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Evaluation of neurobehavioral and neuroinflammatory end-points in the post-exposure period in rats sub-acutely exposed to manganese

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Abstract

Manganese (Mn) can cause manganism, a neurological disorder similar to Parkinson's Disease (PD). The neurobehavioral and neuroinflammatory end-points in the Mn post exposure period have not been studied yet. Rats were injected on alternate days with 8 doses of MnCl₂ (25 mg/Kg) or saline, then euthanized 1, 10, 30 or 70 days following the last dose. Whole-blood (WB) (p<0.05), urine (p<0.05) and brain cortical (p<0.0001) Mn levels were significantly increased 24h after the last dose. Decreases in the rats' ambulation were noted 1, 10 and 30 days after the last Mn dose (p<0.001; p<0.05; p<0.001, respectively) and also in the rearing activity at the four time-points (p<0.05). Cortical glial fibrillary acid protein immunoreactivity (GFAP-ir) was significantly increased at 1, 10, 30 (p<0.0001) and 70 (p<0.001) days after the last Mn dose, as well as tumor necrosis α (TNF- α) levels (p<0.05) but just on day 1. Taken together, the results show that, during the 70-day clearance phase of Mn, the recovery is not immediate as behavioral alterations and neuroinflammation persist long after Mn is cleared from cortical brain compartment.

Keywords

Manganese neurotoxicity; neurobehavioral assays; TNF- α ; GFAP; neuroinflammation

1. Introduction

Manganese (Mn) is an abundant naturally occurring essential element that is required for normal mammalian physiological function, including growth and development of bone and cartilage (Hurley, 1981), connective tissue (Greger, 1999; Keen et al., 1999), as well as

optimal brain function (Golub et al., 2005; Michalke et al., 2007). Despite its essentiality, excess Mn is known to cause neurotoxicity (Cotzias et al., 1968). Excessive exposure to Mn is inherent to occupational, environmental and iatrogenic sources (Bertinet et al., 2000; Dickerson, 2001; Fitzgerald et al., 1999). Mn intoxication is a progressive disorder (Rosenstock et al., 1971). The initial neurobehavioral symptoms are nonspecific (Mergler, 1999; Verhoeven et al., 2011), as the initial neurobehavioral symptoms may progress to a parkinsonian-like disorder characterized by tremor, bradykinesia and rigidity (Calne et al., 1994; Pal et al., 1999). The diagnosis of manganism is commonly made in the late phases of the disease after the appearance of the parkinsonian symptoms (Alves et al., 1997; Fell et al., 1996; Kim et al., 2009; Reynolds et al., 1994; Rohling and Demakis, 2007; Sadek et al., 2003) by determination of whole blood (WB) and urine Mn levels (Fitzgerald et al., 1999; Nagatomo et al., 1999; Takagi et al., 2002) and increased signal intensity in T1-weighted magnetic resonance (MR) images (Finkelstein et al., 2008; Mirowitz et al., 1991). Although WB Mn analysis is the preferred screening method, the high variability in normal Mn levels (4.2 to 16.5 µg/L in WB and 0.40 to 0.85 µg/L in serum) (Jankovic, 2005), render it inadequate for individual biological monitoring (Apostoli et al., 2000). Generally, WB Mn concentrations do not accurately reflect Mn concentration in targeted tissues, particularly the brain (Zheng et al., 2011). Furthermore, Mn-induced parkinsonism may develop long after exposure cessation when Mn blood concentrations are within the normal range (Huang et al., 1993).

Locomotor activity depends on mesolimbic and mesocortical dopaminergic (DAergic) neuronal transmission (Fink and Smith, 1980) with the involvement of motor control centers such as the globus pallidus, substantia nigra, and deep cerebellar nuclei (Oberlander et al., 1987). Brain Mn deposition is predominant in the basal ganglia (Aschner, 1999; Finkelstein et al., 2008; St-Pierre et al., 2001). Mn accumulation is associated with striatal dopamine (DA) depletion (Desole et al., 1994; Slood et al., 1996). Thiruchelvam et al. (2000) also noted alteration in dopaminergic (DAergic) system 7 days after Mn last dose (Thiruchelvam et al., 2000).

Excessive Mn exposure affects the glia in the central nervous system (CNS), specifically the astrocytes in nuclei of the basal ganglia (Normandin and Hazell, 2002). Astrocytes (Hazell, 2002) and microglia (Zhang et al., 2009) play a major role in the etiology of manganism (Milatovic et al., 2009; Zhao et al., 2009). Mn-induced injury is characterized by astrocytic hypertrophy (Norton et al., 1992; O'Callaghan et al., 1995) and accumulation of glial fibrillary acidic protein (GFAP) (Eng et al., 1986), representing Alzheimer type II astrocytosis (Hazell et al., 2006; Liu et al., 2006; Normandin and Hazell, 2002). Microglial activation contributes to the activation of astrocytes, by the production and secretion of proinflammatory cytokines, such as tumor necrosis α (TNF-α) (Crews, 2012). Given that chronic inflammatory processes have a role in neurodegeneration (McGeer and McGeer, 1995), in particular manganism, the objectives of the present study were a) to determine *in vivo*, whether neuroinflammation and neurobehavioral alterations recede during the clearance phase of Mn; b) to evaluate *in vivo*, internal indices of Mn exposure, such as WB and urine Mn during the Mn clearance phase.

2. Materials and methods

2.1. Materials

Manganese chloride tetrahydrate (MnCl₂·4H₂O; 99,9%) for Graphite Furnace Atomic Absorption Spectrometry (GFAAS), hydrogen peroxide (H₂O₂, 30%), ethylenediaminetetraacetic acid (EDTA), N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with trimethylchlorosilane (TMCS) 1%, 3-phenylbutyric acid, ethoxyamine hydrochloride,

hydrochloride acid (HCl), tris(hydroxymethyl)aminomethane (Tris), sodium azide, sodium dodecyl sulfate, sodium deoxycholate, aprotinin, IGEPAL and phenylmethylsulfonyl fluoride were purchased from Sigma Aldrich (St. Louis, MO). Nitric acid (HNO₃; 65%) and magnesium matrix modifier for GFAAS were purchased from Merck (Darmstadt, Germany). Rabbit anti-mouse IgG1-FITC (sc-358946) and GFAP (F-7) mouse monoclonal IgG1 (sc-166458) were purchased from Santa Cruz Biotechnology (CA, USA). The blocking solution and the common antibody diluents were purchased from Biogenex (San Ramon, CA, USA). The universal blocking reagent buffered casein solution with preservative was purchased from Biogenex (Fremont, CA, USA). The fluorescence mounting medium was purchased from Vector Laboratories Inc. (Burlingame, CA). The phosphate buffered saline (PBS) was purchased from Cellgro - Mediatech, Inc (Manassas, VA). The ethyl acetate lichenol was purchased from Merck (Darmstadt, Germany). The ELISA Development Kit for TNF- α quantification was purchased from Peprotech (Rocky Hill, NJ). The bicinchoninic acid assay (BCA assay) reagent was purchased from Thermo Scientific (Rockford, IL).

2.2. Animals and Treatment

Six-week-old male Wistar rats (163 $\pm$ 11g) were obtained, specific pathogen free, from Charles River Laboratories (Barcelona, Spain) and maintained under standard environmental conditions. The animals were housed in a room, with 12-12 h light/dark cycle, 50–70 % humidity at 24°C. Experiments were performed according with the guiding principles of the European Community Council Directive (89/609/EEC) for the care and use of laboratory animals. The rats were randomly divided into two groups; one group (n = 20) received eight intraperitoneal (ip) injections of MnCl₂ 25 mg/kg, as MnCl₂·4H₂O, and the control group (n = 16) was injected with eight doses of sterile saline, on alternate days. From each group, 5 Mn exposed and 4 unexposed, were euthanized with pentobarbital (20 mg/kg) at four time-points (1, 10, 30 and 70 days after the Mn last dose). A previous study from our lab has shown that Mn (also 8 doses 25 mg/kg) caused altered behavioral function and increased brain oxidative and inflammatory responses in rats (Santos et al., 2012). Urine was collected during 24 hours in metabolic cages, at the four time-points (1, 10, 30 and 70 days after the last dose), 24 hours before sacrifice. The cerebral hemispheres were immediately removed and frozen in liquid nitrogen. WB was drawn from the left ventricle of the heart, collected into heparinized tubes, at the four time-points (1, 10, 30 and 70 days after the last dose). The samples were stored at –80°C until biochemical and chemical determinations.

2.3. Behavioral Assays

Locomotor activity was evaluated in an open-field apparatus. Animals were individually placed at the center of the open-field apparatus (60 cm $\times$ 90 cm $\times$ 30 cm, divided into six equal squares). Spontaneous ambulation (number of segments crossed with the four paws) and rearing (exploratory activity expressed by the number of rearings on the hind limbs) were recorded for 5 min (Markel et al., 1989; Marreilha Dos Santos et al., 2011). Neurobehavioral assessments were carried out 24h before the 4 time-points of rats' sacrifice (1, 10, 30 and 70 days after the treatment period), in Mn-exposed and control groups, as well in all the rats before the treatment.

2.4. Analysis of Mn in brain cortex, WB and urine

Prior to GFAAS, the brain cortex and WB samples were digested with an oxidizing acid mixture of 4:1 (v/v) 65% suprapure HNO₃:H₂O₂, using a microwave-assisted acid digestion. Urine samples were digested in a water bath with an acid mixture of 1:1 (v/v) 65% suprapure HNO₃:HCl for 60 min. Brain cortex, blood and urine Mn concentrations were determined by GFAAS, using a PerkinElmer Analyst™ 700 atomic absorption spectrometer

equipped with AA WinLab 32 software, using previously published methods (Santos et al., 2012). The 24 hour urine samples were normalized to creatinine levels.

2.5. Analysis TNF- α in brain

Brain hemispheres were homogenized according to a protocol described by Vargas et al. (2005). TNF- α protein was quantitated in brain homogenate using a standard dual-antibody solid phase immunoassay as previously described (Thomas Curtis et al., 2011). Total cell protein was determined using the BCA assay (Davis et al., 2002) in order to normalize TNF- α levels (pg/mg total protein).

2.6. Brain cortical GFAP immunohistochemistry

Frozen brains were sectioned sagittally and mounted on superfrost/plus slides, and stored at -20°C . Prior to analysis, the slides were blocked for 30 min in 30% formaldehyde and washed with PBS. Sections were then incubated in a blocking buffer for 30 min, followed by overnight incubation with mouse monoclonal anti-GFAP (1:1000) in a common antibody diluent at 4°C . Sections were washed with PBS for 5 min and incubated for 1 h at room temperature in rabbit anti-mouse IgG1-FITC (1:400). Sections were washed three times for 5 min each with PBS and coverslipped with a mounting medium for fluorescence. The relative densities of staining in the cortex were assessed using ImageJ® software (Gavet and Pines, 2010).

2.7. Statistical Analysis

The data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc multiple comparison test. The neurobehavioral assays data were analysed by the student's t-test. Biochemical data for each control group were compiled into a single control. All analyses were carried out with GraphPad Prism 4.02 software for Windows (GraphPad Software, San Diego, CA, USA). GFAP-ir was correlated to peripheral biomarkers predictive of Mn-induced neurotoxicity using Pearson correlation coefficients. Results were considered statistically significant at values of $p < 0.05$. The Image J software was used to calculate optical the density, and Prism 4.0 (GraphPad, San Diego, CA, USA) was used to calculate and plot the results.

3. Results

Mn levels in brain cortex, WB and urine at various time-points after MnCl_2 or saline injections are shown in Figures 1 A, B and C, respectively. Brain cortex and WB Mn levels were significantly increased (*vs.* control) on days 1 ($p < 0.0001$; $p < 0.05$, respectively) and 10 ($p < 0.0001$) after the last Mn dose, while urinary Mn levels ($p < 0.05$) were significantly increased (*vs.* control) only 24h after the last Mn dose. No significant effect on body weight loss could be detected during or after Mn treatment (results not shown). Figure 2A shows a significant decrease (*vs.* control) in ambulation at 1 ($p < 0.001$), 10 ($p < 0.05$) and 30 ($p < 0.0001$) days after the last Mn dose compared with the respective control group. Figure 2B shows a significant decrease in rearing of Mn exposed rats at all time-points ($p < 0.05$) compared with the control group.

As shown in Figure 3, cortical glial fibrillary acid protein immunoreactivity (GFAP-ir) of the Mn-exposed group was significantly higher (*vs.* control) at 1, 10, 30 ($p < 0.0001$) and 70 days ($p < 0.001$) after the last Mn dose, and decreased in a time-dependent manner during the 70-day recovery period. In Figure 4, TNF- α levels were significantly ($p < 0.05$) increased (*vs.* control) 1 day after the last Mn dose.

4. Discussion

This is the first study to our knowledge to focus on the recovery from Mn-induced neurotoxicity and the fate of Mn in blood and brain tissues after the cessation of repeated Mn exposure. We determined GFAP-ir and TNF- α to assess the recovery from Mn-induced neuroinflammation in rat brains. Our data showed that Mn led to lasting functional impacts, even when brain Mn levels returned to normal. Astrogliosis (Figure 3) persisted although Mn had been cleared from the cortex (Figure 1A). These results are in agreement with reports on increased GFAP expression in both rats and mice (Baek et al., 2007; Kern and Smith, 2011). TNF- α levels were significantly increased (vs. control) only 1 day after the last Mn dose which corroborates previous studies that established the ability of Mn to increase the production of TNF- α levels in the rat (Zhao et al., 2009).

Brain Mn was significantly increased (vs. control) at days 1 and 10 after the last Mn dose (Figure 1A); although, at day 30, brain cortical Mn levels remained increased, the difference was not significant (Figure 1A). Other studies have shown that Mn accumulates in the rat cortex (Bock et al., 2009; Fitsanakis et al., 2011; Guilarte et al., 2006). Brain Mn levels peaked 1 day after last Mn dose (Figure 1A), while WB Mn peak concentrations were observed 10 days after the last dose (Figure 1B). Maynard and Cotzias (1955) showed that after a single dose of ^{54}Mn , its disappearance from the blood was rapid, suggesting that Mn is entrapped and then gradually released from peripheral tissues to blood (Maynard and Cotzias, 1955). Chronic or repeated exposures give rise to significant increases in WB-Mn that might result from the slow release from Mn stores (Lucchini et al., 1995). In a study by Roels et al. (1997) a single administration of MnO_2 gave rise to the onset of increased blood Mn concentration at 48 to 72 h after intratracheal dosing with MnO_2 and after 168 h the peak value (200% increase) was achieved (Roels et al., 1997).

Urinary Mn levels represent < 1% of the daily absorbed amount of Mn and about 6% of the total excreted amount (Saric, 1986). Urinary Mn was significantly increased only at day 1 after the last Mn dose compared to controls (Figure 1C). The biological half-time of Mn in urine is estimated to be less than 30 h following cessation of exposure (Bounds, 2009). Our results corroborate previous studies that show that on group basis, urine Mn concentration appears to reflect recent exposure, whilst WB Mn concentration more closely correlates with brain Mn accumulation (Blanc, 1990; Roels et al., 1987a; Roels et al., 1987b).

During the 70 days Mn clearance phase, we noted impairment of behavioral function in the open-field test. The open field test is designed to measure behavioral responses such as locomotor activity, hyperactivity, and exploratory behaviors (Prut and Belzung, 2003; Walsh and Cummins, 1976). The recovery of Mn induced neurotoxicity was not fully complete as 70 days after Mn last dose we noted a decrease in rearing activity ($p < 0.05$) compared to control group (Figure 3). Similar results have been described in other studies (Torrente et al., 2005; Vezer et al., 2007). We conclude that both WB and urine Mn together are suitable and complementary biomarkers of brain Mn accumulation after repetitive exposure. The recovery from Mn-induced neurotoxicity was not fully complete even after brain Mn levels were indistinguishable from control.

In addition, a persistent brain neuroinflammatory response was observed during the Mn clearance phase. Further studies should be performed to clarify the effects of novel therapeutic anti-inflammatory approaches that target cellular events that mediate neurotoxicity, along with the chelation therapy.

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Highlights

- Whole blood (WB) and urine Mn are suitable indicators of brain Mn accumulation and exposure assessment;
- WB and urine Mn levels are not suitable biomarkers of neuroinflammation;
- Cortical neuroinflammation, and behavioural changes persist even after Mn is largely cleared from the brain;
- Increased cortical GFAP-ir is persistent long-after Mn is cleared from the brain.

C

Figure 1.

Mn concentrations in rats exposed to 8 doses of MnCl₂ (25 mg/Kg, on alternate days) or saline (controls) sacrificed 1, 10, 30 or 70 days after the last dose; Data for each control group were compiled into a single control. (A) brain Mn concentration (n=5); (B) WB-Mn concentrations (n=5); (C) urine Mn concentrations (n=4). Bars represent mean $\pm$ SEM (n=5). * $p < 0.05$, *** $p < 0.0001$ indicate statistical difference from control group by one-way ANOVA followed by Bonferroni's multiple comparison tests.

A

B

Figure 2.

Neurobehavioral evaluation in rats exposed to 8 doses of MnCl_2 (25 mg/Kg, on alternate days) or saline (controls) sacrificed 1, 10, 30 or 70 days after the last dose; (A) ambulation, number of crossings in the open field; (B) number of rearings in the open field. Bars represent mean $\pm$ SEM (n=5). * $p < 0.05$ ** $p < 0.001$ *** $p < 0.0001$ indicate statistical difference from the control group by one-way ANOVA followed by Bonferroni's multiple comparison tests.

Figure 3.

Cortical astrocytic GFAP levels [expressed as relative fluorescence units (RFU)] in rats exposed to 8 doses of MnCl_2 (25 mg/Kg, on alternate days) or saline (controls) sacrificed 1, 10, 30 or 70 days after the last dose; Data for each control group were compiled into a single control. Bars represent mean $\pm$ SEM (n=3). *** $p < 0.0001$, ** $p < 0.001$ indicate statistical difference from the control by one-way ANOVA followed by Bonferroni's multiple comparison tests.

Figure 4.

TNF- α concentrations in rats exposed to 8 doses of MnCl₂ (25 mg/Kg, on alternate days) or saline (controls) sacrificed 1, 10, 30 or 70 days after the last dose; Data for each control group were compiled into a single control. Bars represent mean $\pm$ SEM (n=5). * p<0.05 indicates statistical difference from control by one-way ANOVA followed by Bonferroni's multiple comparison tests.