Molecular mechanisms of Mn induced neurotoxicity: RONS generation, genotoxicity, and DNA-damage response

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Scope: In industrial countries dietary manganese (Mn) intake is well above the estimated average requirement. Moreover, exposure to high Mn levels is known to cause adverse neurological effects in humans, which are yet mechanistically not well understood.

Methods and results: This study aimed to identify early modes of action of Mn induced toxicity in mammalian brain cells. In primary porcine brain capillary endothelial cells induction of reactive oxygen and nitrogen species was identified as the most sensitive endpoint (≥0.5 μM MnCl₂). In cultured human astrocytes MnCl₂ was rapidly bioavailable, induced a slight increase of cellular reactive oxygen and nitrogen species levels and a slight decrease of ATP levels (1–100 μM MnCl₂), while no genotoxic effects were observed. However, MnCl₂ (≥1 μM) efficiently disturbed DNA-damage-induced poly(ADP-ribosyl)ation in human astrocytes, which indicates sensitization of cells to genotoxic treatment. Additionally, we determined Mn levels in infant formula, which are generally massively supplemented with Mn and thus might pose an important source for Mn overexposure.

Conclusion: The observed inhibition of DNA-damage-induced poly(ADP-ribosyl)ation in human astrocytes by exposure-relevant Mn concentrations indicate that in terms of Mn the existing guidelines for infant formula but also drinking water should be critically reconsidered.

Keywords: Infant formula / Manganese / Neurotoxicity / Oxidative stress / Poly(ADP-ribosyl)ation

1 Introduction

Manganese (Mn) is a widely distributed essential trace element. It is necessary for brain development and the regulation of numerous biochemical and cellular reactions, for example as constituent of important metalloenzymes, such as arginase, pyruvate carboxylase, or superoxide dismutase [1,2]. Mn occurs naturally in water, air, soil, and food and exists as both inorganic and organic species, with the inorganic Mn²⁺ and Mn³⁺ species being more prevalent. For the general population dietary intake is the major source for Mn [3]. Highest Mn concentrations are seen in herbal food, including grain, rice, nuts, and tea.

The ubiquitous presence of Mn in food accounts for the fact that in industrial countries dietary Mn intake (2–5 mg/day) is well above the estimated average requirement. As a consequence Mn deficiency is practically nonexistent in the general population [4]. Nevertheless, in case of high occupational (welding, mining), medical (total parenteral nutrition, contrast agents), and environmental (dietary
supplements, pesticides) Mn exposure, excessive levels of Mn can accumulate in the brain, especially in the substantia nigra [5, 6]. This has been shown to result in a specific clinical central nervous system disorder (referred to as manganism), which shares multiple clinical analogies with Parkinson’s disease [6–11]. To date neither Mn uptake in the brain nor the molecular mechanisms behind Mn induced neurotoxic effects are fully understood [12]. On the cellular level Mn is believed to exert toxicity via a number of mechanisms, including impairment in iron homeostasis, excitotoxicity, disruptive effects on the neurochemistry of neurotransmitters (γ-aminobutyric acid, dopamine, glutamate), protein aggregation, mitochondrial dysfunction as well as direct and indirect formation of reactive oxygen and nitrogen species (RONS) [5, 13, 14].

Excessive production of RONS can lead to reactions with macromolecules, such as DNA, lipids, and proteins. It has been proposed that DNA damage contributes to neurological dysfunction, including Parkinson’s disease, underscoring the critical importance of DNA repair for neural homeostasis [15, 16]. Defective responses to DNA damage and an impairment of genomic stability are to date increasingly linked with diseases such as Alzheimer’s and Parkinson’s disease [17–19]. In this regard, the role of the DNA damage response protein poly(ADP-ribose) polymerase-1 (PARP-1) in CNS disorders was investigated in the last years [20, 21]. In response to DNA strand breaks two members of the PARP superfamily, PARP-1 and PARP-2, are rapidly activated and transfer ADP-ribosyl units from NAD+ onto themselves and other target proteins, thus producing protein-coupled ADP-ribose polymers of up to 200 units. PARP-1 is responsible for about 90% of cellular poly(ADP-ribose) (PAR) formation [22, 23]. Poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosylhydrolase-3 (ARH3) contribute to PAR degradation [21]. PARP-1 is a 116-kDa protein that is involved in several biological pathways and poly(ADP-ribose)ylation affects proteins involved in transcription, replication, telomere maintenance, genomic stability, chromatin organization, and DNA repair [24–26]. Overactivation of PARP-1 results in cellular NAD+ depletion, energy failure, and ultimately cell death [27–29]. PARP-1 inhibition [28–30] might decrease genomic stability due to a disturbance of DNA repair pathways. To date little is known about the effects of Mn on the cellular DNA damage response. Recently, we identified Mn-induced inhibition of H2O2-stimulated poly(ADP-ribose)ylation in human cervix carcinoma cells as a highly sensitive endpoint for Mn cellular toxicity, although the underlying mechanism awaits further evaluation [31].

This study aimed to investigate whether Mn is capable to inhibit damage-induced poly(ADP-ribose)ylation in brain cells. In parallel, further potentially sensitive modes of action, including oxidative stress and genotoxicity were studied, thereby also taking into account the cellular bioavailability of Mn.

2 Material and methods

2.1 Preparation of MnCl2 stock solution

MnCl2 (>99.95% purity, Sigma-Aldrich, Deisenhofen, Germany) stock solutions in sterile distilled water were prepared shortly before each experiment to prevent oxidation.

2.2 Cell culture and incubation with the test compounds

Astrocytic cultures (CCF-STTG1 (CCL-185TM)) obtained from the American Type Culture Collection (Bethesda, MD, USA) and freshly isolated porcine brain capillary endothelial cells (PBCECs) were used as in vitro model systems. CCF-STTG1 cells were cultured in RPMI 1640 (Biochrom, Berlin, Germany), supplemented with 10% FCS (PAA Laboratories, Pasching, Austria), 1.4 mM l-glutamine (Biochrom), 100 U/mL penicillin, and 100 µg/mL streptomycin (PAA (RPMI culture medium), under human cell culture standard conditions at 37°C with 5% CO2 in air and 100% humidity. Logarithmically growing cells were treated with MnCl2 as described for the respective experiments. PBCECs were isolated, cultivated, and cryoconserved according to [32]. For the respective experiments on day 2 in vitro (DIV2) PBCECs were gently thawed and seeded (250 000/cm2) on rat tail collagen-coated 96-well culture plates or on gelatin-covered glass coverslips in plating medium (Medium 199 Earle supplemented with 10% newborn calf serum, 0.7 mM l-glutamine, 100 µg/mL gentamycin, 100 µ/mL penicillin, 100 µg/mL streptomycin (all Biochrom)) at 37°C with 5% CO2 and 100% humidity. At reaching confluence (DIV4), the plating medium was replaced by serum-free culture medium (DMEM/Ham’s F 12 (1:1) containing 4.1 mM l-glutamine, 100 µg/mL gentamycin, 100 U/mL penicillin, 100 µg/mL streptomycin (all Biochrom)), and 550 nM hydrocortisone (Sigma-Aldrich) to induce differentiation. Incubation with MnCl2 was performed on DIV7.

2.3 Cellular bioavailability

Mn levels in CCF-STTG1 cells were measured after 2–48-h incubation with MnCl2 by inductively coupled plasma emission spectrometry (ICP-OES, iCAP 6300, Thermo Fisher Scientific), as reported previously [31, 33]. Briefly, the MnCl2 exposed cells were trypsinized, collected by centrifugation, washed with ice-cold PBS, and the cell number as well as cell volume were measured by an automatic cell counter (Casy®TTC, Roche Innovatis AG) in each sample. After digesting the cells with 65% HNO3/30% H2O2 (1:1) at 95°C for at least 12 h samples were diluted with water and Mn was quantified by ICP-OES.
For Mn efflux studies CCF-STTG1 cells (2 × 10^4) were exposed to MnCl₂ for 24 h and subsequently washed with RPMI culture medium. After 0.5–24 h postincubation with fresh, non-Mn incubated RPMI culture medium and after quantification of the respective cell numbers and volumes, total Mn amounts were determined by ICP-OES.

### 2.4 Cellular RONS level

The ability of Mn to increase the cellular RONS level was determined by a carboxy-DCFH-DA based reader test system, applying three different incubation protocols. Thus, 24-h preincubation with MnCl₂ before dye loading were carried out, as well as incubation with MnCl₂ immediately after dye loading or a combination of both. In all sets of experiments the RONS generation was monitored up to 24 h after dye loading and was normalized to control cells (dye-loaded cells without MnCl₂ treatment) to consider naturally occurring and procedure induced RONS. Briefly, 48 h after seeding (42,000 cells/well of a 96-well plate) CCF-STTG1 cells were preincubated for 24 h with MnCl₂. In PBCECs 24-h preincubation with MnCl₂ was carried out on DIV6. After preincubation and before dye loading CCF-STTG1 cells were washed twice with phenol-red free medium (Biochrom). PBCECs were washed with serum-free culture medium. CCF-STTG1 cells were exposed to 15 μM carboxy-DCFH-DA (5&6)-Carboxy-2′,7′-dichlorodihydrofluorescein-diacetate (Invitrogen, OR, USA)) for 15 min and PBCECs were incubated with 10 μM carboxy-DCFH-DA for 10 min at 37°C. Subsequently cells were washed and finally incubated with H₂O₂ (positive control) or MnCl₂. Intracellular oxidation of carboxy-DCFH, which correlates with the intracellular RONS level, was determined (ex. 485 nm/em. 535 nm) by a microplate reader (Infiniti Pro M200, Tecan, Salzburg, Austria) immediately after incubation; kinetics were constructed up to 24 h after incubation. Data were always applied to a control (dye-loaded cells without a RONS generator), to exclude an interfering fluorescence of the matrix.

### 2.5 Determination of DNA strand breaks

DNA strand breaks were determined by the alkaline unwinding technique [31]. Logarithmically growing CCF-STTG1 cells (200 000) were exposed to MnCl₂ for 2, 24 or 48 h. In case of combination experiments with H₂O₂, after preincubation with MnCl₂, cells were coincubated with 100 μM H₂O₂ for 5 min. Thereafter the culture medium was removed, cells were washed with cold PBS and lesions were quantified and calculated as described earlier [31].

### 2.6 Formation of micronuclei

To investigate the induction of micronuclei, logarithmically growing CCF-STTG1 cells were seeded in 6-well plates on Alcian blue (Sigma-Aldrich) coated glass coverslips. After 48 h cells were incubated with MnCl₂, 5 h after MnCl₂ incubation cytochalasin B (Sigma-Aldrich) was coincubated (final concentration 1 μg/ml). Forty-three hours after cytochalasin B exposure, cells were fixed with an ice-cold fixation solution (90% methanol/10% PBS, –20°C) for 10 min, dried in the air, stained with acridine orange (125 mg/L in PBS) (Roth, Karlsruhe, Germany) for 10 s and finally analyzed by fluorescence microscopy (Zeiss, Oberkochen, Germany). Per coverslip at least 1000 binucleated cells were counted; analysis was carried out after coding of slides.

### 2.7 Measurement of energy related nucleotides

The impact of a 2-h incubation of MnCl₂ on the levels of the cellular energy related nucleotides (ATP, ADP, ADP-ribose, AMP, NAD⁺, NADH) were quantified by a reliable ion-pair RP HPLC based method [33]. Briefly, 10 × 10⁶ MnCl₂ exposed cells were trypsinized, resuspended in cold PBS containing 5% FCS, and cell number was determined by an automatic cell counter (Casy® TTT). The nucleotides were extracted by adding 300 μL 0.5 M KOH and pulling the pellet 10 times through a 23-gauge needle. Subsequently, the extracts were neutralized with 60-μL phosphoric acid (10%). After centrifugation the nucleotides were separated and quantified immediately by ion-pair RP HPLC/DAD.

### 2.8 Cellular levels of poly(ADP-ribosylation)

Cellular poly(ADP-ribosylation) was measured as described earlier [34] with minor modifications as indicated later. CCF-STTG1 cells were seeded on Alcian blue coated glass coverslips, cultured for 48 h and incubated with MnCl₂ for 2–48 h. PBCECs were cultured on gelatin-covered (Sigma-Aldrich) glass coverslips and incubated with MnCl₂ on DIV6 for 2–48 h. Poly(ADP-ribosylation) was stimulated by 10-min incubation with 250 μM H₂O₂. Subsequently, cells were rinsed with PBS (containing 1 mM MgCl₂) and fixed with ice cold methanol (–20°C, 8 min). After fixation cells were rinsed twice with Tris-buffered saline (TBS) and blocked in TBS/0.3% Tween20/1% BSA (Roth) (TTB) at 30°C for 30 min. Incubation with the primary anti-PAR antibody 10H [35] was carried out in blocking solution (1:300) at 30°C for 45 min, followed by repeated washing steps with TBS containing 0.3% Tween20. The secondary, Alexa 488-conjugated anti-mouse antibody (Invitrogen) (dilution 1:250 in TTB) was applied accordingly. Coverslips were washed again and embedded in Vectashield mounting medium containing 1 μg/ml DAPI (Vector Laboratories Inc., Burlingame, CA, USA). Fluorescence analyses were performed applying a Zeiss Axio ImagerM2 wide field fluorescence microscope (Zeiss). At least 300 cell nuclei per slide were selected by DAPI staining. In the selected areas the relative Alexa-488 fluorescence intensities were quantified using Axio Vision (Version 4.5) imaging software (Zeiss).
In case of nicotinic acid (NA) (VWR, Darmstadt, Germany) incubation studies, CCF-STTG1 cells were cultured on cover slips for 36 h, preincubated with 15 or 30 μM NA for 12 h and coincubated with MnCl₂ for 2 h. Subsequently, cellular PAR stimulation and quantification was carried out as described earlier.

### 2.9 Relative PARP-1 gene expression

Real time RT-PCR was performed for quantification of PARP-1 and PARG mRNA levels. Briefly, 24 h after seeding of 2 × 10⁵ astrocytes, cells were incubated with MnCl₂ for 2–48 h. All further steps were performed exactly as described earlier [36]. The respective forward (for) and reverse (rev) primers PARP-1 for 5′-CTTGCCCTGACCAACTGTCTG-3′, PARG-1 rev 5′-GCAAGACACTCTGATCATCTG-3′ (efficiency 101.3%); PARP-1 rev 5′-CCTACTGGTGTGGTGACCATT-3′, PARG rev 5′-CGTAAGTGACATGCAATCGT-3′ (efficiency 92.1%); GA PDH for 5′-CTGCAACACACACATCTTAG-3′, GAPDH rev 5′-GGCATGGACTGTGGTCATGAG-3′ (efficiency 104.2%) were applied. The thermal cycling program consisted of the following steps: 1.5 min at 95°C to activate polymerase, 40 cycles of 30 s at 95°C, 1 min at 60°C, and 15 s at 72°C. GAPDH expression was not significantly changed by MnCl₂ in the observed concentration range and thus can be used in this approach as an adequate reference gene.

### 2.10 Total PARP-1 protein level

Total cellular PARP-1 protein level was quantified by SDS-PAGE/Western blot analysis as described previously [31] with slight modifications. After 2–48-h incubation with MnCl₂, CCF-STTG1 cells were trypsinized, resuspended with PBS containing 5% FCS, and cell numbers were measured by an automatic cell counter in each sample. After centrifugation, cells were resuspended on ice in proteinase inhibitor solution (1 mmol/L EDTA, 10 mmol/L sodium bisulfite, 0.01 mmol/L pepstatin, 0.1 mmol/L PMSF in PBS (Sigma-Aldrich)) and proteins were denatured by adding 100 μL of 95°C hot SDS-PAGE loading buffer (10 min at 95°C). Afterwards respective protein aliquots (each referring to 70 000 cells) were analyzed by 12% denaturing SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Munic, Germany). After blocking with 5% dry milk solution in PBS containing 0.1% Tween20 (PBS-T) at room temperature, membranes were incubated with a primary antibody against PARP-1 (Enzo Life Sciences GmbH, Lörach, Germany) in blocking solution (1:1000) overnight at 4°C, followed by incubation with HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, USA) for 1 h at room temperature; actin (antibody 1:1500) served as loading control. Immunoreactive bands were detected by chemiluminescence using ECL prime detection reagents (GE Health-care) and a chemiluminescence imaging system (Chemidoc™ XRS, Bio-Rad, Munic, Germany). Protein levels were quantified by densitometric analysis with Quantity One software (Bio-Rad) and normalized to controls.

### 2.11 Activity of recombinant PARP-1

Activity of recombinant PARP-1 (expressed in a baculovirus system [37]) was quantified by a further established immunoslot-blot technique based on a test system published recently [38]. Briefly, after 2-min preincubation of PARP-1 (0.69 ng/μL (61 nM)) with MnCl₂ in preincubation buffer (0.08 M HEPES pH 7.9, 10-nM MgCl₂, 0.2 mM EDTA pH 7) at room temperature, the PARP-1 reaction was carried out for 5 min at 37°C in reaction buffer (200 μM NAD⁺, 8.9-nmol/mL GGAATCCC (Eurofins MWG Operon, Ebersberg, Germany), 0.08 M HEPES pH 7.9, 10 nM MgCl₂, 0.2 mM EDTA pH 7). Poly(ADP-ribose)ylation was stopped by adding an equal volume of 20% trichloroacetic acid (TCA), followed by a further dilution with 10% TCA in order to transfer 5% of each reaction to the membrane. Respective aliquots of 100 μL were transferred to a PVDF membrane by a slot blotter (Minifold I system, 24 mm²/slot; VWR), followed by a washing step with 10% TCA. The membrane was blocked overnight at 4°C in TNT (10 mM Tris pH 8, 150 mM NaCl, Tween20 0.05%)/5% dry milk and PAR polymers were detected by immunoblot analysis using a monoclonal PAR-antibody (10H) (1 h 1:1000, in blocking solution at RT) and a HRP-conjugated secondary antibody (1 h 1:1000 in blocking solution at RT). The immunoreactive bands were quantified as described earlier.

### 2.12 Mn content in infant formula

Mn concentrations in infant formula were quantified by ICP-OES (ICP-OES Optima 7000DV, Perkin Elmer, Waltham, USA) after microwave digestion [39] (Anton Paar, Ostfildern, Germany). Approximately, 0.6 g of the substance was weighed into a perfluoroalkoxy alkane (PFA) microwave vessel and gallium (internal standard (10 mg/L)) (Sigma-Aldrich), 0.5 mL hydrochloric acid and 3 mL nitric acid were added. The applied microwave program was a ramp up from 100 to 600 W within 5 min, constant power for 5 min, followed by further increase to 1000 W, which was constant for the next 10 min. Afterwards digested samples were diluted with distilled water in a 25 mL glass flask and the obtained solutions were analyzed by ICP-OES (Table 1). LOD for Mn was 0.072 μg/L calculated according to the 3σ-criterion [40]. Determinations of blank and certified reference material (CRM 414 (plankton) (Community Bureau of Reference of the Commission of the European Communities)) were performed periodically after 15 samples each.
Table 1. Inductively coupled plasma emission spectrometry parameters (ICP-OES Optima 7000™ DV, Perkin Elmer) used for the measurements of the infant formulas

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Value</th>
</tr>
</thead>
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<td>Power</td>
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<tr>
<td>Nebulizer gas flow</td>
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</tr>
<tr>
<td>Flow rate</td>
<td>0.9 mL/min</td>
</tr>
<tr>
<td>Nebulizer</td>
<td>Concentric glass nebulizer (Mienhard)</td>
</tr>
<tr>
<td>Spray chamber type</td>
<td>Cyclone</td>
</tr>
<tr>
<td>Position</td>
<td>Axial (position: x = 0 mm, y = 15 mm)</td>
</tr>
<tr>
<td>Wavelength</td>
<td>Manganese: 257.610 mm Gallium: 417.206 mm</td>
</tr>
</tbody>
</table>

2.13 Statistical analysis

All experiments were at least carried out three times at three different days, with at least three independent measurements. Using the raw data the mean SD was calculated and a statistical analysis was performed by using the unpaired Student's t-test. As indicated in the respective figure legends significance levels are *p < 0.05, **p < 0.01, and ***p < 0.001.

3 Results

3.1 Cytotoxicity and cellular bioavailability in cultured astrocytes

The cytotoxicity of MnCl₂ in CCF-STTG1 cells after 24- and 48-h incubation has been studied by our group and published before [33]. Briefly, regarding the endpoints cell number and cell volume incipient cytotoxic effects were observed at 1000 μM after 24-h incubation (cell number: 85 ± 2%) and at 500 μM after 48-h incubation (cell number: 76 ± 7%), respectively. To avoid strong cytotoxic effects, the highest concentration applied in this study was 500 μM MnCl₂. Mn bioavailability was assessed by measuring cellular Mn by ICP-OES. Comparing extracellular and cellular Mn concentrations after 2-h incubation with 1 and 10 μM MnCl₂, an accumulation was observed in cells. At 50–500 μM, however, the respective cellular Mn concentrations were lower as compared to the extracellular concentrations administered (Fig. 1A). Similar effects occurred after 24 h (partly shown in Fig. 1B and C) and 48-h incubation with MnCl₂ [33].

The experiments regarding time dependency of Mn cellular bioavailability showed that in case of incubation with 50 or 500 μM MnCl₂, cellular Mn reached a maximum after 0.5 h (Fig. 1B) and 1 h (Fig. 1C) of incubation, respectively. In the subsequent 23.5 or 23 h of incubation with MnCl₂, cellular Mn levels were stable.

In a set of efflux experiments after 24-h incubation, the culture medium was replaced with fresh culture medium, not supplemented with MnCl₂. Cells loaded with 50 μM MnCl₂ were able to release the absorbed Mn nearly completely within 0.5 h after medium replacement (Fig. 1B), whereas after incubation with 500-μM MnCl₂ the efflux was not complete within 12 h (Fig. 1C).

3.2 Impact on the cellular RONS level

In CCF-STTG1 cells MnCl₂ showed only a moderate increase of the cellular RONS level in case of all three incubation

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Figure 1. Cellular bioavailability of MnCl₂ in CCF-STTG1 cells. Cellular Mn levels after 2-h incubation with MnCl₂; an additional cutout is displayed for the concentration range 0–50 μM MnCl₂ (A). Time dependent cellular bioavailability of Mn after 0.25–24-h incubation with 50 μM (B) or 500-μM (C) MnCl₂ and efflux profile of the Mn loaded cells after medium replacement by fresh, not Mn-incubated culture media (marked by the dashed line). Shown are mean values of at least four independent determinations ± SD.
protocols. Comparing the incubation protocols, highest, but still very slight, effects (10–15% increase) were achieved after combined 24-h pre- and postincubation with MnCl₂ (Fig. 2A, data not shown for pre- or postincubation only). The decrease in the RONS-related fluorescence level at 500 μM MnCl₂ was due to the cytotoxicity of MnCl₂, which caused a disturbance of the cell monolayer. The operational reliability and sensitivity of the test system was always ensured by treating cells with 200 μM H₂O₂ as positive substance, excluding among others that the stress of the cells during the test procedure might have affected the results. A total of 200 μM H₂O₂ time-dependently increased the cellular RONS levels, reaching a maximum of 250% after 1 h of incubation (data not shown). In order to elucidate the RONS generation in primary brain cells, PBCECs were used in the next set of experiments; brain capillary endothelial cells are known to be highly sensitive to Mn induced toxic effects [14, 41]. In PBCECs the RONS increasing potential of MnCl₂ was much stronger and faster than in astrocytes. Strongest effects were induced after a 24-h pre- and postincubation with 0.5–10 μM MnCl₂ (Fig. 2B, data not shown for pre- or postincubation only). At higher, still noncytotoxic, MnCl₂ concentrations (50, 100 μM) PBCECs seem to be able to cope with Mn induced oxidative stress.

3.3 Determination of DNA strand breaks and micronuclei formation

Genotoxic effects on DNA and chromosomal level were studied by the alkaline unwinding technique [31] and the cytokinesis-block micronucleus assay [42], respectively. After 2-, 24-, and 48-h incubation MnCl₂ did not significantly induce DNA strand breaks in CCF-STTG1 cells (Fig. 3A). After 48 h of incubation MnCl₂ did also not increase the number of micronuclei in the cultured astrocytes; the respective cytokinesis-block proliferation indexes of ~1.8 indicated no impact of Mn on cell proliferation up to 250 μM MnCl₂ (Fig. 3B).

3.4 Effects on poly(ADP-ribosyl)ation

After short-term (2 h) and long-term (24, 48 h) incubation MnCl₂ exerted no effect on poly(ADP-ribosyl)ation in both nonstimulated CCF-STTG1 cells and PBCECs (data not shown). Since in unstressed cells the presence of PAR is in generally quite low, in the next set of experiments cellular poly(ADP-ribosyl)ation was stimulated by H₂O₂, to study the

Figure 2. Effect of MnCl₂ on the cellular reactive oxygen and nitrogen species level of CCF-STTG1 cells (A) and porcine brain capillary endothelial cells (B). Cellular reactive oxygen and nitrogen species level as measured by carboxy-DCF fluorescence after 24-h preincubation with MnCl₂, dye loading and subsequent MnCl₂ postincubation. Shown are mean values (+ SD) of at least eight measurements, which were applied to dye-loaded control cells.

Figure 3. Generation of DNA strand breaks (A) and micronuclei induction (B) in CCF-STTG1 cells after incubation with MnCl₂. (A) DNA strand breaks after 2-, 24-, and 48-h incubation with MnCl₂ as quantified by the alkaline unwinding technique. Five-minute incubation with 100-μM H₂O₂ served as positive control. (B) Five-hour MnCl₂ preincubation and 48-h incubation of cytochalasin B in the continued presence of MnCl₂. A total of 10 J/cm² UVC irradiation served as positive control. Shown are each mean values of at least three independent determinations with three measurements each + SD.
impact of Mn on this DNA damage related signaling reaction. In cultured human astrocytes a preincubation with subcytotoxic concentrations of MnCl₂ significantly decreased the extent of H₂O₂-induced poly(ADP-ribosyl)ation. In doing so, inhibition largely depended on incubation time with MnCl₂. Thus, after 2-h MnCl₂ incubation already 1 µM MnCl₂ significantly inhibited H₂O₂-stimulated poly(ADP-ribosyl)ation (Fig. 4A). After long-term incubation (24, 48 h) inhibition occurred only at higher concentrations (Fig. 4B and C).

In H₂O₂-stimulated primary PBCECs, MnCl₂ significantly inhibited poly(ADP-ribosyl)ation after 2-h incubation (Fig. 4B). In contrast after 24- or 48-h incubation no significant inhibition was observed (data not shown).

Since the impact of Mn on H₂O₂-induced poly(ADP-ribosyl)ation was stronger in cultured astrocytes than in PBCECs all further experiments related to this signaling reaction were carried out in CCF-STTG1 cells. Time course experiments, investigating the level of poly(ADP-ribosyl)ation after 10-, 20-, 30-, and 60-min H₂O₂ incubation in the absence and presence of MnCl₂, revealed that the observed decrease in H₂O₂-stimulated poly(ADP-ribosyl)ation is not due to a delay of the signaling reaction (Fig. 4D).

Supplementation of cultured astrocytes with the NAD⁺ precursor NA indicated that the observed Mn-induced inhibition of H₂O₂-stimulated poly(ADP-ribosyl)ation results not from a diminished NAD⁺ concentration (data not shown).

3.5 PARP-1 and PARG gene expression

In CCF-STTG1 cells, after all incubation times, MnCl₂ did not decrease PARP-1 mRNA expression, but even significantly increased PARP-1 mRNA levels after 2-h incubation (Table 2). Similar to PARP-1, PARG gene expression was increased after short-term incubation (Table 2).
Table 2. Effect of MnCl₂ on PARP-1 and PARG gene expression. CCF-STTG1 cells were incubated with MnCl₂ for 2-48 h. Relative gene expression was determined by real time RT-PCR; mean values of at least three independent determinations with three measurements each referring to the control and normalized to GAPDH ± SD

<table>
<thead>
<tr>
<th>MnCl₂ [µM]</th>
<th>PARP-1 gene expression normalized to GAPDH</th>
<th>PARG gene expression normalized to GAPDH</th>
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<tr>
<td></td>
<td>2 h</td>
<td>24 h</td>
</tr>
<tr>
<td>0</td>
<td>1.00 ± 0.07</td>
<td>1.00 ± 0.11</td>
</tr>
<tr>
<td>1</td>
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<td>1.10 ± 0.13</td>
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<tr>
<td>10</td>
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<td>1.15 ± 0.18</td>
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<tr>
<td>50</td>
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<td>0.98 ± 0.16</td>
</tr>
<tr>
<td>100</td>
<td>1.70 ± 0.20***</td>
<td>1.18 ± 0.13</td>
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</table>

3.6 Cellular PARP-1 protein level

Western blot experiments demonstrated no impact of MnCl₂ on total PARP-1 protein level in the cultured astrocytes after 2-, 24-, and 48-h incubation with up to 500 µM MnCl₂ in the supernatant of recombinant PARP-1 with MnCl₂ did not diminish the PARP-1 activity in the range of 0.5-4000 µM (Fig. 5C). In order to come closer to the cellular system and because of the controversial discussions in literature about the existing Mn species in biological media [43, 44], in a next step CCF-STTG1 cells were incubated with MnCl₂ for 2 h. Then an aliquot of the supernatant was preincubated with purified recombinant PARP-1. However, also under these conditions, Mn did not affect the activity of recombinant PARP-1 (data not shown).

3.7 Effects on poly(ADP-ribosyl)ation of recombinant PARP-1

To determine whether Mn alters the activity of recombinant PARP-1, a nonradioactive immuno-slot-blot assay was further established (details given in Experimental Procedures) based on an existing test system [38]. The applicability of the established assay was verified by the PARP inhibitor 3-aminobenzamide (Fig. 5B). Two-minute preincubation of recombinant PARP-1 with MnCl₂ did not diminish the PARP-1 activity in the range of 0.5-4000 µM (Fig. 5C). In order to come closer to the cellular system and because of the controversial discussions in literature about the existing Mn species in biological media [43, 44], in a next step CCF-STTG1 cells were incubated with MnCl₂ for 2 h. Then an aliquot of the supernatant was preincubated with purified recombinant PARP-1. However, also under these conditions, Mn did not affect the activity of recombinant PARP-1 (data not shown).

3.8 Effect on the level of energy related nucleotides

A 2-h incubation with MnCl₂ did not significantly affect the cellular levels of the nicotinamide adenine nucleotides, NAD+ and NADH (Fig. 6A). ATP (1-100 µM MnCl₂) and ADP-ribose (≥250 µM MnCl₂) levels were significantly

Figure 5. Effect of Mn on poly(ADP-ribosyl) polymerase-1 (PARP-1) protein level in CCF-STTG1 cells after 2-h MnCl₂ incubation and a representative western blot (A). Impact of 3-aminobenzamide (B) or MnCl₂ (C) on the activity of isolated PARP-1. After 2-min preincubation of PARP-1 with 3-aminobenzamide or MnCl₂ at room temperature, PARP-1 activity was carried out for 5 min and poly(ADP-ribosyl) was quantified by an immuno-slot-blot assay. Shown are mean values of at least four independent determinations ± SD.
decreased after 2-h MnCl₂ incubation in CCF-STIG1 cells, while ADP (≥100 μM MnCl₂) and AMP (≥250 μM MnCl₂) levels were significantly increased (Fig. 6B).

3.9 Effects on H₂O₂-induced DNA strand break formation

After 2-h incubation a significant increase in DNA strand breaks after combined treatment of 250 μM MnCl₂ or 500 μM MnCl₂ with 100 μM H₂O₂ was observed when compared to 100 μM H₂O₂ alone (Fig. 6C). This rules out that the observed inhibition of cellular H₂O₂-stimulated poly(ADP-ribosyl)ation is due to a lower number of DNA strand breaks induced after combined treatment of MnCl₂ and H₂O₂ as compared to H₂O₂ alone. 24- or 48-h preincubation with MnCl₂ did not significantly affect the number of strand breaks induced by H₂O₂ (data not shown).

3.10 Mn content in infant formula

Mn content in breast milk is generally quite low (3–10 μg/L) [45, 46]. In contrast, Mn concentrations in infant formulas can vary dramatically, depending on the protein source and fortification in the manufacturing process. Here we determined total Mn levels in three cow milk-based infant formula as well as one goat milk and one soy milk-based infant formula. All three cow-based formula contained about 20-fold more Mn than breast milk. In goat milk based infant formula

113.8 ± 0.5 μg/L Mn were determined. Highest levels were examined in a soy formula, which contained a 100-fold higher Mn content than breast milk (Fig. 7).

4 Discussion

The aim of this study was to study the impact of Mn treatment on human astrocytes by analyzing several critical biological
incubation and remained at these subequimolar
perturbation might diminish ATP-dependent neuroprotective actions
and ATP production [59,61,62]. As a consequence, ATP depletion
dysfunction, thereby impairing oxidative phosphorylation
mitochondria has been reported to result in mitochondrial
ATP decreased slightly, followed by an increase in the ATP
levels, at Mn concentrations of up to 100 μM in human astrocytes. In addition to the slight increase in RONS
discussed differences in Mn bioavailability between rat and
MnCl₂ increase in the cellular RONS levels was observed after
in this study in cultured human astrocytes only a slight
dependent increase of RONS in cultured rat astrocytes [49],
In contrast to the published massive time- and concentration-
disturbing the cellular oxidative defense systems [14, 54–56].
Various authors observed RONS generation as one
possible mode of action of Mn-induced neurotoxicity. Various
endothelial cells, PC12 cells and astrocytes [14,57–60]. In human
in brain associated cultured rat cells, including capillary endothelial cells, poly(ADP-ribosyl)ation is rapidly activated in response to DNA strand breaks and is involved in many cellular processes including genomic stability, chromatin modulation, DNA repair, replication, telomere maintenance, and transcription [68]. Regarding the disturbance of this essential DNA damage signaling reaction, astrocytes are more sensitive than capillary endothelial cells, which is in contrast to the situation regarding induction of oxidative stress by Mn.

The observed decrease of cellular H₂O₂-induced poly(ADP-ribosyl)ation neither resulted from a delay of the signaling reaction nor from diminished formation of DNA strand breaks. Mn did not decrease PARP-1 gene expression or PARP-1 protein level after any time points investigated. After 2-h incubation PARP-1 gene expression was even increased, which suggests a counter-regulatory mechanism in order to maintain cellular poly(ADP-ribosyl)ation capacity. Likewise, after long-term incubation astrocytes seem to be able to adapt to Mn and to cope with Mn-induced negative effects regarding damage-induced poly(ADP-ribosyl)ation. This might also explain the observed attenuation of the disturbance of the signaling reaction with an increase in MnCl₂ incubation time. Likewise PARP-1, PARG gene expression was affected by MnCl₂. Thus, it cannot be excluded that the observed inhibition of H₂O₂-induced poly(ADP-ribosyl)ation is partly due to an increase of PARG expression.

Both the determination of the cellular levels of NAD⁺/NADH and the supplementation studies with the NAD⁺ precursor NA [69, 70], clearly ruled out that the Mn-induced inhibition of H₂O₂-induced poly(ADP-ribosyl)ation is due to a limitation of the substrate NAD⁺. Although cellular NA supplementation resulted in a threefold increase of cellular H₂O₂-induced cellular poly(ADP-ribosyl)ation, Mn still exerted a similar disturbance of the signaling reaction.
In this study neither MnCl₂ nor any other Mn-species, formed in the culture media after incubation with MnCl₂, diminished the activity of recombinant PARP-1. However, we cannot exclude that in living cells reduced damage-induced poly(ADP-ribosyl)ation might be due to an interaction of Mn with the cellular PARP-1 activity. Thus, inside the cell Mn²⁺ or Mn³⁺ species might be formed, e.g. Mn-citrate complexes, which could attack sensitive target sites of PARP-1, including its three zinc structures. The two N-terminal zinc fingers are the major contributors for DNA binding activity of PARP-1 to single and double strand breaks and in each zinc finger structure zinc is coordinated by three cysteine and one histidine residues (Cys₂His₁Cys₁). The third PARP-1 zinc binding structure (Cys₄) is involved in protein–protein interactions that orchestrate PARP-1 activation [71]. Moreover, in the cellular system PARP-1 activity might also be disturbed by Mn-induced RONS as well as a disturbance of the cellular redox state [33, 72].

A further putative scenario, involving the replacement of Zn-ions from easy accessible structural sites in enzymes by Mn [73, 74], could be that newly synthesized PARP-1 is incorporating Mn at one or more of the three zinc-binding motives. This would probably negatively impact on the enzymatic activity and could also lead to accelerated proteasomal degradation of PARP-1. To compensate, cells enhance transcription of PARP-1, resulting in normal steady-state levels of the protein. Of note, PARP-1 negatively autoregulates its own promoter by binding and stabilizing a weak stem-loop structure. This would explain the reduction in cellular PAR-synthesis upon stimulation with constant PARP-1 protein levels and despite enhanced transcription. Also, activity of recombinant PARP-1 in vitro would not be affected as this has already been properly folded, preventing fast replacement of Zn by Mn.

Dysregulation of PARP-activity is well known to cause severe cellular toxicity, both in case of overactivation and disturbance. In neurological disorders, such as stroke or neurotrauma, PARP inhibitors are discussed to hamper PARP overactivation, thereby protecting brain cells from energy depletion and consequently cell death [20, 75]. On the other hand, quite recently Lee and colleagues reported that PARP-1 inhibition may cause disturbances in gene expression, which stimulates a genetic network that enhances oncogenic potential [76]. Moreover, PARP-1 inhibition is well known to result in diminished DNA repair, thereby causing genomic instability [28, 29]. Numerous in vitro and in vivo studies reveal evidence that PARP-1 contribute to several DNA repair pathways including single strand break repair, base excision repair and double strand break repair [24, 26, 77–80]. As a consequence of PARP-1 inhibition, cells are hypersensitive to alkylating agents and ionizing radiation. Thus, an increase in DNA strand break formation or chromosomal aberrations was observed, resulting in sister chromatid exchanges or higher micronuclei frequency [81–84]. Consistent with diminished DNA strand break protection by PARP-1, in this study a 2-h incubation with MnCl₂ (250–500 µM) resulted in a significantly increased amount of H₂O₂-induced strand breaks. Likewise a defective response towards DNA strand breaks has been shown in neurological disorders including Parkinson’s disease, emphasizing the importance of DNA repair in neuronal homeostasis [16]. Whether the Mn-induced chromosomal aberrations and sister chromatid exchanges in vitro [65, 85] and in vivo [67] might be due to an inhibition of poly(ADP-ribosyl)ation is still unknown. PARP-1 is also a nuclear epigenetic regulator of mitochondrial DNA repair and transcription, and its inhibition results in an impairment of mitochondrial homeostasis and related bioenergetics [86]. Additionally inhibition of poly(ADP-ribosyl)ation might negatively affect other CNS proteins like alpha-synuclein and sirtuins [79, 87–89]. Alpha-synuclein accumulation and impairment of sirtuin function have been reported to be involved in neurodegenerative diseases [90, 91].

For an estimation of the exposure relevance of our findings, in literature there are only limited data available regarding Mn concentrations in mammalian brain tissues. To the best of our knowledge no data exist about Mn levels in astrocytes of humans. Physiological levels of Mn in brain tissues are thought to range from 2 to 8 µM and to increase several fold upon overexposure in rodents and humans [12]. In this study damage-induced poly(ADP-ribosyl)ation was significantly inhibited already by 1 µM MnCl₂. Taking into account the cellular bioavailability of Mn in the tested astrocytes, 1 µM MnCl₂ refers to an effective cellular Mn level of 14 µM, which is in the range of brain levels upon overexposure.

Disturbance of DNA-damage-stimulated poly(ADP-ribosyl)ation is likely to result in increased numbers of DNA damage harboring, dysfunctional brain cells and might lead to neurological dysfunction. Since PARP-1 activation is also associated with neurite outgrowth and long-term memory, chronic PARP-1 inhibition might additionally compromise neurogenesis and learning abilities [92, 93]. In this context, recent epidemiological studies have reported associations between elevated dietary Mn exposure and neurobehavioral and neurocognitive deficits in children [94–97]. Infants, in particular neonates, are likely at greater risk for Mn neurototoxicity, which is partly due to their immature and therefore leaky blood brain barrier, but also results from their immature Mn excretion [98–100]. Important diet-related routes for Mn overexposure in neonates and infants include total parenteral nutrition [101] but also industrially manufactured baby formula [46, 102] and contaminated drinking water [94]. In this study we observed up to 100-fold higher Mn levels in infant formula than in breast milk. These levels are in accordance with published data [45, 46, 102, 103], where Mn content in human breast milk ranged between 3 and 10 µg/L, while that of soy- and cow-based formula was determined as 200–300 µg/L and 30–75 µg/L, respectively. However, the meaning of this massive Mn fortification of infant formula seems to be disputable. In the past high Mn fortification has been justified with strong differences in Mn bioavailability between breast milk and formula. Nevertheless, today it is known that in infants bioavailability of the naturally occurring Mn species in...
breast milk and the supplemented Mn species in formula is similar [45, 46].

Finally both the apparent Mn overexposure of formula consuming infants and the observed Mn-related disturbance of damage-induced poly(ADP-ribosyl)ation in human astrocytes at exposure-relevant concentrations indicate that in terms of Mn the existing guidelines for infant formula but also drinking water [10] should be critically reconsidered.

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The authors have declared no conflict of interest.

5 References

[7] Ellingsen, D. G., Hetland, S. M., Thomassen, Y., Mitochondrial DNA deletion in formula is similar to that in breast milk and the supplemented Mn species in formula is similar [45, 46].

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