion, indicating that GSH status likely represents a key factor in the neuropathologic and cell specific effects of MeHg.

Previous in vivo studies in rodent cerebellum and in vitro studies in sub cellular fractions (Ali et al., 1992) in cell lines (Sarafian et al., 1994) and primary cultures (Shanker et al., 2004; Shanker and Aschner, 2003) have indicated that ROS are mediators of MeHg-induced neurotoxicity. In addition, GSH modulators have been reported to influence MeHg-induced ROS in cerebellar astrocytes (Shanker et al., 2005). The present study is in accordance with the previous studies, as we also observed an increase in CMH_2DCFDA fluorescence in both cell types with MeHg treatment. The results indicate that depletion of cellular thiols by DEM induced and augmented MeHg-induced accumulation of ROS. CMH_2DCFDA measurements indicated that neurons were more affected by MeHg treatment as well as DEM induced ROS than astrocytes. MeHg-induced ROS was however, ameliorated with NAC treatment in both cell cultures as indicated by decrease in CMH_2DCFDA fluorescence. Thus, GSH modulators affect MeHg-induced ROS generation differentially in neurons and astrocytes.

4.2. MeHg accumulation

The unique aspect of the present study is that intracellular GSH concentrations were directly correlated both in astrocytes and neurons not only with MeHg-induced oxidative stress, but also with cell-associated MeHg content. Since the demethylation of MeHg is a slow process (Magos et al., 1985) it will not occur at any significant rate during the 30 min incubation period used in the present study and the ^{14}C measurements thus represent MeHg content (Simmons-Willis et al., 2002; Morken et al., 2005; Koh et al., 2002).

The GSH modulator-DEM increased MeHg accumulation in both cell types, indicating that depletion of thiol groups predisposes the cells to MeHg-induced cytotoxicity. However, the tendency of DEM to increase Hg accumulation was more considerable in neurons ($p < 0.001$) versus astrocytes ($p < 0.02$). It was also observed that cell associated-MeHg was approximately five times higher in neurons than astrocytes. These data suggest that cerebellar neurons were more sensitive to thiol depletion and MeHg-induced neurotoxicity than astrocytes. No significant differences were observed in either cell type between MeHg group and NAC pretreated MeHg group. The non-significant increase in cell associated-MeHg with NAC may be due to binding of MeHg to the cysteine group of NAC, which may prevent binding of MeHg to other sensitive target sites. Previous studies have indicated that MeHg uptake increases when it is present as L-cysteine complex but not as D-cysteine, NAC, penicillamine or GSH complexes (Wang and Clarkson, 2000; Simmons-Willis et al., 2002) suggesting the active role of L-neutral amino acid transporter in MeHg uptake. The MeHg-thiol interactive chemistry is responsible for variable accumulation of MeHg. Increased MeHg accumulation increases MeHg-induced toxicity as indicated by the more pronounced production of ROS, depletion of GSH and reduction of MTT, when both neurons and astrocytes are co-incubated with MeHg and DEM.

4.3. Effects on mitochondrial function (MTT-reduction)

The MTT data supports MeHg's differential effects in neurons versus astrocytes. Cerebellar neurons are more sensitive to MeHg than cerebellar astrocytes, consistent with previous reports (Sanfeliu et al., 2001). Our present data asserts that this cellular specificity reflects increased neuronal accumulation of MeHg in neurons versus astrocytes. Previous studies have indicated the protective effects of NAC against thimerosal (James et al., 2005) and MeHg hydroxide (Gatti et al., 2004) induced reduction of MTT activity in different cell lines. Effectiveness of amino acids and antioxidants, such as cysteine and GSH against MeHg-induced decrease in MTT activity has also been previously reported in primary cell cultures (Sanfeliu et al., 2001). Our present MTT data corroborates these studies and demonstrates the efficacy of NAC supplementation in primary neuronal and astroglial cell

cultures. In both cell types, DEM and MeHg treatment induced a reduction in MTT activity. The combined effect of MeHg and DEM was, however, synergistic and not additive. Previous studies have proposed that decrease in intracellular GSH by DEM causes inhibition of MeHg efflux from brain endothelial cells (Kerper et al., 1996). Our present data shows that DEM causes increase in intracellular concentration of MeHg available for reduction in MTT activity.

In summary, the present study demonstrates that maintenance of adequate GSH levels protects against MeHg-induced oxidative stress in primary cell cultures of neurons and astrocytes. The difference in the GSH status provides a possible explanation for the differences between MeHg-induced neuronal and astroglial toxicity. The limited GSH availability might be one of the mechanisms responsible for making neurons more susceptible to MeHg toxicity versus astrocytes. It affirms that modulation of GSH levels effectively change the intracellular concentration of MeHg which in turn will alter the risk of MeHg-induced oxidative stress.

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Paper III

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Role of glutathione in determining the differential sensitivity between the cortical and cerebellar regions towards mercury-induced oxidative stress

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Abstract

Certain discrete areas of the CNS exhibit enhanced sensitivity towards MeHg. To determine whether GSH is responsible for this particular sensitivity, we investigated its role in MeHg-induced oxidative insult in primary neuronal and astroglial cell cultures of both cerebellar and cortical origins. For this purpose, ROS and GSH were measured with the fluorescent indicators, CMH_2DCFDA and MCB. Cell associated-MeHg was measured with ^{14}C-radiolabeled MeHg. The intracellular GSH content was modified by pretreatment with NAC or DEM. For each of the dependent variables (ROS, GSH, and MTT), there was an overall significant effect of cellular origin, MeHg and pretreatment in all the cell cultures. A trend towards significant interaction between origin × MeHg × pretreatment was observed only for the dependent variable, ROS (astrocytes $p = 0.056$; neurons $p = 0.000$). For GSH, a significant interaction between origin × MeHg was observed only in astrocytes ($p = 0.030$). The cerebellar cell cultures were more vulnerable ($astrocytes_{mean} = 223.77$; $neurons_{mean} = 138.06$) to ROS than the cortical cell cultures ($astrocytes_{mean} = 125.18$; $neurons_{mean} = 107.91$) for each of the tested treatments. The cell associated-MeHg increased when treated with DEM, and the cerebellar cultures varied significantly from the cortical cultures. Non-significant interactions between origin × MeHg × pretreatment for GSH did not explain the significant interactions responsible for the increased amount of ROS produced in these cultures. In summary, although GSH modulation influences MeHg-induced toxicity, the difference in the content of GSH in cortical and cerebellar cultures fails to account for the increased ROS production in cerebellar cultures. Hence, different approaches for the future studies regarding the mechanisms behind selectivity of MeHg have been discussed.

Keywords: Neurotoxicity; In vitro; Methylmercury; Glutathione; Reactive oxygen species; Cortical; Cerebellar

Abbreviations: MeHg, methylmercury; CNS, central nervous system; GSH, glutathione; ROS, reactive oxygen species; CMH_2DCFDA, chloro methyl derivative of di-chloro di-hydro fluoresceindiacetate; MCB, monochlorobimane; MTT, [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; NAC, *N*-acetyl cysteine; DEM, Di-ethyl maleate; FCS, fetal calf serum; DMEM, Dulbecco's minimum essential medium; HEPES, *N*-2-hydroxy-ethylpiperazine *N*′-2-ethansulfonic acid; BSA, bovine serum albumin; LSD, least significant difference

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1. Introduction

Methylmercury (MeHg) is an important environmental neurotoxicant capable of posing risks to human health (Aschner and Syversen, 2005). It is a hazardous trace metal that is released into the environment from both natural and anthropogenic sources (EPA, 1997; ATSDR, 1999). MeHg accumulates in aquatic organisms and biomagnifies through the food web from 10,000 to 100,000 times (EPA, 1997; Wiener et al., 2003). Thus, the major dietary route of human exposure to MeHg is via the ingestion of seafood for adults and via maternal milk for infants (Manfroi et al., 2004). Major human epidemics in Japan (Takeuchi, 1982; Eto, 2000) and Iraq (Bakir et al., 1973) have established the toxicity of MeHg in the nervous system. It is critical that we ascertain the neurobiological processes which may be detrimentally affected by this environmental agent.

MeHg has a high affinity for binding to all thiol groups (Hughes, 1957), indicating that more than one mechanism may contribute to the expression of MeHg-induced neurotoxicity. However, the existence of multiple mechanisms of toxicity are difficult to reconcile with the specific pattern of neurological damage associated with MeHg, such as in the cerebellar granule cell layer and in the calcarine region of the occipital cortex in humans (Hunter and Russell, 1954; Choi et al., 1978; Takeuchi, 1982; Eto, 1997) and in rodent brains (Nagashima, 1997). The mechanisms associated with enhanced sensitivity in these areas of the CNS remain unknown. Cerebellar granule cells are predominately glutamatergic, whereas cortical neurons are mainly GABAergic, while expressing glutamate receptors also (Yu et al., 1984; Hertz and Schousboe, 1987). Major mechanisms of MeHg neurotoxicity involve the spontaneous release of glutamate and GABA in neurons (Atchison and Hare, 1994) and the inhibition of the uptake of glutamate and GABA in astrocytes (Aschner et al., 2000) and in cortical slices of adult rat brains (Farina et al., 2003). Neuronal dysfunction has been proposed to be secondary to disturbances in astrocytes (Allen et al., 2001). However, the sparing of Purkinje cells and the sensitivity of granule cells in the cerebellum cannot be attributable solely to the vulnerability of cerebellar astrocytes towards MeHg, for under these circumstances, both Purkinje and granule cells would be expected to respond in a similar fashion. Therefore, it is important to identify the unique characteristics of cells that express high resistance to MeHg.

Molecular oxygen is essential for many biological events associated with aerobic metabolism, and this results in the constant formation of ROS (Powis et al., 1995). Conversely, GSH is the most abundant thiol tripeptide present in mammalian cells for scavenging reactive oxygen species (Roberts et al., 1980; Fang et al., 2002). MeHg causes a reduction in the amount of intracellular GSH (Miura and Clarkson, 1993; Sarafian et al., 1994; Choi et al., 1996; Gatti et al., 2004) and the augmentation of ROS formation (Ali et al., 1992; Yee and Choi, 1996; Sorg et al., 1998; Sarafian, 1999; Gasso et al., 2001; Sanfeliu et al., 2001; Shanker and Aschner, 2003). However, little is known concerning this relationship in specific anatomical regions of the brain. Previous work from our laboratory (Kaur et al., 2006) prompted us to investigate whether GSH is responsible for the differences induced by MeHg in cerebellar and cortical cultures. MCB has been previously used for measuring GSH levels in tissue (Kamencic et al., 2000), glia (Chatterjee et al., 1999, 2000), neuroblastoma and neuronal cultures (Tauskela et al., 2000; Sebastia et al., 2003). MCB is a cell permeable, nonfluorescent bimane which conjugates to GSH, forming a fluorescent compound with the help of the enzyme, glutathione-*S*-transferase. Application of CMH_2DCFDA dye for measuring ROS (Liu et al., 2001; Shanker et al., 2004; Shimazawa et al., 2005) is more useful than other DCF dyes (Carter et al., 1994; Oyama et al., 1994), since the trapped fluorescent adduct inside the cell is better retained in live cells.

The present study was designed to assess the differences between the oxidative effects of MeHg in cerebellar and cortical primary cultures of neurons and astrocytes from mice brains. This comparative approach allowed us to test the effect of differences in GSH content on the generation of MeHg-induced ROS in these regions of the brain.

2. Materials and methods

2.1. Materials

Twenty-four-well plastic tissue culture plates were purchased from Falcon (Becton Dickinson Labware). FCS used for cerebellar cultures and cortical astrocytes was acquired from Seralab Ltd. (Sussex, UK). Dialyzed serum for culturing cortical neurons was obtained from GIBCO BRL, Life Technologies (Roskilde, Denmark). The culture medium was acquired from Sigma Aldrich (Germany). The fluorescent indicator CMH_2DCFDA was purchased from Molecular Probes, Inc. (Eugene, OR). NAC, DEM and MCB were acquired from Sigma Aldrich (Norway). Radiolabeled ^{14}C-MeHg (Cat. no. ARC-1302) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). MeHgCl (Cat. no. 23308) was purchased from K&K Laboratories (Plainview, NY, USA). All other chemicals were of analytical grade.

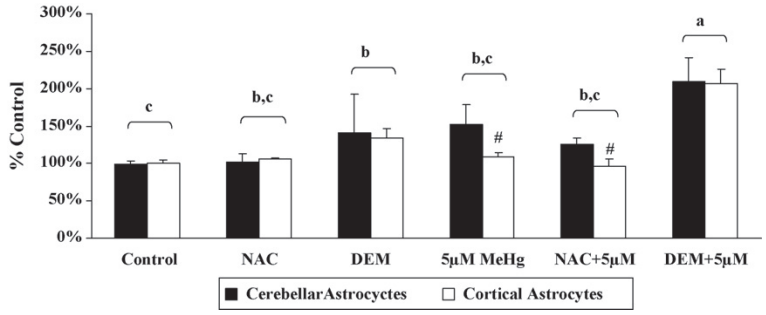

Fig. 5. Cellular ROS detected by CMH_2DCFDA fluorescence in primary astroglial cultures from the cerebellum and the cerebrum. Results are expressed as mean ± standard deviation ($n = 8$ replicates for each cell type in two independent experiments). The letters (a, b, and c) indicate the interaction between MeHg × pretreatment and data not showing common letters are significantly different from each other ($p = 0.000$); #significant difference between the cerebellum and the cerebrum for each type of treatment (origin × MeHg $p = 0.007$; origin × MeHg × pretreatment $p = 0.056$). Values were corrected for protein content in each well and determined as a percentage of fluorescence with respect to control cells.

action between origin × MeHg (astrocytes $p = 0.007$; neurons $p = 0.000$, Table 1) indicated that the amount of ROS produced in the cerebellar cultures varied significantly from the cortical cultures. Interaction between origin × MeHg × pretreatment (astrocytes $p = 0.056$; neurons $p = 0.000$) indicated that treatment with MeHg resulted in the increased production of ROS in cerebellar astrocytes and neurons from both the cerebellum and the cortex with respect to the control groups. This interaction also underlines that cerebellar cultures exposed to MeHg produce more ROS ($astrocytes_{mean} = 151.915$; $neurons_{mean} = 411.770$) than cortical cultures ($astrocytes_{mean} = 108.384$; $neurons_{mean} = 133.729$). In particular, ROS generated by MeHg in cerebellar neurons varied significantly from ROS generated in the cortical neurons, and this difference was sustained upon DEM treatment. However, in astrocytes, an increase in the amount of ROS was observed in the DEM-pretreated MeHg group when compared to the MeHg group. The amount of ROS generated in cerebellar cultures which were treated with NAC and MeHg varied significantly from the amount of ROS generated in the cortical cultures. In addition, for neurons, the NAC-pretreated MeHg group varied from the MeHg group.

3.3. Depletion of GSH and ROS accumulation induces cytotoxicity

Exposure to MeHg (5 μM) resulted in a reduction of MTT activity in all of the cultures when compared to the control (Fig. 7, astrocytes; Fig. 8, neurons).

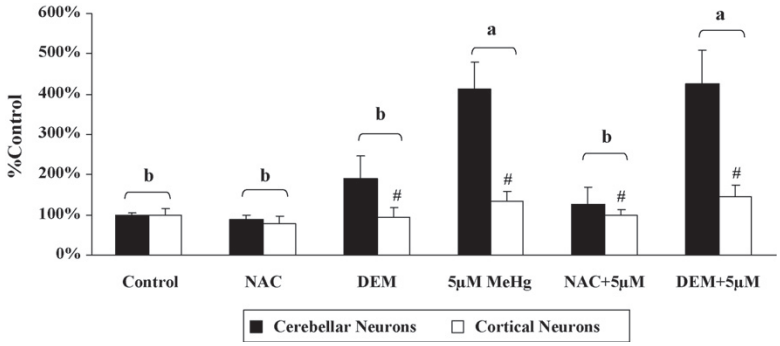

Fig. 6. Cellular ROS detected by CMH_2DCFDA fluorescence in primary neuronal cultures from the cerebellum and the cerebrum. Results are expressed as mean ± standard deviation ($n = 8$ replicates for each cell type in two independent experiments). The letters (a and b) indicate the interaction between MeHg × pretreatment, and data not showing common letters are significantly different from each other ($p = 0.000$); #significant difference between the cerebellum and the cerebrum for each type of treatment (origin × MeHg × pretreatment $p = 0.000$). Values were corrected for protein content in each well and determined as a percentage of fluorescence with respect to control cells.

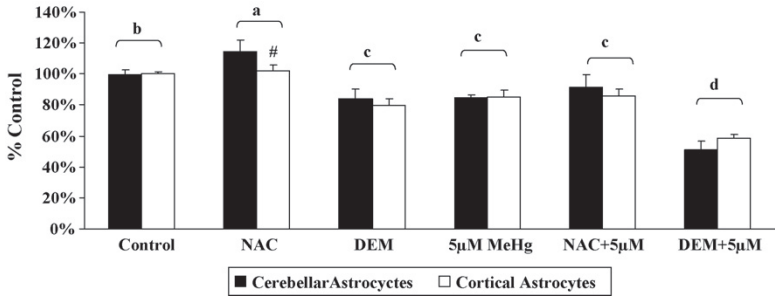

Fig. 7. Cytotoxicity detected by reduction of MTT in primary astroglial cultures from the cerebellum and the cerebrum. Results are expressed as mean ± standard deviation ($n = 8$ replicates for each cell type in two independent experiments). The letters (a, b, c, and d) indicate the interaction between MeHg × pretreatment, and data not showing common letters are significantly different from each other ($p = 0.000$); #significant difference between the cerebellum and the cerebrum for each type of treatment (origin × pretreatment $p = 0.000$). Values are presented as a percentage of activity in control cells.

The MTT activity was further reduced upon DEM and MeHg treatment in all of the cultures when compared to the DEM group as well as the MeHg group. For the dependent variable, MTT, non-significant interactions between the origin × MeHg × pretreatment (Table 1) were observed for both neurons and astrocytes. However, significant interactions between the origin × MeHg (neurons $p = 0.000$, Table 1) indicated that there was a significant difference in the MTT activity in cortical neurons when compared to cerebellar neurons upon MeHg treatment. No significant difference in the MTT activity was observed between cerebellar and cortical astrocytes when treated with MeHg. However, we observed that cortical astrocytes are more viable than cerebellar astrocytes when continuously exposed to a low dose of MeHg during maturation (data not shown). Increased MTT activity was observed with NAC pretreatment in cerebellar cultures as compared to cortical cultures. The MTT activity in the NAC-pretreated MeHg group did not vary significantly from the MeHg group in any of the cell cultures.

3.4. MeHg accumulation

Depletion of GSH with DEM treatment led to increased MeHg accumulation in both cortical and cerebellar cultures (Table 2). However, no significant changes were observed in cell associated-MeHg in NAC pretreated cortical astrocytes and neurons from the cerebellum or the cortex when compared to the control. The MeHg accumulation in cortical cultures treated with either NAC or DEM differed significantly from the MeHg accumulation in the cerebellar cultures. It was observed that cerebellar cultures accumulated MeHg to a greater extent than cortical cultures when modified by NAC or DEM.

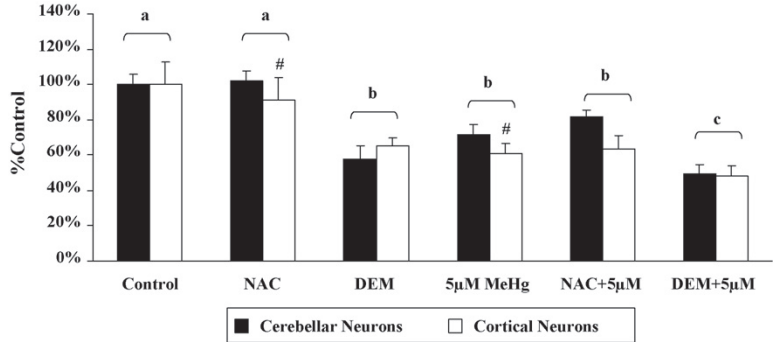

Fig. 8. Cytotoxicity detected by the reduction of MTT in primary neuronal cultures from the cerebellum and the cerebrum. Results are expressed as mean ± standard deviation ($n = 8$ replicates for each cell type in two independent experiments). The letters (a, b, and c) indicate the interaction between MeHg × pretreatment, and data not showing common letters are significantly different from each other ($p = 0.000$); #significant difference between the cerebellum and the cerebrum for each type of treatment (origin × MeHg $p = 0.003$; origin × pretreatment $p = 0.000$). Values are presented as a percentage of activity in control cells.

Table 2
Effect of GSH modulators on MeHg accumulation in primary cultures of cerebellar and cortical origin as measured by ^{14}C radiolabeled MeHg at 50 min

Cell type	Region	MeHg	MeHg + NAC	MeHg + DEM
Astrocytes	Cerebellar	1.06 ± 0.09 d	1.21 ± 0.22 bc	1.61 ± 0.25 a
	Cortical	1.11 ± 0.14 cd	1.00 ± 0.14 d	1.32 ± 0.11 b
Neurons	Cerebellar	1.37 ± 0.18 b	1.31 ± 0.20 b	1.55 ± 0.22 a
	Cortical	1.04 ± 0.16 c	1.06 ± 0.24 c	1.34 ± 0.26 b

Values are expressed as μgHg/mg protein, mean ± standard deviation. Experiments were conducted in 15 replications for each cell types in three independent experiments. The letters (a, b, c, and d) indicate the interaction between origin × pretreatment and data not showing common letters are significantly different from each other. Values were corrected for protein content in each well.

3.5. MTT timeline studies

A significant effect of time and MeHg concentration ($p = 0.000$) was observed for astrocytes (Fig. 9) from both the cortex and the cerebellum, which indicated that an increase in MeHg concentration and exposure time results in reduced mitochondrial activity. There was a significant difference between the control and 5 and 10 μM MeHg treated astrocytes ($p = 0.000$). The timeline for astrocytes indicated that the 15 min interval varied significantly from the 30 min ($p = 0.018$) and 60 min ($p = 0.000$) intervals. However, there was a non-significant interaction between the origin × MeHg treatment ($p = 0.078$) in astrocytes. For neurons (Fig. 10.), a significant effect of origin, time and MeHg concentration ($p = 0.000$) was observed. Additionally, a significant interaction between the origin × MeHg group ($p = 0.000$) as well as the time × MeHg group ($p = 0.003$) was observed for neurons. These significant interactions indicated that the cortical neurons varied from the cerebellar neurons both at 5 and 10 μM concentrations of MeHg. The time × MeHg interaction for neurons indicated that all of the time intervals (15, 30 and 60 min) varied significantly from each other. However, both neurons and astrocytes demonstrated non-significant interactions for the origin × MeHg × time treatment.

3.6. Effect of biological material on MTT activity

The effect of two different cell concentrations on MTT activity was observed (Table 3) in order to signify the importance of optimal and consistent cell seeding densities while comparing cells from different regions for investigating MeHg cytotoxicity. The significant interaction between the origin × MeHg × cell density for astrocytes ($p = 0.002$) indicated that the MTT activity in cortical astrocytes treated with MeHg varied significantly from the MTT activity in MeHg treated cerebellar astrocytes only at a lower cell density. Moreover, the MTT activity in cerebellar astrocytes at a low cell density varied significantly for the respective culture at a high cell density. For neurons, the significant differences for the fixed factors such as origin ($p = 0.010$), cell density

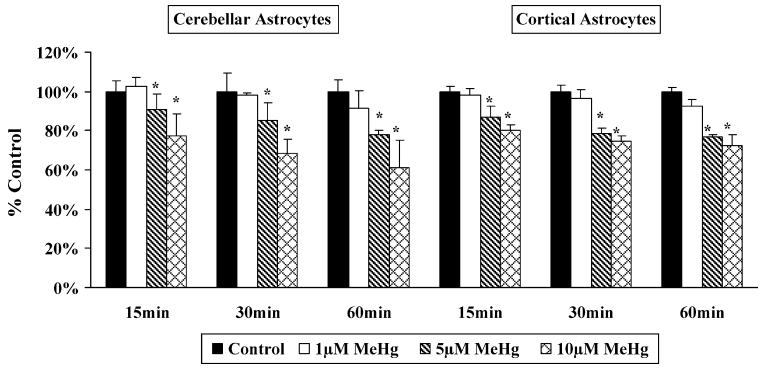

Fig. 9. Study of MTT timeline in primary cerebellar and cortical astrocytes. Results are expressed as mean ± standard deviation ($n = 4$ replicates for each cell type in two independent experiments) where '*' indicates the significant difference when compared to the control group (MeHg $p = 0.000$). No significant interaction between origin × MeHg and origin × MeHg × time was observed. Values were corrected for protein content in each well and determined as a percentage of activity with respect to control cells.

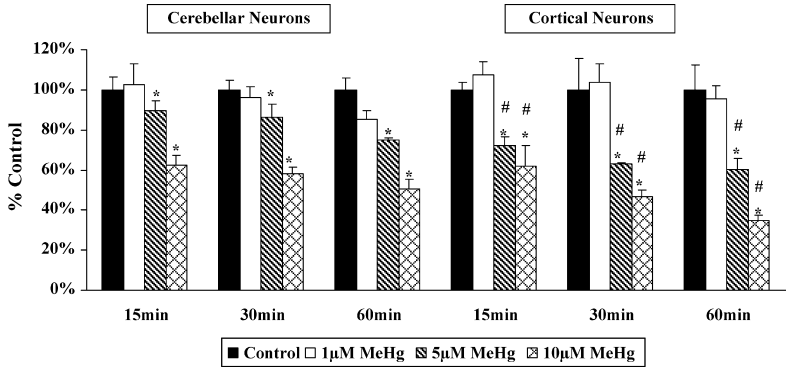

Fig. 10. Study of MTT timeline in primary cerebellar and cortical neurons. Results are expressed as mean ± standard deviation ($n = 4$ replicates for each cell type in two independent experiments) where '*' indicates the significant difference when compared to the control group (MeHg $p = 0.000$); #significant difference between the cerebellum and the cerebrum for each type of treatment (origin × MeHg $p = 0.000$). No significant interaction between origin × MeHg × time was observed. Values were corrected for protein content in each well and determined as a percentage of activity with respect to control cells.

Table 3
Effect of different cell densities on MTT activity in cerebellar and cortical cultures

Cell type	Region	Low cell density		High cell density	
		Control	5 μM MeHg	Control	5 μM MeHg
Astrocytes	Cerebellar	100 ± 10 a	70 ± 8 d	100 ± 3 ab	84 ± 2 c
	Cortical	100 ± 8 a	90 ± 5 bc	100 ± 1 a	85 ± 4 c
Neurons	Cerebellar	100 ± 1	63 ± 4	100 ± 5	72 ± 5
	Cortical	100 ± 3	50 ± 6	100 ± 13	61 ± 6

Low cell density = 0.5 cerebella or cerebra/24-well culture plate (astrocytes); 0.8×10^6 cells/2 cm^2 (neurons) and high cell density = 1 cerebella or cerebra/24 well plate (astrocytes); 1.5×10^6 cells/2 cm^2 (neurons). Values were determined as a percentage of activity with respect to control cells. Results are expressed as mean ± standard deviation ($n = 8$ replicates for each cell type in two independent experiments). The letters (a, b, c, and d) indicate the interaction between origin × MeHg × seeding density for astrocytes and data not showing common letters are significantly different from each other. No significant interaction between origin × MeHg × seeding density was observed for neurons. However, please note that all the MeHg treated cultures differed significantly from the control cultures.

($p = 0.035$) and MeHg ($p = 0.000$) as well as significant interactions between the origin × MeHg ($p = 0.010$) and cell density × MeHg ($p = 0.035$) were observed. These findings indicated that the cortical neurons were significantly different from the cerebellar neurons, and low cell density was significantly different from high cell density. However, no significant interaction between origin × MeHg × cell density was observed for neurons.

4. Discussion

The susceptibility of cells from the cerebrum and the cerebellum towards MeHg-induced toxicity has been compared in very few studies (Mundy and Freudenrich, 2000; Gasso et al., 2001; Adachi and Kunimoto, 2005). Moreover, these studies have not compared the ability of cerebellar and cerebral cultures to generate MeHg-induced ROS with respect to the amount of GSH present in each of these cultures. Therefore, the present study explores the role of GSH in exhibiting the differential effects of MeHg in cerebellar and cortical cultures.

This study corroborates previous studies (Yee and Choi, 1996; Sanfeliu et al., 2001; Shanker and Aschner, 2003; Shanker et al., 2004, 2005; Kaur et al., 2006) confirming that MeHg depletes GSH content in both cortical and cerebellar cultures. It further reports that treatment with MeHg is associated with greater depletion of GSH in cerebellar astrocytes, indicating that cortical astrocytes are more resistant to MeHg-induced depletion of GSH. Conversely, cortical astrocytes were more vulnerable when their GSH content was modified with NAC or DEM. This result might be attributed to the presence of a higher GSH content in cortical astrocytes as compared to cerebellar astrocytes, as observed in the present study, possibly indicating that DEM is able to further deplete the available GSH content, whereas NAC

is not able to upregulate the already saturated state of GSH in these cultures. However, non-significant interactions between MeHg × pretreatment do not tell us whether these differences between cortical and cerebellar astrocytes are maintained in NAC or DEM-pretreated MeHg groups. In neurons, non-significant interactions between origin × MeHg and origin × pretreatment for GSH indicated that cortical neurons did not vary significantly from cerebellar neurons for either of the tested treatments. However, modulation of GSH content with NAC or DEM pretreatment did significantly influence the MeHg-induced GSH loss in neurons. This indicates that an increase in GSH content with NAC pretreatment prevents MeHg-induced GSH loss in neurons, whereas a decrease in GSH content with DEM pretreatment augments this loss.

In accordance with the previous studies (Ali et al., 1992; Yee and Choi, 1996; Sorg et al., 1998; Sarafian, 1999; Sanfeliu et al., 2001; Shanker and Aschner, 2003; Shanker et al., 2004, 2005), the present study demonstrates that treatment with MeHg induces ROS in both cerebellar and cortical cultures. It further reports that modulation of the GSH content influences MeHg-induced ROS in these cultures and treatment with MeHg is associated with greater production of ROS in cerebellar cultures. The higher amount of GSH in cortical astrocytes after exposure to MeHg might explain the decreased production of ROS in this cell type when compared to cerebellar astrocytes. However, the non-differential status of GSH in cortical and cerebellar neurons after MeHg exposure does not provide an explanation for the increased susceptibility of cerebellar neurons, indicating the importance of factors other than GSH in protecting cortical cultures from MeHg-induced ROS.

The present study reports that cortical neurons were more sensitive to MeHg-induced cytotoxicity, which is in accordance with the observation by Miyamoto et al. (2001). The present study also indicates that NAC alone is more effective for increasing mitochondrial activity in cerebellar versus cortical cultures, emphasizing the need for a better understanding of NAC transporters in various brain regions. Moreover, the DEM-pretreated MeHg group caused a reduction in MTT activity when compared to the MeHg group, suggesting that, after depletion of thiol groups by DEM, more MeHg is available for inhibiting mitochondrial enzymes.

The present study supplements our knowledge regarding the effects of intracellular GSH concentrations on cell associated-MeHg. The depletion of GSH by DEM increased the accumulation of MeHg in cell cultures from both the cerebrum and the cerebellum. The reason for this increased cell associated-MeHg content after DEM treatment warrants further investigation. Increased cell associated-MeHg in cerebellar cultures treated with NAC or DEM might explain the increased vulnerability to MeHg toxicity in cerebellar cultures as compared to cortical cultures. However, previous studies using silver nitrate, which detects Hg^{2+} (Leyshon-Sørland et al., 1994), have postulated that the differential and selective vulnerability of cells is not simply due to the preferential accumulation of MeHg since Purkinje cells accumulate more Hg than granule cells. However, assessment of ^{14}C labeled-MeHg provides a measurement of the presence of organic mercury only.

The importance of comparable cell densities for the study of MeHg toxicity for comparing different cell types is of prime importance and appears not to be considered in previous studies (Mundy and Freudenrich, 2000; Gatti et al., 2004). The present study reports an increase in MTT activity in MeHg treated primary cultures of cerebellar astrocytes when seeded from low to high concentrations. Previous studies have reported the effects of cell density on neuronal survival (Barbin et al., 1984; Dal et al., 1988) and MTT activity (Erl et al., 2000; Fujita et al., 2001). Therefore, in cell-based assays, particular attention should be given to optimal and consistent cell seeding densities.

Since GSH content only partially explained the differential MeHg-sensitivity of cerebellar and cortical cultures, the role played by other factors should be considered. Specific neurotransmitter receptors have been suggested to contribute to the selective vulnerability of granule cells (Atchison, 2005). It has been proposed that MeHg inhibits $GABA_A$ receptors and that the presence of the α6 subunit in cerebellar granule cells (Laurie et al., 1992; Mathews et al., 1994; Yuan and Atchison, 2003) makes them more vulnerable to MeHg toxicity than Purkinje cells which contain the α1 subunit (Wisden et al., 1992; Puia et al., 1994). Astrocytes express a large variety of ion channels and neurotransmitter receptors (Verkhratsky and Steinhauser, 2000). However, cerebellar astrocytes do not contain the α6 subunit of the $GABA_A$ receptor (Bovolin et al., 1992), making the question of the enhanced sensitivity of cerebellar versus cortical astrocytes even more intriguing. Additionally, granule cells express the highest density of muscarinic M3 receptors within the cerebellar cortex (Neustadt et al., 1988), and downregulation of these receptors has been reported to protect against MeHg-induced cell death (Limke et al., 2004). Furthermore, it has been reported (Basu et al., 2005) that receptors for muscarinic acetylcholine (mACh) binding are more sensitive in the cerebellum. Another receptor likely to play a role in distinguishing cell types is the *N*-methyl-D-aspartate

receptor (NMDA), since its enhanced sensitivity has been reported to cause the increased vulnerability of cortical neurons (Miyamoto et al., 2001). The resistant Purkinje cells, however, contain AMPA-type receptors as opposed to the NMDA receptors in the cerebellum. Alteration of intracellular calcium has been investigated for comparing the sensitivities of different types of cells (Denny and Atchison, 1996; Marty and Atchison, 1997; Mundy and Freudenrich, 2000; Edwards et al., 2005). Additionally, the inhibition of ryanodine receptors by MeHg could lead to alterations in calcium signaling, which might be responsible for the sensitivity of cerebellar cultures since the cerebellum contains many of these types of receptors (Roegge and Schantz, 2006). Identifying these potentially selective targets during MeHg poisoning should provide a basis for future research for determining the mechanism(s) responsible for MeHg neurotoxicity.

Conflict of interest

The authors confirm that they do not have any financial, personal or institutional interests which may be in conflict with the content or use of the present study.

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Paper IV

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Paper V

Docosahexaenoic acid may act as a neuroprotector for methylmercury-induced neurotoxicity in primary neural cell cultures.

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Running title: DHA attenuates MeHg-induced ROS.

Abstract

The ability of docosahexaenoic acid (DHA) to modulate methylmercury (MeHg)-induced neurotoxicity was investigated in primary astrocytes and neurons from the cerebellum. Gas chromatography measurements indicated increased DHA content in both cell types after 24 hr supplementation. After individual or combined treatment with MeHg (10μM) and DHA (30 and 90μM), the cell associated-MeHg measurements were done using ^{14}C-labelled MeHg. In addition, mitochondrial activity was evaluated by MTT reduction, glutathione (GSH) content was measured with the fluorescent indicator monochlorobimane (MCB) and reactive oxygen species (ROS) were detected with the fluorescent indicator -chloro methyl derivative of di-chloro di-hydro fluorescein diacetate (CMH_2DCFDA). For all the tested treatments i.e. DHA, MeHg or DHA + MeHg treatment, the neurons differed significantly ($p<0.001$) from astrocytes exhibiting increased ROS production and decreased MTT activity. After MeHg and 30μM DHA treatment there were no changes in MTT or GSH content but significant decrease ($p<0.001$) in ROS was observed in both the cell types when compared to MeHg alone. The cell associated-MeHg measurements indicated reduced MeHg-accumulation in both cell types ($p<0.05$) upon 30μM DHA exposure. Taken together, this study, for the first time establishes that DHA pretreatment effectively reduces cell-associated MeHg and prooxidant response from MeHg in both cerebellar astrocytes and neurons and thus supports the hypothesis that fish-derived nutrients offer possible neuroprotection from MeHg.

Keywords: Neurotoxicology, *In vitro*, Primary cell cultures, Glutathione, Reactive oxygen species.

Abbreviations: DHA, Docosahexaenoic acid; MeHg, methylmercury; MTT, [3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide]; GSH, glutathione; MCB, monochlorobimane; ROS, reactive oxygen species; CMH_2DCFDA, chloro methyl derivative of di-chloro di-hydro fluoresceindiacetate; PUFA, poly unsaturated fatty acids; GFAP, glial fibrillary acidic protein; ALA, Alpha linolenic acid; FAF-BSA, Fatty acid free bovine serum albumin; DMEM, Dulbecco's modified eagle's medium-low glucose; HEPES, N-2-hydroxy-ethylpiperazine N′-2-ethansulfonic acid; BSA, bovine serum albumin; FCS, fetal calf serum; GC, gas chromatography; LA, linoleic acid; DPA, docosapentaenoic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; GPx, glutathione peroxidase .

1. Introduction

Methylmercury (MeHg) is an important environmental neurotoxicant that affects the developing and mature nervous system (Atchison, 2005; Clarkson et al., 1988). The major dietary route of human exposure to MeHg is via the ingestion of seafood for adults (Clarkson, 1997; Kamps et al., 1972; Spry and Wiener, 1991) and via maternal milk for infants (Franco et al., 2006; Manfroi et al., 2004). MeHg causes a reduction in intracellular glutathione (GSH) (Choi et al., 1996; Miura and Clarkson, 1993; Sarafian et al., 1994). Previous work from our laboratory (Kaur et al., 2007b) reported that the B35 neuronal cell line responded to MeHg by increasing GSH levels, an effect that was opposite to what had been reported for in situ primary neurons (Sanfeliu et al., 2001). Since cerebellum is the major target organ of MeHg exposure which is accompanied with the loss of granule cell layer (Eto and Takeuchi, 1978; Vendrell et al., 2007); neural cells from cerebellum were selected for the present study as a cellular model for studying MeHg-induced neurotoxicity.

The nervous system is highly enriched in long-chain poly unsaturated fatty acids (PUFAs). Fish and maternal milk are the major dietary sources of PUFAs (Larque et al., 2002; Rodriguez et al., 1999). Docosahexaenoic acid (DHA; 22:6*n*-3), in particular, is the most abundant polyunsaturated fatty acid in the brain and it is essential for the normal brain function (Kim, 2007; Salem et al., 1999; Uauy et al., 2001). Accretion of DHA in the central nervous system occurs actively during the developmental period (Scott and Bazan, 1989). DHA can be biosynthesized from alpha-linolenic acid (ALA) through chain elongation and desaturation processes (Sprecher, 2000). While neurons are highly enriched with DHA, they cannot produce it because of lack of desaturase activity; only astrocytes have the capacity to synthesize DHA (Moore et al., 1991).

Astrocytes are in close contact with neurons and readily release DHA into the extracellular fluid under basal and stimulated conditions, thus providing a source for neuronal DHA (Garcia and Kim, 1997; Kim et al., 1999; Moore, 2001). Considering astroglia support neurons by providing neurotrophic factors, the supply of DHA by astrocytes can also be trophic.

MeHg has been reported to augment reactive oxygen species (ROS) formation (Ali et al., 1992; Gasso et al., 2001; Sanfeliu et al., 2001; Sarafian, 1999; Shanker and Aschner, 2003; Sorg et al., 1998; Stringari et al., 2008; Yee and Choi, 1996). However, the ability of DHA to induce ROS is controversial. Several contrasting studies documenting the apoptotic (Albino et al., 2000; Chen and Istfan, 2000; Diep et al., 2000) and antiapoptotic (Akbar and Kim, 2002; Kim et al., 2000; Kishida et al., 1998; Rotstein et al., 1997; Yano et al., 2000) effects of DHA have been reported. In addition, contrary to the ability of DHA to decrease the level of lipid peroxide (Gamoh et al., 1999; Hashimoto et al., 2002, 2005); it has also been reported to cause free radical-mediated peroxidation in the brain (Leonardi et al., 2005; Montuschi et al., 2004; Tsai et al., 1998). Therefore, it is of importance to ascertain whether the balance between the oxidative and reductive cellular processes is disturbed following combined exposure to DHA and MeHg (DHA + MeHg).

The current advice on human mercury contaminated seafood intake is based on population data where the primary source of MeHg is either fish diet (Davidson et al., 1998; Harada, 1995; Myers et al., 1997), combined fish and whale meat diet (Grandjean et al., 1997) or wheat (Bakir et al., 1973). In particular the Seychelles (Myers et al., 1997) and the Faroes (Grandjean et al., 1997) studies differ with respect to possible

neurodevelopmental effects of MeHg exposure. The sources for these differences in the above mentioned studies have not been clearly identified (CENR, OSTP and The White house, 1998). The risk evaluation of the combined exposure to a neurotoxicant and neuroprotectant remains unsettled. Therefore, it is important to assess dietary nutrients as well as neurotoxic exposures in determining the risks and benefits of fish consumption (Myers et al., 2007). Our aim has been to provide an experimental rationale for the possible neuroprotective components present in the fish diet. Our findings contribute to a better understanding on the neurodevelopmental discrepancies found in the Seychelles (Davidson et al., 1998) and Faroes studies (Grandjean et al., 1997).

2. Materials and methods

2.1. Materials

24-well plastic tissue culture plates were purchased from Falcon (Becton Dickinson Labware, USA). Fetal bovine serum (Cat. No. 04-007-1A) used for cerebellar cultures was purchased from Biological Industries, In Vitro AS (Denmark). The culture medium (Cat. No. D5030) was acquired from Sigma Aldrich (Germany). Fatty acid free bovine serum albumin (FAF-BSA, Cat. No. A0281), DHA (Cat. No. D2534), 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 purchased from Molecular Probes, Inc. (Eugene, OR, USA). Radiolabeled ^{14}C-MeHg (Cat. no. ARC-1302) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). MeHgCl which was 99% pure (Cat. No. 23308) was purchased from K&K Laboratories (Plainview, NY, USA). All other chemicals were of analytical grade.

2.2. Animals

NMRI mice were purchased from Møllegaard Breeding Center (Copenhagen, Denmark). The animals had free access to food and water and were kept under the following constant conditions: temperature (22°C), humidity (60%) and 12 hr light/dark cycles. The animals were handled in compliance with the NTNU Animal Care and Use Committee.

2.3. Cell cultures

Primary cultures of cerebellar astrocytes were prepared from 7-day-old mice as described earlier (Hertz et al., 1989). Briefly, cerebella obtained from the brains of

mice were directly passed through Nitex nylon netting (80 μm pore size) into DMEM containing 20% (v/v) fetal calf serum (FCS). The cell suspension was then plated at a density of 1 cerebellum per 24-well culture plate, which was coated previously with poly-D-lysine. The medium was changed two days after plating and subsequently twice a week, gradually decreasing the FCS concentration to 10%. At 14 days in culture, dibutyryl-cAMP was added to the medium for one week to promote the morphological differentiation of astrocytes. The cerebellar astrocytes multiply during this period and were used for the experiment 21 days post isolation.

Primary cultures of cerebellar neurons were prepared from 7-day-old mice according to the method described by (Schousboe et al., 1989). Briefly, mild trypsinized cerebella were triturated in a DNase solution containing a soybean trypsin inhibitor. The cells were suspended in DMEM (19 mM KCl, 31 mM glucose, and 0.2 mM glutamine) supplemented with p-aminobenzoate, insulin, penicillin, garamycin and 10% (v/v) FCS. Cells were seeded at a density of 1.5×10^6 cells/$2cm^2$ in 24-multiwell plates, which were previously coated with poly-D-lysine. The cell were then incubated in a humidified 5% CO_2 / 95% air atmosphere at 37°C and used one week post isolation as previously described (Kaur et al., 2007a).

2.4. Cell culture and DHA modification

Cell morphology was frequently checked using phase contrast microscopy. A stock solution of 630 and 1890μM DHA was prepared in media with FFA-BSA and was used to modify the fatty acid content of the primary cells (shown in Table 2 and 3). The stock solution was added directly to the cells such that the final concentration given to the cells was 30 and 90μM DHA. The control cells received the same

amount of normal media with FFA-BSA. The cerebellar astrocytes and neurons received DHA on day twenty and seven respectively; and were then used for the experiment on day twenty one and eight i.e. 24 hrs after incubation with DHA. The concentrations of DHA were identical to those used in the previous study in cell lines (Kaur et al., 2007b) as a major aim of this study is to compare those effects in primary cells. The amount of protein present at the day of the experiment was between 100-125 μg protein per well for both cerebellar astrocytes and neurons. The seeding density was chosen to facilitate the fluorescence measurements.

2.5. Treatments

A stock solution of 1mM MeHgCl was prepared in 5mM Na_2CO_3. From this stock, a working solution was prepared in HEPES buffer [122 mM NaCl, 3.3 mM KCl, 0.4 mM $MgSO_4.7H_2O$, 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 the day of the experiment, the cells were washed once with HEPES buffer and incubated with 10μM MeHg for 50 min. For the last 20 min, the cells were incubated with either fluorescent probes CMH_2DCFDA (7μM) or MCB (40μM) or with colorimetric reagent MTT (2.4mM). We selected the same MeHg concentration for a short exposure time since we wanted to compare the effects in primary cells as opposed to the previously used cell lines (Kaur et al., 2007b).

2.6. Determination of fatty acid content in media and primary cells

Fatty acid composition of total lipids was analyzed by using a Trace Gas Chromatography (GC) Ultra gas chromatograph (Thermo Electron Corporation, Waltham Massachusetts, USA) with a Merlin microseal septumless SSL injector. The

gradient temperature program started at 100°C, rising to 220°C (at 50°C/min), holding 220°C for 1 minute then rising to 250°C (at 80°C/min), then holding for 1.5 minutes. The GC was equipped with a Thermo UFM Ultrafast wax column (length: 5 m, id: 0.1 mm). Lipids from the samples were extracted and then saponified and methylated with NaOH and BF_3 (both in methanol) under heat, respectively. Fatty acids were detected by Flame Ionization Dectector and identified by retention time using standard mixtures of methyl esters (Nu-Chek, Elyian, USA), and then quantified using Chromeleon software (version 6.80, Dionex). The amount of fatty acid per weight of the tissue or media was calculated using 19:0 methyl ester as an internal standard.

2.7. Determination of free thiol levels

The content of intracellular free thiols was determined by using the fluorescent indicator, MCB (40μM) as described previously (Kaur et al., 2006, 2007a). The final values of fluorescence were corrected for intracellular protein in each well and expressed as a percent of fluorescence in control wells.

2.8. Detection of intracellular ROS accumulation

Intracellular ROS accumulation was monitored as described previously (Kaur et al., 2006, 2007a) using CMH_2DCFDA (7μM) which upon oxidation yields a fluorescent adduct dichloro fluorescein (DCF). The final values were corrected for intracellular protein in each well and expressed as a percent of fluorescence in control wells.

2.9. Cytotoxicity activity

Cytotoxicity was determined by colorimetric MTT (2.4mM) reduction assay (Carmichael et al., 1987; Dahlin et al., 1999) by measuring the absorbance obtained

at 570 nm using a Sunrise absorbance reader (Tecan, Austria). Cytotoxicity was then expressed as a percent of MTT activity in control wells.

2.10. Cellular MeHg accumulation

The cell associated-MeHg studies were done with ^{14}C-labeled 10μM MeHg (82 nCi/μg Hg) for 50 min as described previously (Kaur et al., 2007a,b). The radioactivity in cells was counted in a 1450 Micro Beta Trilux Liquid scintillation counter (Wallac, Perkin Elmer Life Sciences, Norway) and corrected for cellular protein.

2.11. Estimation of protein

Protein concentration was determined by the folin reagent with BSA as a standard (Lowry et al., 1951).

2.12. Data analysis

All results are given as mean ± standard deviation. Differences between groups were analyzed statistically with one-way ANOVA followed by the Tukey HSD *post hoc* test for multiple comparisons and $p<0.05$ was considered statistically significant. In addition, a three-way ANOVA was done to evaluate the interactive effects between different parameters. Cell type (cerebellar astrocytes or neurons), MeHg treatment and DHA pretreatment were considered as three fixed factors for the dependent variables, DCF, MCB and MTT. For the dependent variable, fatty acids, cell type, type of fatty acid and DHA pretreatment were considered as three fixed factors. The three-way ANOVA was done according to the SPSS Base 14.0 User's Guide, GLM Univariate Analysis.

3. Results

The interactive effect between cell type, MeHg treatment and DHA pretreatment for primary cells is shown in Table 1. For both cell types, a significant interactive effect ($p=0.000$) between all three parameters was noted for the dependent variables ROS and MTT. This indicates that neurons differed significantly from astrocytes exhibiting increased ROS and decreased MTT activity after DHA, MeHg or DHA + MeHg treatment. For the dependent variable GSH, a significant interactive effect between cell type and DHA pretreatment, as well as cell type and MeHg treatment was noted. Observation under the microscope indicated that treatment with DHA for 24 hrs did not induce any morphological changes in the cells.

3.1. Modification of fatty acids by treatment with DHA

The fatty acid composition for the incubation media and for the primary cells is shown in Tables 2 and 3, respectively. The significant interaction between type of fatty acid × DHA pretreatment in the incubation media and primary cells (Table 1) indicated that pretreatment with DHA for 24 hrs resulted in a concentration-dependent cellular uptake of DHA from the incubation media. In addition, significant interaction between the effect of cell type × type of fatty acid in primary cells indicated that astrocytes varied significantly and had increased fatty acid content as compared to neurons. However, the non-significant interaction between the cell type × type of fatty acid × DHA pretreatment in the media and cells (Table 1) indicated that the DHA content in media used for pretreatment and the DHA content in cells after pretreatment was similar for both astrocytes and neurons. The incubation media (Table 2) contained the majority of DHA with small amounts of other PUFAs, such

as linoleic acid (LA) and ALA. However, the levels of LA and ALA in the incubation media did not vary significantly between the control, 630 and 1890 μM DHA media. The cerebellar astrocytes treated with DHA (Table 3) varied significantly ($p<0.05$) from the cerebellar neurons in the levels of total, sum of identified, DPA (docosapentaenoic acid), AA (arachidonic acid), LA, ALA, stearic and palmitic fatty acids. Furthermore, the level of DHA, AA and EPA (eicosapentaenoic acid) were also modified in cerebellar astrocytes after 90μM DHA treatment when compared to the control group. The increase of DHA in cerebellar astrocytes was associated with an increase in EPA and decrease in AA. After DHA treatment, the ratio of DHA/AA increased significantly ($p<0.001$) in astrocytes and neurons, the most prominent increase being observed in the neurons.

3.2. Modification of mitochondrial activity by treatment with MeHg and DHA

Exposure to MeHg (10μM) resulted in a more pronounced reduction of MTT activity in cerebellar neurons as compared to cerebellar astrocytes (Fig. 1). For the dependent variable, MTT, significant interactions for all the tested parameters were observed (Table 1), indicating that cerebellar neurons exhibited a greater reduction in MTT activity at all the tested treatments and differed significantly from the cerebellar astrocytes. In cerebellar astrocytes a significant increase ($p<0.05$) in MTT activity was observed after 30 and 90μM DHA treatment. On the other hand, the cerebellar neurons treated with 90μM DHA, exhibited a reduction in mitochondrial activity ($p<0.001$) when compared to control group. For both astrocytes and neurons treated with 30μM DHA + MeHg, no further reduction in MTT activity was observed when compared to MeHg group. Only in cerebellar neurons treated with 90μM DHA +

MeHg, a greater reduction in mitochondrial activity ($p<0.001$) was observed when compared with MeHg alone.

3.3. Modification of cellular thiols by treatment with MeHg and DHA

The GSH levels in cerebellar astrocytes and neurons were quantified with fluorescence intensity measurements of the bimane-GSH adduct (Fig. 2). The significant interactions for the dependent variable GSH (Table 1) indicated that cerebellar astrocytes varied significantly from the cerebellar neurons after MeHg treatment (cell type × MeHg $p=0.000$) and DHA pretreatment (cell type × DHA pretreatment $p=0.000$). Treatment with DHA alone induced a reduction in GSH activity ($p<0.001$) only in cerebellar neurons at 90μM dose when compared to control group. Additionally, 90μM DHA + MeHg treatment was able to further reduce the GSH content in cerebellar neurons when compared with MeHg alone ($p<0.001$). However, the cerebellar neurons at 30μM DHA and cerebellar astrocytes at both 30 and 90μM DHA did not exhibit this trend of decreased GSH content after combined DHA + MeHg treatment as compared to the group treated with MeHg only.

3.4. Depletion of GSH content induces ROS generation

ROS levels in primary cultures quantified by the fluorescence intensity of the oxidized product, DCF, are shown in Fig. 3. A significant interaction between cell type × MeHg × pretreatment (Table 1) indicated that the amount of ROS produced in cerebellar astrocytes varied significantly ($p=0.000$) from the cerebellar neurons. The cerebellar neurons, when treated with MeHg alone or MeHg + DHA, exhibited increased production of ROS ($p<0.001$) as compared to cerebellar astrocytes. In the cerebellar neurons, treatment with 90μM DHA alone resulted in induction of ROS

($p<0.05$). However, after DHA + MeHg exposure there was a decrease in ROS production in cerebellar neurons ($p<0.001$) when compared to MeHg treated group. In addition, the amount of ROS produced in cerebellar astrocytes in the DHA + MeHg treated group did not vary significantly from the control group. The DHA + MeHg treatment resulted in 60% decrease in ROS in cerebellar neurons ($p<0.001$) as compared to 30% decrease in cerebellar astrocytes when compared to MeHg treated group.

3.5. DHA modulation affects cell associated-MeHg

In both the cell types, treatment with 30μM DHA led to significant ($p<0.05$) decrease in cell-associated MeHg when compared to MeHg treated group (Table 4). The 90μM DHA concentration resulted in decreased cell-associated MeHg only in cerebellar neurons. In both cell types, the 30 and 90μM DHA treated groups did not vary significantly from each other with respect to the cell-associated MeHg. The cerebellar astrocytes varied significantly ($p<0.001$) and exhibited decreased cell-associated MeHg when compared to cerebellar neurons for all the tested treatments.

4. Discussion

Dietary exposure to MeHg has been linked to adverse neurological effects and is a public health concern particularly among fish-eating populations (Crump et al., 1998; Grandjean et al., 1997; Kjellstrom et al., 1989). However, findings by Hibbeln et al. (2007), using the Avon Longitudinal Study of Parents and Children (ALSPAC), indicated that lower intake of seafood during pregnancy is associated with higher risk of suboptimal neurodevelopmental outcomes. In addition, studies at Seychelles (Davidson et al., 1998; Myers et al., 1997), Peru (Marsh et al., 1995) and US (Mozaffarian and Rimm, 2006; Oken et al., 2005) have also indicated possible opposing effects of overall fish consumption (i.e., providing DHA) and MeHg exposure. These studies have raised the question of the rationale behind the decision of FDA's fish advisories (US EPA and FDA, 2004) to limit seafood consumption. Therefore, more studies aimed at analyzing the combined effects of MeHg and DHA exposure are necessitated. The innovative aspect of this study is the analysis of effects inherent to each of these compounds individually or when simultaneously administered to primary neural cells. Overall, the present study clearly establishes the ability of DHA to afford reduction towards normalization of MeHg-induced ROS.

Previous work from this laboratory (Kaur et al., 2007b) reported increased MeHg-induced ROS after DHA pretreatment using C6-glial and B35-neuronal cells. It was reported that C6-glial cells had increased sensitivity towards MeHg and DHA-induced oxidative stress as compared to the B35-neuronal cells. However, it has been well known that astrocytes contain higher concentrations of GSH than neurons (Kranich et al., 1996), and GSH provides a major cellular defense against MeHg-induced ROS (Yee and Choi, 1996). In addition, the rate limiting precursor for GSH

synthesis, cysteine, is provided by astrocytes to neurons for the neuronal synthesis of GSH (Kranich et al., 1998; Sagara et al., 1993; Wang and Cynader, 2000). Therefore, the previously reported results on increased GSH content in B35 cells upon MeHg exposure was contrary to other observations from a range of neuronal models. This observation warranted a study using primary cells to provide a better understanding and characterization of the effects of DHA and MeHg treatment.

The present study reports that MTT activity in cerebellar neurons was attenuated to a greater degree at all the experimental conditions when compared to astrocytes. This indicates that mitochondrial dehydrogenase enzyme is inhibited more significantly in cerebellar neurons after MeHg exposure as compared to astrocytes, which is in accordance with other studies (Morken et al., 2005; Sanfeliu et al., 2001). In addition, the well documented effects of MeHg-induced GSH depletion (Carty and Malone, 1979; Hughes, 1957; Mokrzan et al., 1995) and ROS generation (Sanfeliu et al., 2001; Sorg et al., 1998) were corroborated by cerebellar neurons and astrocytes where neurons exhibit more pronounced GSH depletion and ROS production.

The relationship between DHA levels and oxidative stress remains controversial. It has been reported that DHA enrichment either increases (Song et al., 2000; Brand et al., 2000) or decreases (Bechoua et al., 1999; Yavin et al., 2002) sensitivity to free radicals. Moreover, the combined effects of DHA and MeHg exposure on ROS levels have not been investigated in primary neural cells. Here, we note that combined exposure to DHA and MeHg was effective in attenuating MeHg's effect alone, reducing ROS levels in cerebellar astrocytes to levels indistinguishable from controls. Similar effects were seen in cerebellar neurons, where treatment with DHA and

MeHg significantly reduced ROS levels in comparison to MeHg treatment alone. The ability of DHA to reduce the ROS levels in primary cells is contrary to our results in transformed cell lines (Kaur et al., 2007b). It has been previously reported (Grammatikos et al., 1994; Tsai et al., 1998) that the growth of cancerous cells is more significantly inhibited by DHA as compared to non-cancerous cell type. They also reported that the cellular response to exogenous long-chain PUFAs is modified during the course of malignant transformation. Therefore the choice of cellular model for studying the effects of DHA is critical.

The reduced ROS levels after DHA + MeHg exposure in cerebellar astrocytes and neurons did not induce any significant changes in MTT or GSH production when compared with MeHg alone. Only in neurons at 90μM DHA concentration, there was a significant reduction in MTT activity and GSH content which was associated with increased ROS production. However after combined exposure to 90μM DHA and MeHg, there was no further increase in ROS in this group. This could be due to the presence of higher DHA/AA ratio in neurons as compared to astrocytes. It has been reported that a higher DHA/AA ratio prevents the generation of lipid peroxides (Gamoh et al., 1999; Hashimoto et al., 2005). In addition, it has been reported that toxicity of DHA is due to decrease in glutathione peroxidase (GPx) levels (Ding and Lind, 2007). Therefore, the protective effects of DHA against MeHg-induced ROS could be due to increased GPx levels, which, in turn, could lead to disposal of exogenous peroxides by conversion of reduced GSH to the oxidized form, GSSG (Eklow et al., 1984; Wendel, 1980). Since, the fluorescent compound MCB has been reported to react with the reduced form of GSH (Haugland, 1996), the decreased GSH and ROS levels might be explained by the increased activity of GPx by DHA.

We further noted a concentration-dependent accumulation of DHA in both primary neurons and astrocytes. However, the total lipid content of the primary cells was not modified after exposure to 30 and 90μM DHA. The concentration of DHA in adult rat brain and retina has been reported to range from 17% to >33% of the weight of the total fatty acids (Hamano et al., 1996; Stubbs and Smith, 1984). In the present study, DHA treatment resulted in DHA constituting 24-41% of the total fatty acids in the primary cultures. The amount of mg DHA/gm test material in primary cells is similar to the levels previously reported in cell lines (Kaur et al., 2007b). Apart from DHA, the cerebellar astrocytes varied significantly from the cerebellar neurons in the content of total, sum of identified, DPA, AA, EPA, LA, ALA, stearic and palmitic fatty acids. In general, astrocytes had higher fatty acid content than neurons in all the tested groups. These differences can be attributed to the supporting role played by astrocytes and the dependence of neurons on long-chain PUFA's derived from neighboring astrocytes (Moore et al., 1990; Spector and Moore, 1992).

Our results show that DHA treatment was associated with an increase in EPA and decrease in AA, probably due to reverse metabolism (retroconversion) of DHA to EPA leading to a compensatory decrease in AA which is in accordance with the previous reports in astrocytes (Champeil-Potokar et al., 2004, 2006) and other cell types (Alessandri et al., 2003). Since MeHg has been reported to increase the release of AA (Shanker et al., 2002), decrease in AA levels with DHA could be one of the mechanisms behind the protective effects of DHA. Moreover, the increase in AA metabolites is intimately integrated with decrease in GPx activity (Bryant et al., 1982; Vericel et al., 1992; Weitzel and Wendel, 1993). This phenomenon further

strengthens our proposed mechanism of increased GPx activity behind decreased GSH and ROS levels in 90μM DHA + MeHg treated cerebellar neurons.

The same 10μM MeHg concentration for a short exposure time (50min) was selected in order to compare the effects in primary cells to the previously used cell lines (Kaur et al., 2007b). The MeHg content observed in the present study is 10-20 times that of *in vivo* studies (Eto, et al., 2001) or same as in extended *in vitro* exposure studies (Sakaue et al., 2006) since the effective dose during *in vitro* exposure must be considerable higher than the *in vivo* situation when the target concentration is used as a dose indicator. Exposure to 10μM MeHg for a 50min period resulted in Hg levels in primary astrocytes analogous to the levels observed in the C6-glial cell line (Kaur et al., 2007b). However, the Hg levels found in primary neurons were higher than the levels found in B35-neuronal cell line (Kaur et al., 2007b). This may explain the increased cytotoxicity in primary neurons to MeHg vs. B35 cells. In the present study, DHA treatment led to reduced cell associated-MeHg in neurons at both 30μM and 90μM dose and in astrocytes at 30μM dose. The reduced cell associated-MeHg in astrocytes and neurons correlates with the protective effect of DHA against MeHg-induced ROS.

Primary neurons accumulated more MeHg than astrocytes, which is in accordance with our previous reports (Kaur et al., 2006, 2007a). However, previous studies using silver nitrate have reported that MeHg accumulates preferentially in astrocytes rather than in neurons (Leyshon-Sørland et al., 1994). The possible explanation for this discrepancy is that silver nitrate technique detects Hg^{2+} whereas the assessment of ^{14}C labeled-MeHg provides a measurement of organic mercury only.

In summary, the DHA pretreatment reduced the cell-associated MeHg and prooxidant response from MeHg challenge in primary cells. This was not associated with an increase in MTT activity or GSH content. The protection afforded against ROS in DHA + MeHg treated group could be due to production of less ROS due to reduced cell-associated or intracellular MeHg. The proposed protective mechanisms against MeHg-induced ROS may include the reduction in AA levels and increase in GPx activity which may further influence the downstream transcription factors and signal transduction pathways. Recognition of these protective effects and identification of their mechanisms may improve the basis for risk/benefit assessment of a MeHg-containing fish diet.

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TABLE 1.

F and P values calculated with three-way ANOVA for interactive effects between cell type (cerebellar astrocytes or neurons), MeHg treatment and DHA pretreatment for different parameters investigated in primary astrocytes and neurons from the cerebellum. N.S. indicates not statistically significant effect.

Interactions	**GSH**	**ROS**	**MTT**
Effect of cell type × MeHg	F=49.249 P=0.000	F=136.795 P=0.000	F=4.271 P=0.041
Effect of cell type × DHA pretreatment	F=11.158 P=0.000	F=1.630 P=0.200	F=45.274 P=0.000
Effect of MeHg × DHA pretreatment	F=1.385 P=0.256	F=33.607 P=0.000	F=3.811 P=0.025
Effect of cell type × MeHg × DHA pretreatment	F=2.878 P=0.062	F=8.554 P=0.000	F=12.247 P=0.000
Interactions	**Fatty Acids-media**		**Fatty Acids-cells**
Effect of cell type × type of fatty acid	**N.S.**		F=6.260 P=0.000
Effect of cell type × DHA pretreatment	**N.S.**		**N.S.**
Effect of type of fatty acid × DHA pretreatment	F=219.498 P=0.000		F=4.942 P=0.000
Effect of cell type × type of fatty acid × DHA pretreatment	**N.S.**		**N.S.**

TABLE 2. Fatty acid composition in the media after DHA treatment as measured by GC. N.D. indicates not detectable.

Incubation Media	Fatty Acids	Control	630µM DHA	1890µM DHA
Ast-DMEM (Cerebellar astrocytes)	**Total Fatty Acids**	26.0 ± 9.3	221.4 ± 5.7 [a]	622.4 ± 27.4 [a b]
	Fatty Acids Identified	24.7 ± 8.6	215.4 ± 12.1 [a]	625.8 ± 25.9 [a b]
	DHA (22:6n-3)	N.D.	192.0 ± 17.9	584.1 ± 51.1 [b]
	LA (18:2n-6)	2.4 ± 1.1	1.7 ± 0.6	1.5 ± 0.5
	α linoleic (18:3n-3)	2.0 ± 0.4	2.3 ± 0.8	2.7 ± 0.4
N-DMEM (Cerebellar neurons)	**Total Fatty Acids**	19.6 ± 9.8	237.4 ± 37.3 [a]	659.3 ± 94.8 [a b]
	Fatty Acids Identified	18.5 ± 8.9	235.9 ± 36.3 [a]	652.7 ± 100.1 [a b]
	DHA (22:6n-3)	N.D.	213.0 ± 24.8	617.7 ± 87.1 [b]
	LA (18:2n-6)	N.D.	1.8 ± 0.8	1.5 ± 0.4
	α linoleic (18:3n-3)	2.4 ± 0.3	2.5 ± 0.3	2.9 ± 0.8

Values are expressed as mean ± standard deviation (µg fatty acids/gm test material). Experiments were conducted in 4 replications for each cell types. The superscript (a) indicates $p<0.05$ as compared to control group; (b) indicates $p<0.05$ as compared to 630µM DHA treated group.

TABLE 3. Fatty acid composition in the cells after DHA pretreatment as measured by GC. N.D. indicates not detectable.

Primary Cells	Fatty Acids	Control	30μM DHA	90μM DHA
Cerebellar Astrocytes	**Total Fatty Acids**	2.3 ± 0.7 [c]	2.8 ± 0.7 [c]	3.3 ± 1.1 [c]
	Fatty Acids Identified	2.1 ± 0.6 [c]	2.3 ± 0.6 [c]	3.2 ± 1.2 [c]
	DHA (22:6n-3)	0.16 ± 0.02	0.68 ± 0.2 [a]	1.3 ± 0.2 [a b]
	DPA (22:5n-3)	0.1 ± 0.03 [c]	0.1 ± 0.03 [c]	0.1 ± 0.04 [c]
	AA (20:4n-6)	0.37 ± 0.04 [c]	0.30 ± 0.08 [c]	0.20 ± 0.08 [a c]
	EPA (20:5n-3)	0.02 ± 0.01	0.05 ± 0.02	0.06 ± 0.02 [a]
	LA (18:2n-6)	0.03 ± 0.01	0.03 ± 0.01 [c]	0.03 ± 0.02 [c]
	α linoleic (18:3n-3)	0.01 ± 0.005 [c]	0.01 ± 0.002 [c]	0.01 ± 0.002 [c]
	Stearic (18:0)	0.4 ± 0.07 [c]	0.5 ± 0.03 [c]	0.5 ± 0.14 [c]
	Palmitic (16:0)	0.5 ± 0.1 [c]	0.6 ± 0.2 [c]	0.7 ± 0.1 [c]
	DHA/AA ratio	0.43 ± 0.05 [c]	2.27 ± 1.25 [a c]	6.5 ± 1.52 [a b c]
Cerebellar Neurons	**Total Fatty Acids**	0.9 ± 0.2	1.6 ± 0.7	2.6 ± 0.8
	Fatty Acids Identified	0.8 ± 0.1	1.5 ± 0.7	2.4 ± 0.7
	DHA (22:6n-3)	0.1 ± 0.02	0.47 ± 0.1 [a]	1.05 ± 0.3 [a b]
	DPA (22:5n-3)	0.02 ± 0.007	0.03 ± 0.01	0.03 ± 0.01
	AA (20:4n-6)	0.08 ± 0.03	0.08 ± 0.02	0.10 ± 0.01
	EPA (20:5n-3)	N.D.	N.D.	N.D.
	LA (18:2n-6)	0.009 ± 0.0004	0.011 ± 0.009	0.014 ± 0.006
	α linoleic (18:3n-3)	0.004 ± 0.0007	0.004 ± 0.0005	0.006 ± 0.001
	Stearic (18:0)	0.3 ± 0.01	0.3 ± 0.09	0.3 ± 0.05
	Palmitic (16:0)	0.1 ± 0.04	0.1 ± 0.07	0.2 ± 0.14
	DHA/AA ratio	1.25 ± 0.22	5.88 ± 0.54 [a]	10.5 ± 1.83 [a b]

Values are expressed as mean ± standard deviation (mg fatty acids/gm test material). Experiments were conducted in 4 replications for each cell types. The superscript (a) indicates $p<0.05$ as compared to control group; (b) indicates $p<0.05$ as compared to 30μM DHA treated group; (c) indicates $p<0.05$ when astrocyte v/s neurons for each type of treatment.

TABLE 4.

Effect of DHA on MeHg accumulation in cerebellar astrocytes and neurons as measured by ^{14}C radiolabeled MeHg at 50 min.

Cell Line	10µM MeHg	30µM DHA + MeHg	90µM DHA + MeHg
Cerebellar astrocytes	2.9 ± 0.2 [c]	2.5 ± 0.2 [a c]	2.6 ± 0.2 [c]
Cerebellar neurons	4.6 ± 0.3	4.1 ± 0.2 [a]	4.1 ± 0.2 [a]

Values are expressed mean ± standard deviation (µg Hg/mg protein). Experiments were conducted in 10 replications for each cell types in two independent experiments. The superscript (a) indicates $p<0.05$ as compared to only MeHg treated group; (c) indicates $p<0.05$ when astrocytes v/s neurons for each type of treatment. Values were corrected for protein content in each well.

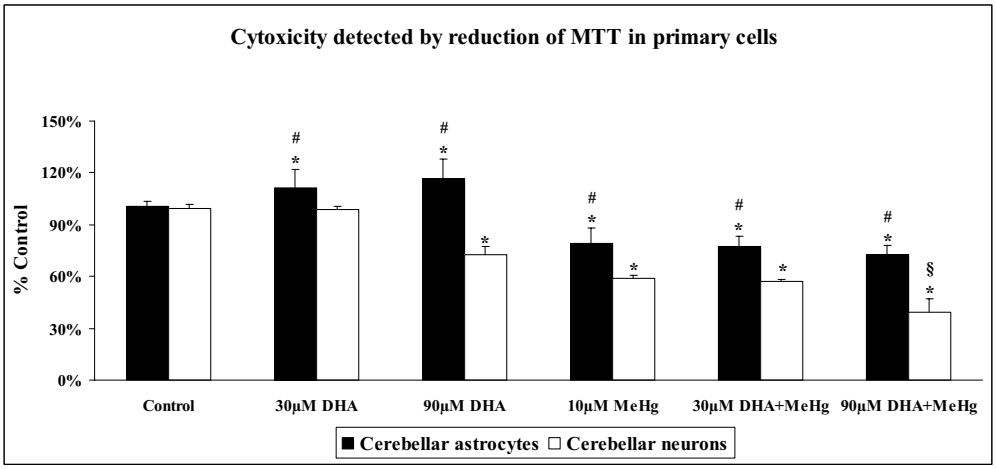
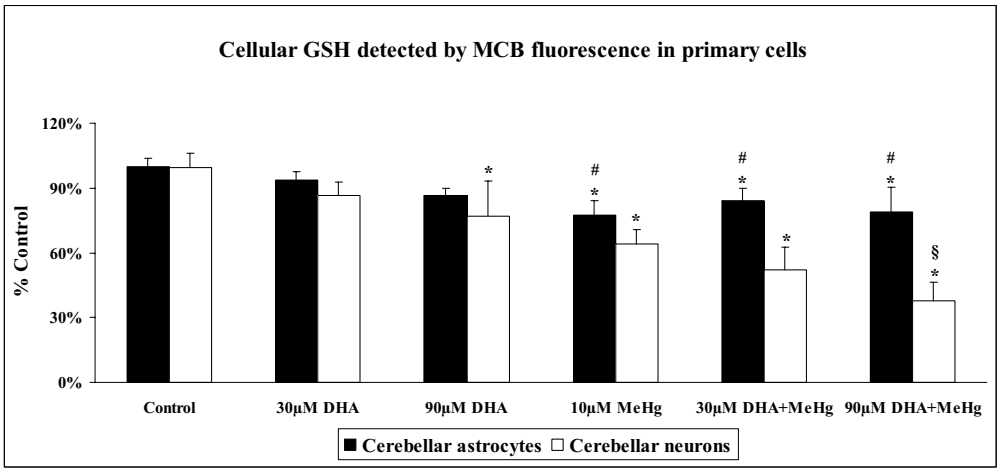

Cytoxicity detected by reduction of MTT in primary cells
% Control
150%
120%
90%
60%
30%
0%
Control
30µM DHA
90µM DHA
10µM MeHg
30µM DHA+MeHg
90µM DHA+MeHg
Cerebellar astrocytes
Cerebellar neurons

FIG. 1.


Cellular GSH detected by MCB fluorescence in primary cells
120%
90%
60%
30%
0%
% Control
Control
30µM DHA
90µM DHA
10µM MeHg
30µM DHA+MeHg
90µM DHA+MeHg
Cerebellar astrocytes
Cerebellar neurons
FIG. 2.

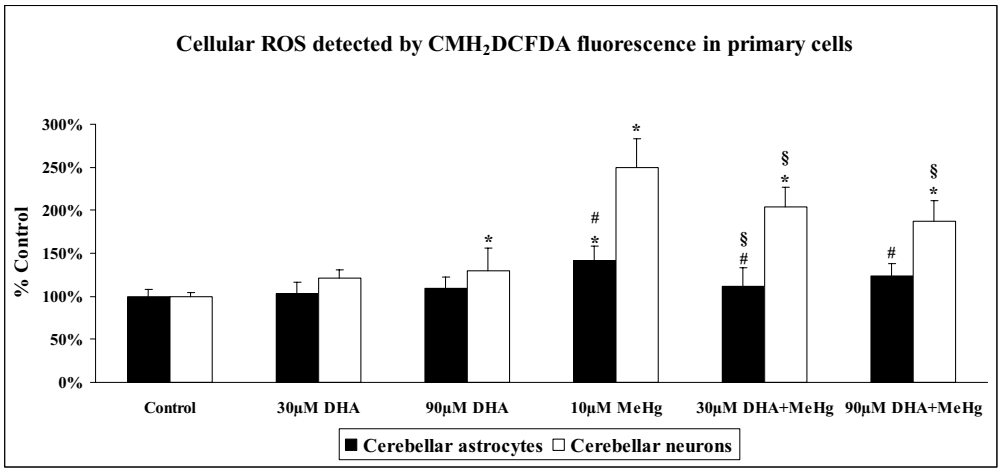

Cellular ROS detected by CMH2DCFDA fluorescence in primary cells
% Control
300%
250%
200%
150%
100%
50%
0%
Control
30µM DHA
90µM DHA
10µM MeHg
30µM DHA+MeHg
90µM DHA+MeHg
Cerebellar astrocytes
Cerebellar neurons

FIG. 3.

Legends

FIG. 1. Cytotoxicity detected by reduction of MTT in cerebellar astrocytes and neurons. Results are expressed as mean ± standard deviation (n=8 replicates for each cell type in two independent experiments). The superscript (*) indicates $p<0.05$ as compared to control group; (#) indicates $p<0.05$ when astrocytes v/s neurons for each type of treatment; (§) indicates $p<0.05$ where MeHg v/s (DHA + MeHg) treated group. Values are presented as a percentage of activity in control cells.

FIG. 2. Cellular GSH detected by MCB fluorescence in cerebellar astrocytes and neurons. Results are expressed as mean ± standard deviation (n=8 replicates for each cell type in two independent experiments). The superscript (*) indicates $p<0.05$ as compared to control group; (#) indicates $p<0.05$ when astrocytes v/s neurons for each type of treatment; (§) indicates $p<0.05$ where MeHg v/s (DHA + MeHg) treated group. Values were corrected for protein content in each well and determined as a percentage of fluorescence with respect to control cells. The average control fluorescence units for cerebellar astrocytes are 29764 ± 283 and for cerebellar neurons are 19982 ± 1368.

FIG. 3. Cellular ROS detected by CMH_2DCFDA fluorescence in cerebellar astrocytes and neurons. Results are expressed as mean ± standard deviation (n=8 replicates for each cell type in two independent experiments). The superscript (*) indicates $p<0.05$ as compared to control group; (#) indicates $p<0.05$ when astrocytes v/s neurons for each type of treatment; (§) indicates $p<0.05$ where MeHg v/s (DHA + MeHg) treated group. Values were corrected for protein content in each well and determined as a percentage of fluorescence with respect to control cells.

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145. Bård Kulseng: A STUDY OF ALGINATE CAPSULE PROPERTIES AND CYTOKINES IN RELATION TO INSULIN DEPENDENT DIABETES MELLITUS.
146. Terje Haug: STRUCTURE AND REGULATION OF THE HUMAN UNG GENE ENCODING URACIL-DNA GLYCOSYLASE.

147. Heidi Brurok: MANGANESE AND THE HEART. A Magic Metal with Diagnostic and Therapeutic Possibilites.
148. Agnes Kathrine Lie: DIAGNOSIS AND PREVALENCE OF HUMAN PAPILLOMAVIRUS INFECTION IN CERVICAL INTRAEPITELIAL NEOPLASIA. Relationship to Cell Cycle Regulatory Proteins and HLA DQBI Genes.
149. Ronald Mårvik: PHARMACOLOGICAL, PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL STUDIES ON ISOLATED STOMACS.
150. Ketil Jarl Holen: THE ROLE OF ULTRASONOGRAPHY IN THE DIAGNOSIS AND TREATMENT OF HIP DYSPLASIA IN NEWBORNS.
151. Irene Hetlevik: THE ROLE OF CLINICAL GUIDELINES IN CARDIOVASCULAR RISK INTERVENTION IN GENERAL PRACTICE.
152. Katarina Tunòn: ULTRASOUND AND PREDICTION OF GESTATIONAL AGE.
153. Johannes Soma: INTERACTION BETWEEN THE LEFT VENTRICLE AND THE SYSTEMIC ARTERIES.
154. Arild Aamodt: DEVELOPMENT AND PRE-CLINICAL EVALUATION OF A CUSTOM-MADE FEMORAL STEM.
155. Agnar Tegnander: DIAGNOSIS AND FOLLOW-UP OF CHILDREN WITH SUSPECTED OR KNOWN HIP DYSPLASIA.
156. Bent Indredavik: STROKE UNIT TREATMENT: SHORT AND LONG-TERM EFFECTS
157. Jolanta Vanagaite Vingen: PHOTOPHOBIA AND PHONOPHOBIA IN PRIMARY HEADACHES

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158. Ola Dalsegg Sæther: PATHOPHYSIOLOGY DURING PROXIMAL AORTIC CROSS-CLAMPING CLINICAL AND EXPERIMENTAL STUDIES
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160. Christina Vogt Isaksen: PRENATAL ULTRASOUND AND POSTMORTEM FINDINGS – A TEN YEAR CORRELATIVE STUDY OF FETUSES AND INFANTS WITH DEVELOPMENTAL ANOMALIES.
161. Holger Seidel: HIGH-DOSE METHOTREXATE THERAPY IN CHILDREN WITH ACUTE LYMPHOCYTIC LEUKEMIA: DOSE, CONCENTRATION, AND EFFECT CONSIDERATIONS.
162. Stein Hallan: IMPLEMENTATION OF MODERN MEDICAL DECISION ANALYSIS INTO CLINICAL DIAGNOSIS AND TREATMENT.
163. Malcolm Sue-Chu: INVASIVE AND NON-INVASIVE STUDIES IN CROSS-COUNTRY SKIERS WITH ASTHMA-LIKE SYMPTOMS.
164. Ole-Lars Brekke: EFFECTS OF ANTIOXIDANTS AND FATTY ACIDS ON TUMOR NECROSIS FACTOR-INDUCED CYTOTOXICITY.
165. Jan Lundbom: AORTOCORONARY BYPASS SURGERY: CLINICAL ASPECTS, COST CONSIDERATIONS AND WORKING ABILITY.
166. John-Anker Zwart: LUMBAR NERVE ROOT COMPRESSION, BIOCHEMICAL AND NEUROPHYSIOLOGICAL ASPECTS.
167. Geir Falck: HYPEROSMOLALITY AND THE HEART.
168. Eirik Skogvoll: CARDIAC ARREST Incidence, Intervention and Outcome.
169. Dalius Bansevicius: SHOULDER-NECK REGION IN CERTAIN HEADACHES AND CHRONIC PAIN SYNDROMES.
170. Bettina Kinge: REFRACTIVE ERRORS AND BIOMETRIC CHANGES AMONG UNIVERSITY STUDENTS IN NORWAY.
171. Gunnar Qvigstad: CONSEQUENCES OF HYPERGASTRINEMIA IN MAN
172. Hanne Ellekjær: EPIDEMIOLOGICAL STUDIES OF STROKE IN A NORWEGIAN POPULATION. INCIDENCE, RISK FACTORS AND PROGNOSIS
173. Hilde Grimstad: VIOLENCE AGAINST WOMEN AND PREGNANCY OUTCOME.
174. Astrid Hjelde: SURFACE TENSION AND COMPLEMENT ACTIVATION: Factors influencing bubble formation and bubble effects after decompression.
175. Kjell A. Kvistad: MR IN BREAST CANCER – A CLINICAL STUDY.
176. Ivar Rossvoll: ELECTIVE ORTHOPAEDIC SURGERY IN A DEFINED POPULATION. Studies on demand, waiting time for treatment and incapacity for work.
177. Carina Seidel: PROGNOSTIC VALUE AND BIOLOGICAL EFFECTS OF HEPATOCYTE GROWTH FACTOR AND SYNDECAN-1 IN MULTIPLE MYELOMA.

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178. Alexander Wahba: THE INFLUENCE OF CARDIOPULMONARY BYPASS ON PLATELET FUNCTION AND BLOOD COAGULATION – DETERMINANTS AND CLINICAL CONSEQUENSES
179. Marcus Schmitt-Egenolf: THE RELEVANCE OF THE MAJOR hISTOCOMPATIBILITY COMPLEX FOR THE GENETICS OF PSORIASIS
180. Odrun Arna Gederaas: BIOLOGICAL MECHANISMS INVOLVED IN 5-AMINOLEVULINIC ACID BASED PHOTODYNAMIC THERAPY

181. Pål Richard Romundstad: CANCER INCIDENCE AMONG NORWEGIAN ALUMINIUM WORKERS
182. Henrik Hjorth-Hansen: NOVEL CYTOKINES IN GROWTH CONTROL AND BONE DISEASE OF MULTIPLE MYELOMA
183. Gunnar Morken: SEASONAL VARIATION OF HUMAN MOOD AND BEHAVIOUR
184. Bjørn Olav Haugen: MEASUREMENT OF CARDIAC OUTPUT AND STUDIES OF VELOCITY PROFILES IN AORTIC AND MITRAL FLOW USING TWO- AND THREE-DIMENSIONAL COLOUR FLOW IMAGING
185. Geir Bråthen: THE CLASSIFICATION AND CLINICAL DIAGNOSIS OF ALCOHOL-RELATED SEIZURES
186. Knut Ivar Aasarød: RENAL INVOLVEMENT IN INFLAMMATORY RHEUMATIC DISEASE. A Study of Renal Disease in Wegener's Granulomatosis and in Primary Sjögren's Syndrome
187. Trude Helen Flo: RESEPTORS INVOLVED IN CELL ACTIVATION BY DEFINED URONIC ACID POLYMERS AND BACTERIAL COMPONENTS
188. Bodil Kavli: HUMAN URACIL-DNA GLYCOSYLASES FROM THE UNG GENE: STRUCTRUAL BASIS FOR SUBSTRATE SPECIFICITY AND REPAIR
189. Liv Thommesen: MOLECULAR MECHANISMS INVOLVED IN TNF- AND GASTRIN-MEDIATED GENE REGULATION
190. Turid Lingaas Holmen: SMOKING AND HEALTH IN ADOLESCENCE; THE NORD-TRØNDELAG HEALTH STUDY, 1995-97
191. Øyvind Hjertner: MULTIPLE MYELOMA: INTERACTIONS BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MICROENVIRONMENT
192. Asbjørn Støylen: STRAIN RATE IMAGING OF THE LEFT VENTRICLE BY ULTRASOUND. FEASIBILITY, CLINICAL VALIDATION AND PHYSIOLOGICAL ASPECTS
193. Kristian Midthjell: DIABETES IN ADULTS IN NORD-TRØNDELAG. PUBLIC HEALTH ASPECTS OF DIABETES MELLITUS IN A LARGE, NON-SELECTED NORWEGIAN POPULATION.
194. Guanglin Cui: FUNCTIONAL ASPECTS OF THE ECL CELL IN RODENTS
195. Ulrik Wisløff: CARDIAC EFFECTS OF AEROBIC ENDURANCE TRAINING: HYPERTROPHY, CONTRACTILITY AND CALCUIM HANDLING IN NORMAL AND FAILING HEART
196. Øyvind Halaas: MECHANISMS OF IMMUNOMODULATION AND CELL-MEDIATED CYTOTOXICITY INDUCED BY BACTERIAL PRODUCTS
197. Tore Amundsen: PERFUSION MR IMAGING IN THE DIAGNOSIS OF PULMONARY EMBOLISM
198. Nanna Kurtze: THE SIGNIFICANCE OF ANXIETY AND DEPRESSION IN FATIQUE AND PATTERNS OF PAIN AMONG INDIVIDUALS DIAGNOSED WITH FIBROMYALGIA: RELATIONS WITH QUALITY OF LIFE, FUNCTIONAL DISABILITY, LIFESTYLE, EMPLOYMENT STATUS, CO-MORBIDITY AND GENDER
199. Tom Ivar Lund Nilsen: PROSPECTIVE STUDIES OF CANCER RISK IN NORD-TRØNDELAG: THE HUNT STUDY. Associations with anthropometric, socioeconomic, and lifestyle risk factors
200. Asta Kristine Håberg: A NEW APPROACH TO THE STUDY OF MIDDLE CEREBRAL ARTERY OCCLUSION IN THE RAT USING MAGNETIC RESONANCE TECHNIQUES

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201. Knut Jørgen Arntzen: PREGNANCY AND CYTOKINES
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203. Asta Bye: LOW FAT, LOW LACTOSE DIET USED AS PROPHYLACTIC TREATMENT OF ACUTE INTESTINAL REACTIONS DURING PELVIC RADIOTHERAPY. A PROSPECTIVE RANDOMISED STUDY.
204. Sylvester Moyo: STUDIES ON STREPTOCOCCUS AGALACTIAE (GROUP B STREPTOCOCCUS) SURFACE-ANCHORED MARKERS WITH EMPHASIS ON STRAINS AND HUMAN SERA FROM ZIMBABWE.
205. Knut Hagen: HEAD-HUNT: THE EPIDEMIOLOGY OF HEADACHE IN NORD-TRØNDELAG
206. Li Lixin: ON THE REGULATION AND ROLE OF UNCOUPLING PROTEIN-2 IN INSULIN PRODUCING ß-CELLS
207. Anne Hildur Henriksen: SYMPTOMS OF ALLERGY AND ASTHMA VERSUS MARKERS OF LOWER AIRWAY INFLAMMATION AMONG ADOLESCENTS
208. Egil Andreas Fors: NON-MALIGNANT PAIN IN RELATION TO PSYCHOLOGICAL AND ENVIRONTENTAL FACTORS. EXPERIENTAL AND CLINICAL STUDES OF PAIN WITH FOCUS ON FIBROMYALGIA
209. Pål Klepstad: MORPHINE FOR CANCER PAIN
210. Ingunn Bakke: MECHANISMS AND CONSEQUENCES OF PEROXISOME PROLIFERATOR-INDUCED HYPERFUNCTION OF THE RAT GASTRIN PRODUCING CELL
211. Ingrid Susann Gribbestad: MAGNETIC RESONANCE IMAGING AND SPECTROSCOPY OF BREAST CANCER
212. Rønnaug Astri Ødegård: PREECLAMPSIA – MATERNAL RISK FACTORS AND FETAL GROWTH

213. Johan Haux: STUDIES ON CYTOTOXICITY INDUCED BY HUMAN NATURAL KILLER CELLS AND DIGITOXIN
214. Turid Suzanne Berg-Nielsen: PARENTING PRACTICES AND MENTALLY DISORDERED ADOLESCENTS
215. Astrid Rydning: BLOOD FLOW AS A PROTECTIVE FACTOR FOR THE STOMACH MUCOSA. AN EXPERIMENTAL STUDY ON THE ROLE OF MAST CELLS AND SENSORY AFFERENT NEURONS

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216. Jan Pål Loennechen: HEART FAILURE AFTER MYOCARDIAL INFARCTION. Regional Differences, Myocyte Function, Gene Expression, and Response to Cariporide, Losartan, and Exercise Training.
217. Elisabeth Qvigstad: EFFECTS OF FATTY ACIDS AND OVER-STIMULATION ON INSULIN SECRETION IN MAN
218. Arne Åsberg: EPIDEMIOLOGICAL STUDIES IN HEREDITARY HEMOCHROMATOSIS: PREVALENCE, MORBIDITY AND BENEFIT OF SCREENING.
219. Johan Fredrik Skomsvoll: REPRODUCTIVE OUTCOME IN WOMEN WITH RHEUMATIC DISEASE. A population registry based study of the effects of inflammatory rheumatic disease and connective tissue disease on reproductive outcome in Norwegian women in 1967-1995.
220. Siv Mørkved: URINARY INCONTINENCE DURING PREGNANCY AND AFTER DELIVERY: EFFECT OF PELVIC FLOOR MUSCLE TRAINING IN PREVENTION AND TREATMENT
221. Marit S. Jordhøy: THE IMPACT OF COMPREHENSIVE PALLIATIVE CARE
222. Tom Christian Martinsen: HYPERGASTRINEMIA AND HYPOACIDITY IN RODENTS – CAUSES AND CONSEQUENCES
223. Solveig Tingulstad: CENTRALIZATION OF PRIMARY SURGERY FOR OVARAIN CANCER. FEASIBILITY AND IMPACT ON SURVIVAL
224. Haytham Eloqayli: METABOLIC CHANGES IN THE BRAIN CAUSED BY EPILEPTIC SEIZURES
225. Torunn Bruland: STUDIES OF EARLY RETROVIRUS-HOST INTERACTIONS – VIRAL DETERMINANTS FOR PATHOGENESIS AND THE INFLUENCE OF SEX ON THE SUSCEPTIBILITY TO FRIEND MURINE LEUKAEMIA VIRUS INFECTION
226. Torstein Hole: DOPPLER ECHOCARDIOGRAPHIC EVALUATION OF LEFT VENTRICULAR FUNCTION IN PATIENTS WITH ACUTE MYOCARDIAL INFARCTION
227. Vibeke Nossum: THE EFFECT OF VASCULAR BUBBLES ON ENDOTHELIAL FUNCTION
228. Sigurd Fasting: ROUTINE BASED RECORDING OF ADVERSE EVENTS DURING ANAESTHESIA – APPLICATION IN QUALITY IMPROVEMENT AND SAFETY
229. Solfrid Romundstad: EPIDEMIOLOGICAL STUDIES OF MICROALBUMINURIA. THE NORD-TRØNDELAG HEALTH STUDY 1995-97 (HUNT 2)
230. Geir Torheim: PROCESSING OF DYNAMIC DATA SETS IN MAGNETIC RESONANCE IMAGING
231. Catrine Ahlén: SKIN INFECTIONS IN OCCUPATIONAL SATURATION DIVERS IN THE NORTH SEA AND THE IMPACT OF THE ENVIRONMENT
232. Arnulf Langhammer: RESPIRATORY SYMPTOMS, LUNG FUNCTION AND BONE MINERAL DENSITY IN A COMPREHENSIVE POPULATION SURVEY. THE NORD-TRØNDELAG HEALTH STUDY 1995-97. THE BRONCHIAL OBSTRUCTION IN NORD-TRØNDELAG STUDY
233. Einar Kjelsås: EATING DISORDERS AND PHYSICAL ACTIVITY IN NON-CLINICAL SAMPLES
234. Arne Wibe: RECTAL CANCER TREATMENT IN NORWAY – STANDARDISATION OF SURGERY AND QUALITY ASSURANCE

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235. Eivind Witsø: BONE GRAFT AS AN ANTIBIOTIC CARRIER
236. Anne Mari Sund: DEVELOPMENT OF DEPRESSIVE SYMPTOMS IN EARLY ADOLESCENCE
237. Hallvard Lærum: EVALUATION OF ELECTRONIC MEDICAL RECORDS – A CLINICAL TASK PERSPECTIVE
238. Gustav Mikkelsen: ACCESSIBILITY OF INFORMATION IN ELECTRONIC PATIENT RECORDS; AN EVALUATION OF THE ROLE OF DATA QUALITY
239. Steinar Krokstad: SOCIOECONOMIC INEQUALITIES IN HEALTH AND DISABILITY. SOCIAL EPIDEMIOLOGY IN THE NORD-TRØNDELAG HEALTH STUDY (HUNT), NORWAY
240. Arne Kristian Myhre: NORMAL VARIATION IN ANOGENITAL ANATOMY AND MICROBIOLOGY IN NON-ABUSED PRESCHOOL CHILDREN
241. Ingunn Dybedal: NEGATIVE REGULATORS OF HEMATOPOIETEC STEM AND PROGENITOR CELLS
242. Beate Sitter: TISSUE CHARACTERIZATION BY HIGH RESOLUTION MAGIC ANGLE SPINNING MR SPECTROSCOPY
243. Per Arne Aas: MACROMOLECULAR MAINTENANCE IN HUMAN CELLS – REPAIR OF URACIL IN DNA AND METHYLATIONS IN DNA AND RNA

244. Anna Bofin: FINE NEEDLE ASPIRATION CYTOLOGY IN THE PRIMARY INVESTIGATION OF BREAST TUMOURS AND IN THE DETERMINATION OF TREATMENT STRATEGIES
245. Jim Aage Nøttestad: DEINSTITUTIONALIZATION AND MENTAL HEALTH CHANGES AMONG PEOPLE WITH MENTAL RETARDATION
246. Reidar Fossmark: GASTRIC CANCER IN JAPANESE COTTON RATS
247. Wibeke Nordhøy: MANGANESE AND THE HEART, INTRACELLULAR MR RELAXATION AND WATER EXCHANGE ACROSS THE CARDIAC CELL MEMBRANE

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248. Sturla Molden: QUANTITATIVE ANALYSES OF SINGLE UNITS RECORDED FROM THE HIPPOCAMPUS AND ENTORHINAL CORTEX OF BEHAVING RATS
249. Wenche Brenne Drøyvold: EPIDEMIOLOGICAL STUDIES ON WEIGHT CHANGE AND HEALTH IN A LARGE POPULATION. THE NORD-TRØNDELAG HEALTH STUDY (HUNT)
250. Ragnhild Støen: ENDOTHELIUM-DEPENDENT VASODILATION IN THE FEMORAL ARTERY OF DEVELOPING PIGLETS
251. Aslak Steinsbekk: HOMEOPATHY IN THE PREVENTION OF UPPER RESPIRATORY TRACT INFECTIONS IN CHILDREN
252. Hill-Aina Steffenach: MEMORY IN HIPPOCAMPAL AND CORTICO-HIPPOCAMPAL CIRCUITS
253. Eystein Stordal: ASPECTS OF THE EPIDEMIOLOGY OF DEPRESSIONS BASED ON SELF-RATING IN A LARGE GENERAL HEALTH STUDY (THE HUNT-2 STUDY)
254. Viggo Pettersen: FROM MUSCLES TO SINGING: THE ACTIVITY OF ACCESSORY BREATHING MUSCLES AND THORAX MOVEMENT IN CLASSICAL SINGING
255. Marianne Fyhn: SPATIAL MAPS IN THE HIPPOCAMPUS AND ENTORHINAL CORTEX
256. Robert Valderhaug: OBSESSIVE-COMPULSIVE DISORDER AMONG CHILDREN AND ADOLESCENTS: CHARACTERISTICS AND PSYCHOLOGICAL MANAGEMENT OF PATIENTS IN OUTPATIENT PSYCHIATRIC CLINICS
257. Erik Skaaheim Haug: INFRARENAL ABDOMINAL AORTIC ANEURYSMS – COMORBIDITY AND RESULTS FOLLOWING OPEN SURGERY
258. Daniel Kondziella: GLIAL-NEURONAL INTERACTIONS IN EXPERIMENTAL BRAIN DISORDERS
259. Vegard Heimly Brun: ROUTES TO SPATIAL MEMORY IN HIPPOCAMPAL PLACE CELLS
260. Kenneth McMillan: PHYSIOLOGICAL ASSESSMENT AND TRAINING OF ENDURANCE AND STRENGTH IN PROFESSIONAL YOUTH SOCCER PLAYERS
261. Marit Sæbø Indredavik: MENTAL HEALTH AND CEREBRAL MAGNETIC RESONANCE IMAGING IN ADOLESCENTS WITH LOW BIRTH WEIGHT
262. Ole Johan Kemi: ON THE CELLULAR BASIS OF AEROBIC FITNESS, INTENSITY-DEPENDENCE AND TIME-COURSE OF CARDIOMYOCYTE AND ENDOTHELIAL ADAPTATIONS TO EXERCISE TRAINING
263. Eszter Vanky: POLYCYSTIC OVARY SYNDROME – METFORMIN TREATMENT IN PREGNANCY
264. Hild Fjærtoft: EXTENDED STROKE UNIT SERVICE AND EARLY SUPPORTED DISCHARGE. SHORT AND LONG-TERM EFFECTS
265. Grete Dyb: POSTTRAUMATIC STRESS REACTIONS IN CHILDREN AND ADOLESCENTS
266. Vidar Fykse: SOMATOSTATIN AND THE STOMACH
267. Kirsti Berg: OXIDATIVE STRESS AND THE ISCHEMIC HEART: A STUDY IN PATIENTS UNDERGOING CORONARY REVASCULARIZATION
268. Björn Inge Gustafsson: THE SEROTONIN PRODUCING ENTEROCHROMAFFIN CELL, AND EFFECTS OF HYPERSEROTONINEMIA ON HEART AND BONE

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269. Torstein Baade Rø: EFFECTS OF BONE MORPHOGENETIC PROTEINS, HEPATOCYTE GROWTH FACTOR AND INTERLEUKIN-21 IN MULTIPLE MYELOMA
270. May-Britt Tessem: METABOLIC EFFECTS OF ULTRAVIOLET RADIATION ON THE ANTERIOR PART OF THE EYE
271. Anne-Sofie Helvik: COPING AND EVERYDAY LIFE IN A POPULATION OF ADULTS WITH HEARING IMPAIRMENT
272. Therese Standal: MULTIPLE MYELOMA: THE INTERPLAY BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MARROW MICROENVIRONMENT
273. Ingvild Saltvedt: TREATMENT OF ACUTELY SICK, FRAIL ELDERLY PATIENTS IN A GERIATRIC EVALUATION AND MANAGEMENT UNIT – RESULTS FROM A PROSPECTIVE RANDOMISED TRIAL
274. Birger Henning Endreseth: STRATEGIES IN RECTAL CANCER TREATMENT – FOCUS ON EARLY RECTAL CANCER AND THE INFLUENCE OF AGE ON PROGNOSIS
275. Anne Mari Aukan Rokstad: ALGINATE CAPSULES AS BIOREACTORS FOR CELL THERAPY
276. Mansour Akbari: HUMAN BASE EXCISION REPAIR FOR PRESERVATION OF GENOMIC STABILITY

277. Stein Sundstrøm: IMPROVING TREATMENT IN PATIENTS WITH LUNG CANCER – RESULTS FROM TWO MULITCENTRE RANDOMISED STUDIES
278. Hilde Pleym: BLEEDING AFTER CORONARY ARTERY BYPASS SURGERY - STUDIES ON HEMOSTATIC MECHANISMS, PROPHYLACTIC DRUG TREATMENT AND EFFECTS OF AUTOTRANSFUSION
279. Line Merethe Oldervoll: PHYSICAL ACTIVITY AND EXERCISE INTERVENTIONS IN CANCER PATIENTS
280. Boye Welde: THE SIGNIFICANCE OF ENDURANCE TRAINING, RESISTANCE TRAINING AND MOTIVATIONAL STYLES IN ATHLETIC PERFORMANCE AMONG ELITE JUNIOR CROSS-COUNTRY SKIERS
281. Per Olav Vandvik: IRRITABLE BOWEL SYNDROME IN NORWAY, STUDIES OF PREVALENCE, DIAGNOSIS AND CHARACTERISTICS IN GENERAL PRACTICE AND IN THE POPULATION
282. Idar Kirkeby-Garstad: CLINICAL PHYSIOLOGY OF EARLY MOBILIZATION AFTER CARDIAC SURGERY
283. Linn Getz: SUSTAINABLE AND RESPONSIBLE PREVENTIVE MEDICINE. CONCEPTUALISING ETHICAL DILEMMAS ARISING FROM CLINICAL IMPLEMENTATION OF ADVANCING MEDICAL TECHNOLOGY
284. Eva Tegnander: DETECTION OF CONGENITAL HEART DEFECTS IN A NON-SELECTED POPULATION OF 42,381 FETUSES
285. Kristin Gabestad Nørsett: GENE EXPRESSION STUDIES IN GASTROINTESTINAL PATHOPHYSIOLOGY AND NEOPLASIA
286. Per Magnus Haram: GENETIC VS. AQUIRED FITNESS: METABOLIC, VASCULAR AND CARDIOMYOCYTE ADAPTATIONS
287. Agneta Johansson: GENERAL RISK FACTORS FOR GAMBLING PROBLEMS AND THE PREVALENCE OG PATHOLOGICAL GAMBLING IN NORWAY
288. Svein Artur Jensen: THE PREVALENCE OF SYMPTOMATIC ARTERIAL DISEASE OF THE LOWER LIMB
289. Charlotte Björk Ingul: QUANITIFICATION OF REGIONAL MYOCARDIAL FUNCTION BY STRAIN RATE AND STRAIN FOR EVALUATION OF CORONARY ARTERY DISEASE. AUTOMATED VERSUS MANUAL ANALYSIS DURING ACUTE MYOCARDIAL INFARCTION AND DOBUTAMINE STRESS ECHOCARDIOGRAPHY
290. Jakob Nakling: RESULTS AND CONSEQUENCES OF ROUTINE ULTRASOUND SCREENING IN PREGNANCY – A GEOGRAPHIC BASED POPULATION STUDY
291. Anne Engum: DEPRESSION AND ANXIETY – THEIR RELATIONS TO THYROID DYSFUNCTION AND DIABETES IN A LARGE EPIDEMIOLOGICAL STUDY
292. Ottar Bjerkeset: ANXIETY AND DEPRESSION IN THE GENERAL POPULATION: RISK FACTORS, INTERVENTION AND OUTCOME – THE NORD-TRØNDELAG HEALTH STUDY (HUNT)
293. Jon Olav Drogset: RESULTS AFTER SURGICAL TREATMENT OF ANTERIOR CRUCIATE LIGAMENT INJURIES – A CLINICAL STUDY
294. Lars Fosse: MECHANICAL BEHAVIOUR OF COMPACTED MORSELLISED BONE – AN EXPERIMENTAL IN VITRO STUDY
295. Gunilla Klensmeden Fosse: MENTAL HEALTH OF PSYCHIATRIC OUTPATIENTS BULLIED IN CHILDHOOD
296. Paul Jarle Mork: MUSCLE ACTIVITY IN WORK AND LEISURE AND ITS ASSOCIATION TO MUSCULOSKELETAL PAIN
297. Björn Stenström: LESSONS FROM RODENTS: I: MECHANISMS OF OBESITY SURGERY – ROLE OF STOMACH. II: CARCINOGENIC EFFECTS OF *HELICOBACTER PYLORI* AND SNUS IN THE STOMACH

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298. Haakon R. Skogseth: INVASIVE PROPERTIES OF CANCER – A TREATMENT TARGET ? IN VITRO STUDIES IN HUMAN PROSTATE CANCER CELL LINES
299. Janniche Hammer: GLUTAMATE METABOLISM AND CYCLING IN MESIAL TEMPORAL LOBE EPILEPSY
300. May Britt Drugli: YOUNG CHILDREN TREATED BECAUSE OF ODD/CD: CONDUCT PROBLEMS AND SOCIAL COMPETENCIES IN DAY-CARE AND SCHOOL SETTINGS
301. Arne Skjold: MAGNETIC RESONANCE KINETICS OF MANGANESE DIPYRIDOXYL DIPHOSPHATE (MnDPDP) IN HUMAN MYOCARDIUM. STUDIES IN HEALTHY VOLUNTEERS AND IN PATIENTS WITH RECENT MYOCARDIAL INFARCTION
302. Siri Malm: LEFT VENTRICULAR SYSTOLIC FUNCTION AND MYOCARDIAL PERFUSION ASSESSED BY CONTRAST ECHOCARDIOGRAPHY
303. Valentina Maria do Rosario Cabral Iversen: MENTAL HEALTH AND PSYCHOLOGICAL ADAPTATION OF CLINICAL AND NON-CLINICAL MIGRANT GROUPS

304. Lasse Løvstakken: SIGNAL PROCESSING IN DIAGNOSTIC ULTRASOUND: ALGORITHMS FOR REAL-TIME ESTIMATION AND VISUALIZATION OF BLOOD FLOW VELOCITY
305. Elisabeth Olstad: GLUTAMATE AND GABA: MAJOR PLAYERS IN NEURONAL METABOLISM
306. Lilian Leistad: THE ROLE OF CYTOKINES AND PHOSPHOLIPASE A_2S IN ARTICULAR CARTILAGE CHONDROCYTES IN RHEUMATOID ARTHRITIS AND OSTEOARTHRITIS
307. Arne Vaaler: EFFECTS OF PSYCHIATRIC INTENSIVE CARE UNIT IN AN ACUTE PSYCIATHRIC WARD
308. Mathias Toft: GENETIC STUDIES OF LRRK2 AND PINK1 IN PARKINSON'S DISEASE
309. Ingrid Løvold Mostad: IMPACT OF DIETARY FAT QUANTITY AND QUALITY IN TYPE 2 DIABETES WITH EMPHASIS ON MARINE N-3 FATTY ACIDS
310. Torill Eidhammer Sjøbakk: MR DETERMINED BRAIN METABOLIC PATTERN IN PATIENTS WITH BRAIN METASTASES AND ADOLESCENTS WITH LOW BIRTH WEIGHT
311. Vidar Beisvåg: PHYSIOLOGICAL GENOMICS OF HEART FAILURE: FROM TECHNOLOGY TO PHYSIOLOGY
312. Olav Magnus Søndenå Fredheim: HEALTH RELATED QUALITY OF LIFE ASSESSMENT AND ASPECTS OF THE CLINICAL PHARMACOLOGY OF METHADONE IN PATIENTS WITH CHRONIC NON-MALIGNANT PAIN
313. Anne Brantberg: FETAL AND PERINATAL IMPLICATIONS OF ANOMALIES IN THE GASTROINTESTINAL TRACT AND THE ABDOMINAL WALL
314. Erik Solligård: GUT LUMINAL MICRODIALYSIS
315. Elin Tollefsen: RESPIRATORY SYMPTOMS IN A COMPREHENSIVE POPULATION BASED STUDY AMONG ADOLESCENTS 13-19 YEARS. YOUNG-HUNT 1995-97 AND 2000-01; THE NORD-TRØNDELAG HEALTH STUDIES (HUNT)
316. Anne-Tove Brenne: GROWTH REGULATION OF MYELOMA CELLS
317. Heidi Knobel: FATIGUE IN CANCER TREATMENT – ASSESSMENT, COURSE AND ETIOLOGY
318. Torbjørn Dahl: CAROTID ARTERY STENOSIS. DIAGNOSTIC AND THERAPEUTIC ASPECTS
319. Inge-Andre Rasmussen jr.: FUNCTIONAL AND DIFFUSION TENSOR MAGNETIC RESONANCE IMAGING IN NEUROSURGICAL PATIENTS
320. Grete Helen Bratberg: PUBERTAL TIMING – ANTECEDENT TO RISK OR RESILIENCE ? EPIDEMIOLOGICAL STUDIES ON GROWTH, MATURATION AND HEALTH RISK BEHAVIOURS; THE YOUNG HUNT STUDY, NORD-TRØNDELAG, NORWAY
321. Sveinung Sørhaug: THE PULMONARY NEUROENDOCRINE SYSTEM. PHYSIOLOGICAL, PATHOLOGICAL AND TUMOURIGENIC ASPECTS
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