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Toxicity of organic and inorganic mercury species in differentiated human neurons and human astrocytes

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A B S T R A C T

Organic mercury (Hg) species exert their toxicity primarily in the central nervous system. The food relevant Hg species methylmercury (MeHg) has been frequently studied regarding its neurotoxic effects in vitro and in vivo. Neurotoxicity of thiomersal, which is used as a preservative in medical preparations, is to date less characterised. Due to dealkylation of organic Hg or oxidation of elemental Hg, inorganic Hg is present in the brain albeit these species are not able to readily cross the blood brain barrier. This study compared for the first time toxic effects of organic MeHg chloride (MeHgCl) and thiomersal as well as inorganic mercury chloride ($HgCl_2$) in differentiated human neurons (LUHMES) and human astrocytes (CCF-STTG1). The three Hg species differ in their degree and mechanism of toxicity in those two types of brain cells. Generally, neurons are more susceptible to Hg species induced cytotoxicity as compared to astrocytes. This might be due to the massive cellular mercury uptake in the differentiated neurons. The organic compounds exerted stronger cytotoxic effects as compared to inorganic $HgCl_2$. In contrast to $HgCl_2$ exposure, organic Hg compounds seem to induce the apoptotic cascade in neurons following low-level exposure. No indicators for apoptosis were identified for both inorganic and organic mercury species in astrocytes. Our studies clearly demonstrate species-specific toxic mechanisms. A mixed exposure towards all Hg species in the brain can be assumed. Thus, prospectively coexposure studies as well as cocultures of neurons and astrocytes could provide additional information in the investigation of Hg induced neurotoxicity.

1. Introduction

Organic mercury (Hg) compounds are important neurotoxicants capable of damaging the developing and adult nervous system [1]. Due to its accumulation in the aquatic food chain, chronic exposure to methylmercury (MeHg) via seafood intake still poses a risk to human health [2]. Ethylmercury (EtHg) containing thiomersal, used as a preservative in medical preparations including vaccines, is of particular concern since it has been linked to autism [3]. Although organic Hg compounds, especially methylmercury (MeHg), have been extensively studied, the mechanisms of Hg species mediated neurotoxicity remain not completely understood [4]. Inorganic Hg^{2+} does not readily cross the blood brain barrier. Probably therefore effects of inorganic Hg^{2+} species on brain cells are not well characterized [5]. Nevertheless, it should be noted that inorganic Hg is present in the brain due to dealkylation of organic species or an oxidation of elemental Hg, which originates e.g., from the outgassing of amalgam fillings [6,7].

In the literature only a few in vitro studies exist, either comparing effects of one Hg species, especially MeHg, in different brain associated cells or comparing different Hg species in one cell type. Sanfeliu et al. performed in vitro cytotoxicity studies in primary proliferating human astrocytes and neurons, indicating an enhanced sensitivity of neurons towards MeHg as compared to astrocytes [8]. In vitro studies in primary proliferating astrocytes and neurons from murine cerebella confirmed these results [9].

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The respective concentrations of MeHg, which cause significant effects on neuronal cell viability, depend on the animal species and the duration of exposure. They ranged from 30 nM in rat cerebellar neurons (48 h incubation) to 5 μM in mouse cerebellar neurons (15 min incubation) [9–11]. Since evidence exists that MeHg accumulates preferentially in astrocytes and neuronal dysfunction is secondary to disturbance of astrocytes, a central role for astrocytes in mediating Hg induced neurotoxicity has been proposed [12]. Pieper et al. compared cytotoxic effects of MeHg, thiomersal and mercuric mercury on human astrocytes (CCF-STTG1) concluding an increased cytotoxic potential with rising alkylation level [13]. Previous studies have demonstrated similar toxicities of EtHg and MeHg in brain-associated cells, whereas inorganic Hg induced effects were restricted to substantially higher concentrations [14,15]. Disturbance of neuronal function is accompanied with altered functions specific for certain neurotransmitters such as glutamate and dopamine [16,17]. Nevertheless, numerous studies are limited to the use of immature, proliferating neurons [8,9,11,18,19] or neurons during differentiation [20–22] to investigate developmental neurotoxicity. Representing a differentiated neural cell culture model, Castoldi et al. investigated effects of MeHg on primary cultures of mitotically inhibited rat granule neurons [23]. Furthermore, neurite outgrowth was affected in a differentiated PC12 cell clone [24]. To our knowledge, only one study exists comparing effects of MeHgCl on cell viability and neurite degeneration in a differentiated human neural cell culture model. In the respective study, MeHgCl inhibited neurite growth of differentiating LUHMES cells, whereas MeHgCl did not affect the mature neurites without inducing cell death [25].

In this study, we apply LUHMES human neuronal cells that can be differentiated within 6 days into mature neurons [26]. Here we compare toxic effects of organic MeHgCl and thiomersal as well as inorganic $HgCl_2$ in human astrocytes (CCF-STTG1) and differentiated human neurons (LUHMES) within one study for the first time.

2. Material and methods

2.1. LUHMES cell culture and differentiation

Cell culture dishes were pre-coated with 50 μg/mL poly-L-ornithine (Sigma–Aldrich, Deisenhofen, Germany) and 1 μg/mL fibronectin (Sigma–Aldrich) in sterile distilled water overnight at 37 °C. Dishes were washed with water and completely air dried before cell seeding. For proliferation, cells were cultured in Advanced Dulbecco's modified Eagle's medium/F12 (Advanced DMEM/F12, Life Technologies GmbH, Darmstadt, Germany) supplemented with 1 × N2 supplement (Life Technologies), 2 mM L-glutamine (Biochrom, Berlin, Germany) and 40 ng/mL recombinant human basic fibroblast growth factor (FGF, R&D Systems, Wiesbaden-Nordenstadt, Germany) at 37 °C in a humidified 95% air, 5% CO_2 atmosphere. In accordance to the published protocol [25,27], cell differentiation was initiated 24 h after seeding the cells at a density of 45,000 cells per cm^2 by replacing the proliferation medium with differentiation medium consisting of Advanced DMEM/F12 containing 1 × N2 supplement, 2 mM L-glutamine, 1 μg/mL tetracycline (Sigma–Aldrich), 1 mM dibutyryl cyclic adenosine monophosphate sodium salt (cAMP, Sigma–Aldrich) and 2 ng/mL recombinant human glial cell-derived neurotrophic factor (GDNF, R&D Systems). After 48 h of differentiation, cells were trypsinized and seeded on pre-coated dishes in a defined density (150,000 cells/cm^2) in differentiation medium. Another 48 h later medium is replaced by fresh differentiation medium for additional 2 days. Differentiated LUHMES cells were exposed to the respective Hg species for 24 or 48 h depending on the respective experiment.

2.2. Astrocyte cell culture

CCF-STTG1 cells were cultured in RPMI 1640 (Biochrom) supplemented with 10% FCS (PAA Laboratories, Pasching, Austria), 1.4 mM L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin (PAA) at 37 °C in a humidified 95% air, 5% CO_2 atmosphere. Cells were seeded in a defined density (30,000 cells/cm^2) in 96 or 24 well cell culture plates, respectively. Since proliferating CCF-STTG1 cells are logarithmically growing with a generation interval of about 48 h, all experiments were carried out 48 h after incubation with the respective test compound.

2.3. Preparation of Hg species stock solutions

Stock solutions of MeHgCl (≥99.9% purity, Sigma–Aldrich), thiomersal (≥97% purity, Sigma–Aldrich) and $HgCl_2$ (≥99.999% purity, Sigma–Aldrich) were prepared in sterile distilled water shortly before each experiment.

2.4. Cytotoxicity testing

The cytotoxicity of the Hg species was evaluated after 24 h and 96 h incubation by quantifying their effects on dehydrogenase activity, lysosomal integrity and cell membrane integrity.

2.4.1. Dehydrogenase activity

Cell metabolic activity was assessed by the resazurin assay. Intracellular oxidoreductases contribute to the reduction of resazurin (blue and nonfluorescent) to resorufin (pink and highly fluorescent) with the participation of NADH as cofactor representing the redox potential of the cells [28]. LUHMES cells were seeded and fully differentiated in 96 well cell culture plates and incubated with the respective Hg species for 24 h. CCF-STTG1 cells were exposed to the compounds for 48 h in 96 well plates. Following incubation with the respective Hg species, medium was replaced by resazurin (7-Hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt, Sigma–Aldrich) containing medium (final concentration: 2.5 μg/mL). The fluorescence was measured after 3 h of incubation at 37 °C at 590 nm with an excitation at 540 nm using a plate reader (BMG Labtech FLUOstar OPTIMA).

2.4.2. Lysosomal integrity

Lysosomal integrity was determined by the neutral red uptake assay. This assay is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in their lysosomes [29]. Differentiated LUHMES were seeded in 96 well culture plates and cultivated under differentiating conditions for 4 days as previously described. Cells were exposed to the respective Hg species for 24 h and 96 h. In case of 96 h incubation, medium was replaced by fresh differentiation medium containing the Hg species in their appropriate concentration after 48 h. Following incubation, the medium was replaced by neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride; Roth, Karlsruhe, Germany) containing medium (final concentration: 66.7 μg/mL in Advanced DMEM/F12) and incubated 3 h at 37 °C. Cells were washed twice with PBS containing 0.5% formaldehyde and the incorporated dye was solubilised in acidified EtOH solution (50% EtOH, 1% acetic acid in PBS). The absorbance was measured at 540 nm.

2.4.3. Cell membrane integrity

The cell membrane integrity was assessed by lactate dehydrogenase (LDH) release in the supernatant and the corresponding cell lysate. Differentiated LUHMES cells were exposed to the respective

test compounds for 24 h, CCF-STTG1 cells for 48 h in 24 well culture plates. The LDH release assay was carried out as described before [30]. Briefly, 40 μL of culture medium and 15 μL of cell lysates were mixed in a 96 well plate with reaction buffer (100 mM HEPES, 0.14 g/L NADH, 1.1 g/L sodium pyruvate, pH 7) and the absorbance was measured at 355 nm every 1.5 min at 37 °C over a period of 60 min.

2.5. Cellular bioavailability

After 24 h exposure to the respective Hg species in 24 well cell culture plates, LUHMES were washed twice with PBS and incubated with 120 μL RIPA-buffer (0.01 M Tris, pH 7.6, 0.15 M NaCl, 0.001 M EDTA, 1% sodium desoxycholate, 0.1% SDS (all Sigma Aldrich)) for 15 min on ice. Cells were scraped off and sonicated and the resulting cell suspension was centrifuged at 10,000 × *g* for 20 min at 4 °C. Total cellular Hg content was quantified by inductively coupled plasma mass spectrometry (ICP-MS; Agilent 8800 ICP-QQQ, Agilent Technologies Deutschland GmbH, Boeblingen, Germany) in an aliquot of the supernatant. An external calibration (1–150 ng/L; Hg standard for ICP, TraceCERT®, Fluka, Deisenhofen, Germany) was prepared in 5% HNO_3 + 5% HCl (v/v, both suprapur, Merck KGaA). Aliquots were diluted in the same solution correspondingly. Samples and calibration standards were spiked with rhodium (final concentration 10 ng/L; Merck KGaA, Darmstadt, Germany) as internal standard. The method exhibits a limit of detection of 0.8 ng/L and a limit of quantification of 2.9 ng/L calculated by the calibration method of the German Standard DIN standard 32645 [31]. Total cellular Hg content was related to the cellular protein level, determined by the Bradford assay. In a further approach, results of cellular Hg content were related to the cell volume and number of cells to calculate the molar cellular Hg concentration. This calculation allows for comparison of the effective cellular Hg concentration and the applied incubation concentration of the respective Hg species. The cell volume was determined using an automatic cell counter (CASY®TTC, Roche Innovatis AG, Bielefeld, Germany). These measurements are based on non-invasive, dye-free electrical current exclusion with signal evaluation via pulse area analysis [32]. The cell volume of differentiated cells was 705 ± 22 fL. The number of seeded cells and the cell volume was not affected after incubation of non-cytotoxic concentrations of the Hg species. Thus, in concentration ranges below the EC_{30} values the respective molar cellular Hg concentrations were estimated in relation to the cell volume of 705 ± 22 fL.

2.6. Apoptosis

Apoptosis was elucidated by detection of caspase-3 activation using cell lysates of LDH release assay in LUHMES and CCF-STTG1 cells as described before with slight modifications [33]. 10 μL/30 μL cell lysate (LUHMES/CCF-STTG1) were mixed with reaction buffer (50 mM PIPES, 10 mM EDTA, 0.5% CHAPS, 10 mM DTT, 80 μM DEVD-AFC) in a black 96 well plate and incubated for 1 h/3 h at 37 °C. Subsequently, fluorescence activity of cleaved 7-amino-4-trifluoromethylcumarin (AFC) was determined (400 nm_{ex}, 520 nm_{em}). Quantification was achieved via a standard calibration curve of AFC (0.3–12.8 μM). Results were related to the respective protein contents, quantified by the bicinchoninic acid (BCA) assay.

Results of the caspase-3 activity assay were confirmed by visualization of apoptotic bodies via fluorescence microscopy. Differentiated LUHMES cells were seeded on pre-coated cover slips and incubated with the Hg compounds for 24 h. Subsequently, cells were rinsed with cold PBS containing 0.2 mg/mL magnesium chloride and fixed with ice cold methanol. After fixation, cells were rinsed twice with Tris-buffered saline (TBS) and coverslips were embedded in Vectashield mounting medium containing 1 μg/mL DAPI (Vector Laboratories Inc., Burlingame, CA, USA). Fluorescence analysis was performed applying a Keyence BZ-X700 fluorescence microscope (Keyence, Neu-Isenburg, Germany).

2.7. Statistical analysis

All experiments were carried out three times, each time on a different day with at least three independent measurements minimum. The mean standard deviation (SD) was calculated using the raw data. A statistical analysis was performed by using ANOVA-OneWay-test followed by Dunnettís multiple comparison test. Significance levels are $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

3. Results

3.1. Cytotoxicity

In differentiated LUHMES cells organic MeHgCl and thiomersal disturbed dehydrogenase activity after 24 h incubation in 26–30 fold lower concentrations as compared to inorganic $HgCl_2$ (Fig. 1A–C). These stronger effects of the organic mercury species were likewise visible in astrocytes (Fig. 1D–F), which were much less sensitive towards all Hg species (Table 1).

Since lysosomal integrity turned out to be the most sensitive cytotoxicity endpoint studied after 24 h incubation, we studied this viability marker additionally after long-term (96 h) exposure (Fig. 2A–C). The cytotoxic potential of $HgCl_2$ increased with incubation time to a higher extent as compared to organic Hg compounds.

LDH release was monitored to elucidate cell membrane integrity and was identified as most insensitive endpoint for all studied Hg species in both cellular systems. In astrocytes and differentiated neurons, organic MeHgCl and thiomersal as well as inorganic $HgCl_2$ significantly increased the LDH release only at concentrations above the respective EC_{30} values for the endpoints lysosomal integrity and dehydrogenase activity (Fig. 3A–F).

3.2. Cellular bioavailability

After incubation of differentiated neurons with the respective Hg species, intracellular total Hg concentrations increased in a concentration and time dependent manner. As indicated in Table 2, the incubation of organic MeHgCl and thiomersal resulted in higher cellular Hg concentrations as compared to the inorganic compound. Table 3 shows the calculated molar cellular Hg concentrations. The results indicate a massive accumulation of Hg in the cells as compared to the applied concentrations of the respective Hg species. Following 24 h incubation with the organic species, the cellular Hg content was about 1000 fold higher as compared to the applied concentrations. Inorganic Hg incubation led to 100–290 fold higher cellular Hg concentrations. These accumulation factors were approximately doubled following 96 h exposure towards the Hg species.

3.3. Apoptosis

Apoptosis was assessed by caspase-3 activity and the formation of apoptotic bodies. A 24 h incubation with both MeHgCl and thiomersal increased caspase-3 activity in differentiated neurons in a concentration dependent manner (Fig. 4A and B). In contrast, inorganic $HgCl_2$ did not cause any significant increase in caspase-3 activity up to an incubation concentration of 20 μM (Fig. 4C). The fluorescence microscopic images clearly support these data and reveal the presence of apoptotic bodies in case of low MeHgCl and thiomersal but not $HgCl_2$ incubation (Fig. 5A–G).

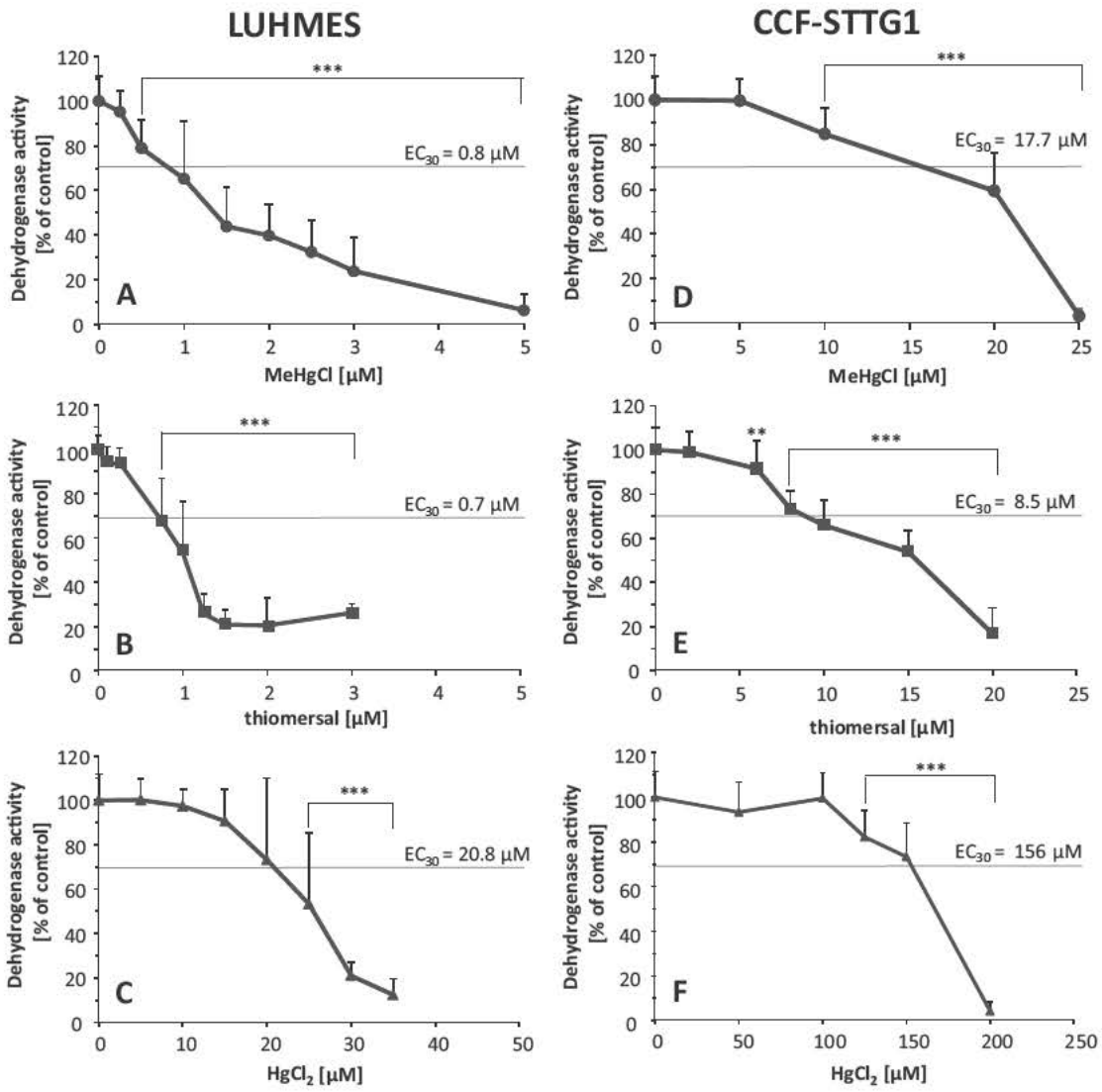

Fig. 1. Effect of MeHgCl (A and D), thiomersal (B and E) and $HgCl_2$ (C and F) on cellular dehydrogenase activity in differentiated LUHMES cells after 24 h incubation (A–C) and in CCF-STTG1 cells after 48 h (D–F). Cellular dehydrogenase activity was determined by the resazurin reduction assay. Shown are mean values of at least 3 independent determinations with 6 replicates +SD. $^{**}p < 0.01$, $^{***}p < 0.001$.

Table 1
Comparison of EC_{30} values regarding different cytotoxicity endpoints in LUHMES after 24 h incubation and in CCF-STTG1 after 48 h incubation with the respective Hg species (* original data from [13]).

Hg species	Cytotoxic endpoint	EC_{30} LUHMES [µM]	EC_{30} CCF-STTG1 [µM]
MeHgCl	Metabolic activity	0.80	17.7
	Lysosomal integrity	0.25	13*
thiomersal	Metabolic activity	0.70	8.5
	Lysosomal integrity	0.10	10*
$HgCl_2$	Metabolic activity	20.8	156
	Lysosomal integrity	16.0	122*

Table 2
Cellular Hg concentrations after incubation with MeHgCl, thiomersal or $HgCl_2$ in LUHMES cells after 24 h and 96 h incubation [µg Hg/mg protein]. Data represent mean values of at least 3 independent determinations with 3 replicates each ± SD. LOQ = limit of quantification.

Hg species	Time [h]	Concentration of incubated Hg species [µM]					
		0.01	0.05	0.1	0.25	1	5
MeHgCl	24	<LOQ	0.030 ± 0.008	0.070 ± 0.010	0.136 ± 0.025	–	–
	96	0.013 ± 0.002	0.061 ± 0.010	0.116 ± 0.031	0.333 ± 0.080	–	–
thiomersal	24	0.005 ± 0.001	0.022 ± 0.067	0.048 ± 0.011	0.111 ± 0.040	–	–
	96	0.012 ± 0.004	0.043 ± 0.010	0.097 ± 0.024	0.680 ± 0.162	–	–
$HgCl_2$	24	–	–	0.012 ± 0.003	0.017 ± 0.004	0.055 ± 0.015	0.228 ± 0.091
	96	–	–	0.022 ± 0.006	0.037 ± 0.010	0.134 ± 0.071	1.581 ± 0.509

Table 3
Cellular Hg concentrations after incubation with MeHgCl, thiomersal or $HgCl_2$ in LUHMES cells after 24 h and 96 h incubation [μM]. Data represent mean values of at least 3 independent determinations with 3 replicates each ± SD. LOQ = limit of quantification; n.c. = not calculated because incubation concentrations are above the EC_{30} value.

Hg species	Time [h]	Concentration of incubated Hg species [μM]					
		0.01	0.05	0.1	0.25	1	5
MeHgCl	24	<LOQ	62.15 ± 11.81	136.15 ± 20.71	n.c.	–	–
	96	26.46 ± 4.43	112.24 ± 7.67	n.c.	n.c.	–	–
thiomersal	24	13.36 ± 2.13	58.71 ± 6.41	n.c.	n.c.	–	–
	96	20.57 ± 7.82	n.c.	n.c.	n.c.	–	–
$HgCl_2$	24	–	–	29.37 ± 5.39	41.38 ± 7.22	130.05 ± 17.09	432.32 ± 84.79
	96	–	–	29.45 ± 8.83	85.79 ± 9.22	n.c.	n.c.

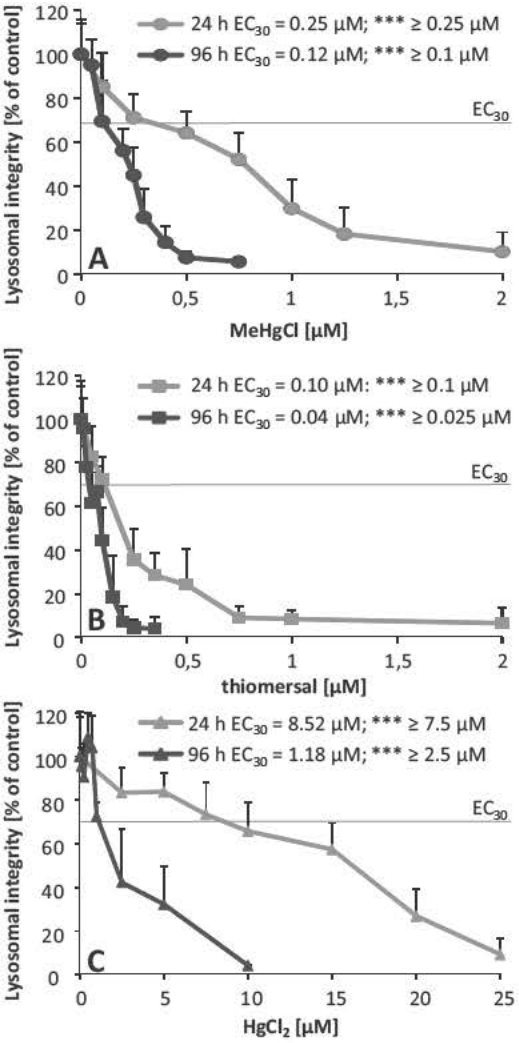

Fig. 2. Effect of MeHgCl (A), thiomersal (B) and $HgCl_2$ (C) on lysosomal integrity in differentiated LUHMES cells after 24 h and 96 h incubation. Lysosomal integrity was determined by the neutral red assay. Shown are mean values of at least 3 independent determinations with 6 replicates +SD. ***$p < 0.001$.

In CCF-STTG1, cells none of the compounds induced caspase-3 activity following 48 h incubation, whereas the positive control staurosporine caused a substantial apoptotic response (Fig. 4D–F).

4. Discussion and conclusion

Available in vitro studies that aim to assess mercury induced neurotoxic effects are either limited to one Hg species or to one cell type. In the present study, we compare toxic effects of three exposure relevant Hg species in two types of brain cells, which have been linked to mercury species induced neurotoxicity before.

Our data clearly demonstrate that all three cytotoxicity related endpoints studied, dehydrogenase activity, lysosomal and membrane integrity, were affected by much lower concentrations of the Hg species in neurons than in astrocytes. This clearly indicates that neurons are more susceptible to mercury species induced cytotoxicity as compared to human astrocytes. This is in accordance with the literature. Sanfeliu et al. demonstrated that effective incubation concentrations of MeHgCl are 2 fold lower after 48 h incubation in primary human neurons versus astrocytes [8]. In our study, organic species exerted cytotoxic effects at 52–100 fold lower concentrations in differentiated human neurons than in astrocytes.

Lysosomal integrity was disturbed in a similar manner by the Hg species in fully differentiated neurons and proliferating, immature LUHMES cells (data not shown). Therefore, the differentiation status itself seems not to account for the enhanced sensitivity of differentiated neurons as compared to proliferating astrocytes.

From all applied viability assays, lysosomal integrity was by far the most sensitive endpoint. Since the uptake of neutral red depends on the cell's capacity to maintain pH gradients through the production of ATP [29], the Hg species seem to disturb the cellular energy status. Fonfria et al. demonstrated both, decreased intracellular ATP levels as well as decreased mitochondrial activity, in murine cerebellar granule cells following incubation of high MeHgCl and $HgCl_2$ concentrations [34] confirming our assumption.

In accordance with the cytotoxic order of the Hg species in astrocytes [13], organic MeHgCl and thiomersal exerted stronger cytotoxic effects as compared to inorganic $HgCl_2$ in neurons with thiomersal being the most cytotoxic compound. These results are in line with the low intracellular Hg contents quantified in neurons following 24 h $HgCl_2$ exposure, whereas an incubation with the organic species resulted in high cellular mercury levels.

Nevertheless, the quantified cellular total Hg concentration following thiomersal incubation is lower at the EC_{30} value as compared to the cellular total Hg level following MeHgCl incubation. This clearly indicates that cytotoxic effects after incubation with the respective species do not always correlate with the total cellular Hg level but are likely to depend on the intracellular mercury species. Follow up speciation analysis could help to identify and clarify the role of the respective Hg species inside the cells.

The estimated cellular molar concentrations indicate a massive Hg accumulation in the differentiated human neurons, especially after incubation with the organic species. With accumulation factors of about 1000 in case of the organic species, Hg accumulation is about 140 fold higher as compared to CCF-STTG1 cells [13]. This might also be one explanation for the enhanced sensitivity of the neurons. Meacham et al. demonstrated Hg accumulations of up to 125 fold higher than the applied MeHgCl concentration in differentiated rat PC12 cells [35]. Compared to our estimations, the upload in these is lower by factor of 10 as compared to the human LUHMES cell line. High cellular Hg levels occurred also in primary rat and

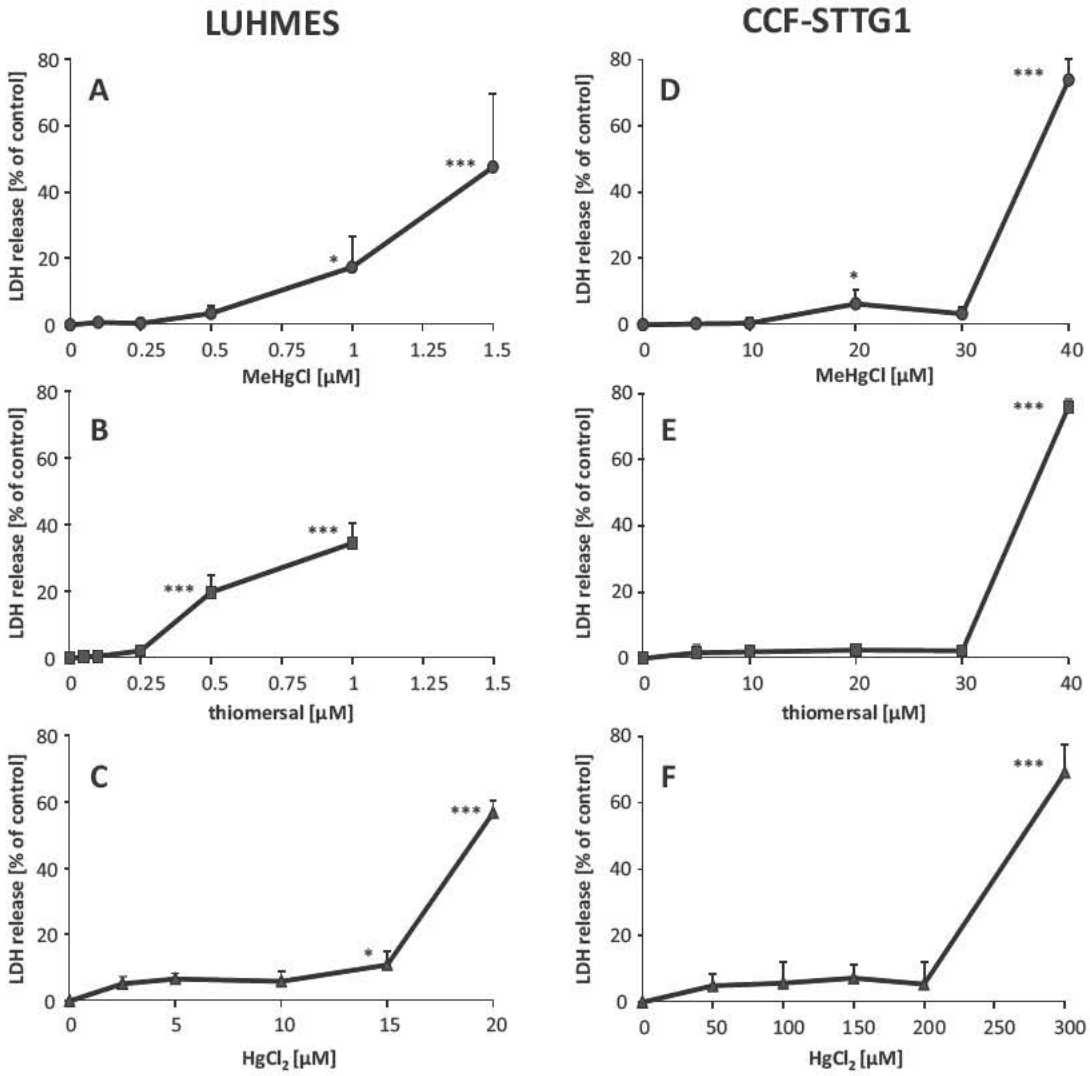

Fig. 3. LDH release after incubation of MeHgCl (A and D), thiomersal (B and E) and $HgCl_2$ (C and F) for 24 h in LUHMES cells (A–C) and for 48 h in CCF-STTG1 cells (D–F). Data represent mean values of at least 3 independent determinations with 4 replicates each +SD. $^{*}p < 0.05$, $^{***}p < 0.001$.

murine cerebellar neurons following MeHgCl exposure [36,37]. Nevertheless, to date it is still not clear why neurons show this enhanced bioavailability for Hg species. Considering brain areas, Hg species seem to persist in cerebellum and cortex in vivo [4,38].

After long-term incubation for all Hg species, but especially for $HgCl_2$, a strong increase in the lysosomal integrity was observed. Since the increase of cellular Hg after 96 h versus 24 h $HgCl_2$ exposure is comparable to the organic compounds, an enhanced bioavailability of mercury with increasing exposure time can be excluded for the intensified sensitivity of LUHMES cells following extended exposure to $HgCl_2$. A delayed deposition of inorganic Hg in the lysosomes, compared with organic Hg species, provides a possible explanation for the enhanced cytotoxicity following long-term exposure that has to be elucidated in further studies. Accordingly, Villegas et al. detected Hg via electron microscopy deposits in neurosecretory neurons of mice after long term oral exposure to $HgCl_2$ in drinking water exclusively within lysosomes [39]. Additionally, accumulation of Hg in hepatic lysosomes was reported for yellow perch, sampled in a Canadian region known as a biological mercury hotspot [40], and for rainbow trout fed with a MeHg supplemented feed diet for 7 weeks [41].

Apoptosis is characterized by cell shrinkage, nuclear and cytoplasmic condensation and intracellular decomposition processes maintaining plasma membrane integrity [42]. Passive cell death via necrosis, on the other hand, is accompanied with swelling of cytoplasm and cell organelles followed by loss of membrane integrity and inflammation [43]. The LDH release correlates with the plasma membrane integrity and is frequently applied as a marker of necrotic cell death. In the present study LDH release was affected only at quite high, already cytotoxic concentrations of the respective Hg species, both in differentiated neurons and astrocytes. In contrast, caspase-3 activation at quite low concentrations of the organic Hg species points clearly towards an induction of apoptotic cell death in differentiated neurons. $HgCl_2$ exposure did not lead to an activation of caspase-3 in these cells. Visualization of apoptotic bodies, as a result of condensation with blebbing of the plasma membrane and subsequent membrane-enclosed fragments [42], confirmed these findings. However, none of the compounds resulted in a caspase-3 activation in the astrocytes. Several studies have provided evidence before that organic and inorganic Hg species can induce apoptosis in different cell types [10,43–47]. In contrast to our studies, Yuntao et al. determined the presence of apoptotic cells by flow cytometry after 6 h of MeHgCl incubation

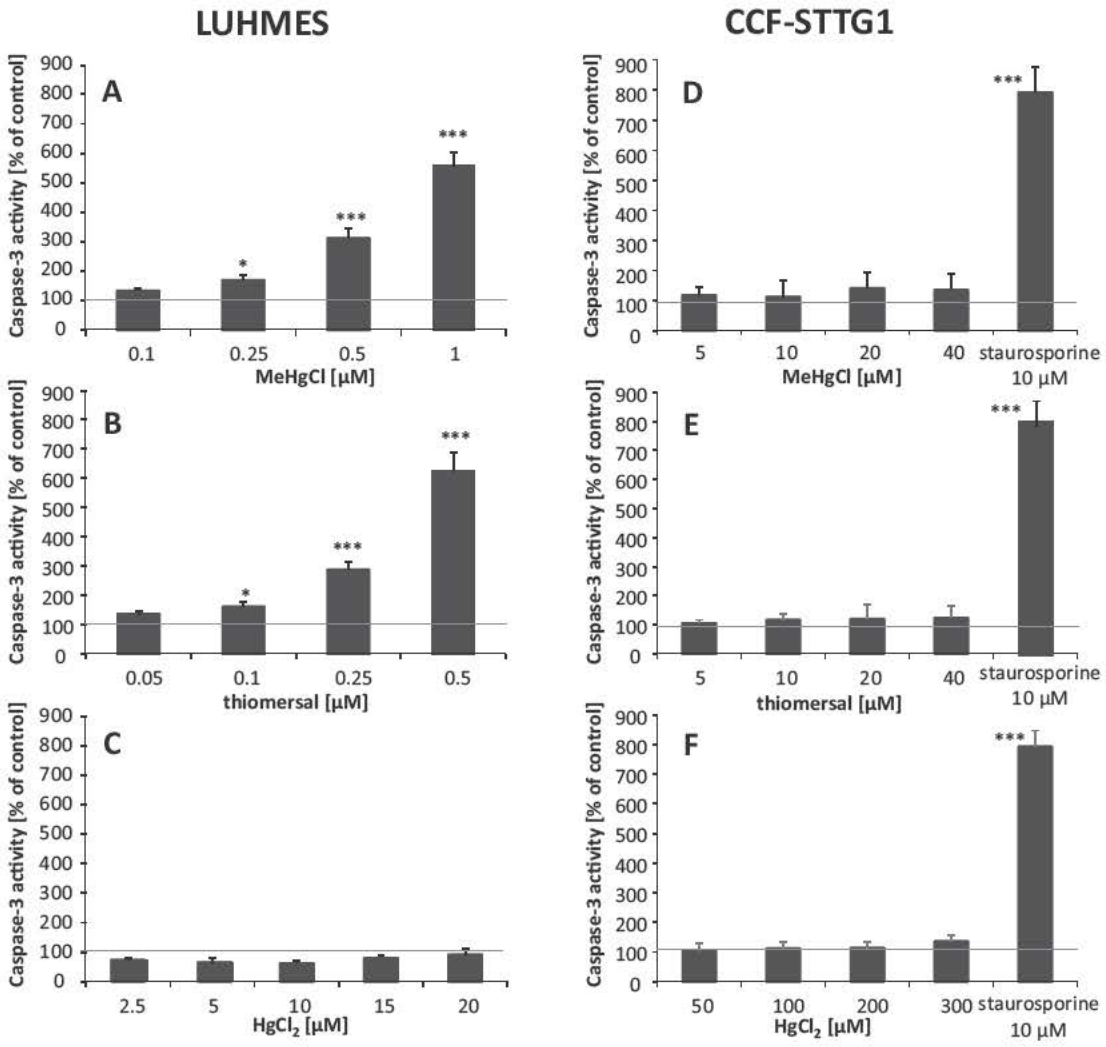

Fig. 4. Caspase-3 activation following MeHgCl (A and D), thiomersal (B and E) and $HgCl_2$ (C and F) exposure for 24 h in differentiated LUHMES cells (A–C) and for 48 h in CCF-STTG1 cells (D–F). Staurosporine (10 µM) served as positive control in CCF-STTG1 cells. Results are presented as percentage of control cells, which were set to 100%. Data represent mean values of at least 3 independent determinations with 4 replicates each + SD. $*p < 0.05$, $***p < 0.001$.

in rat primary astrocytes [48]. In a further study 14.4 µM thiomersal for one hour resulted in caspase-3 activation in normal human astrocytes [45]. Thus, the induction of apoptosis as cell death mechanism seems to be dependent on the respective species of origin and cell type. Intra-lysosomal oxidative processes as a result of oxidative stress may lead to lysosomal labilization and subsequently to apoptosis [43]. Kaur et al. reported increased levels of reactive oxygen species (ROS) following MeHgCl exposure in primary murine astrocytes and neurons with neurons as the more sensitive cell line [9]. Furthermore, the antioxidant glutathione (GSH) pool in astrocytes is generally higher as compared to neurons [9]. Thus, it might be possible, that LUHMES cells are exposed to Hg species mediated oxidative stress in a higher content as compared to CCF-STTG1 cells, resulting in lysosomal destabilization and apoptosis. The positive control, staurosporine, used in CCF-STTG1 cells to prove the ability of these cells to undergo apoptosis, is a well-known, direct inducer of apoptosis by both, caspase dependent as well as caspase independent pathways [49]. In human neural stem cells as well as in murine neural progenitor cells, MeHg was demonstrated to induce apoptosis at low cytotoxic concentrations and durations of exposure [10,50]. In accordance to our data, Castoldi et al. found an induction of apoptosis in mitotically inhibited cerebellar neurons at low MeHg hydroxide concentrations while at higher concentrations the compound induced necrosis [23]. Additionally, incubation of thiomersal for 24 h in cultured human neurons induced apoptosis at low concentrations whereas high concentrations resulted in necrosis [19]. $HgCl_2$ was demonstrated to increase caspase-3 activity in vivo [47]. Studies in human T lymphocyte cells indicate that both organic MeHgCl as well as inorganic $HgCl_2$ induce apoptosis, but each via specific cell death pathways (mitochondrial dependent versus independent) [46,51].

In conclusion, our data clearly indicate that human differentiated neurons are more susceptible to Hg species induced cytotoxicity as compared to human astrocytes, which is likely to result from the higher cellular accumulation of the mercury compounds in the differentiated neurons. Moreover, organic and inorganic Hg species differ in their mechanisms of toxicity in the respective cell types. Whereas in the neurons the organic mercury compounds induced apoptosis, this was not the case in the astrocytes. $HgCl_2$ itself caused no apoptosis in both cell types but exerted, in comparison to the organic mercury compounds, a more pronounced increase of cytotoxicity after long-term exposure. Since in the brain a mixed exposure towards inorganic and organic mercury species occurs, mature neurons and astrocytes are likely to be affected by the Hg species via different toxic mechanisms. Coexposure studies

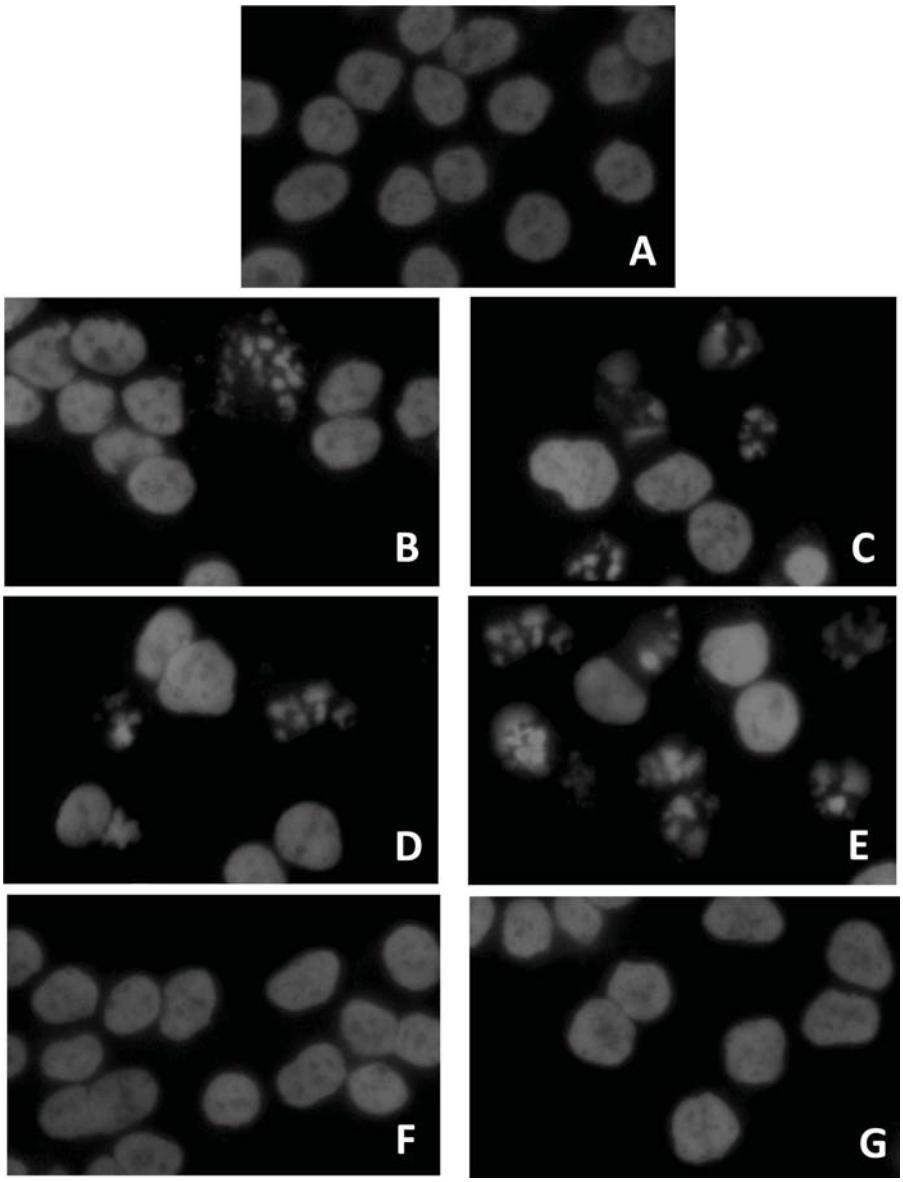

Fig. 5. Fluorescence microscopy images of LUHMES cells with control cells (A), MeHgCl (0.1 μM (B) and 0.25 μM (C)), thiomersal (0.05 μM (D) and 0.25 μM (E)) and $HgCl_2$ (2.5 μM (F) and 10 μM (G)) incubated samples (24 h). Cell nuclei were stained in DAPI containing Vectashield mounting medium.

of the respective Hg species as well as cocultures of neurons and astrocytes are likely to provide additional information if and how the species and the cells interact in the context of Hg mediated neurotoxicity.

Conflicts of interest

The authors have no conflict of interest.

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