Ecotoxicology and Environmental Safety 74 (2011) 615–622

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Manganese effects in the liver following subacute or subchronic manganese chloride exposure in rats

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ARTICLE INFO

Article history:
Received 6 June 2010
Received in revised form
6 August 2010
Accepted 11 August 2010
Available online 1 September 2010

Keywords:
Manganese
Metal concentration
Oxidative damage
Liver
Rat

ABSTRACT

Manganese (Mn) toxicity is most often found in mining and welding industry workers. Accumulation of manganese in the brain can result in a syndrome similar to that of Parkinson's disease. Observations on former Mn-alloy workers suggested that residual effects could last for years after exposure. The objective of this study was to assess effects of Mn in the liver of rats following subacute or subchronic exposure and after recovery. Male Sprague–Dawley rats were exposed to manganese chloride ($MnCl_2$) for 30 days, 90 days, or for 90 days followed by a 30-day post-exposure recovery period. Results showed that $MnCl_2$ exposure resulted in liver injury in rats and the extent of injury correlated positively with exposure time. The effect in mitochondria was stronger than in the membrane or nucleus. Most of the changes in these biomarkers recovered when manganese exposure ceased.

1. Introduction

Manganese (Mn) is an essential trace metal that is found in all tissues and is required for normal amino acid, lipid, protein, and carbohydrate metabolism in vivo (Erikson et al., 2005). Mn is also heavily used in industry (Gerber et al., 2002). While Mn deficiency is extremely rare in humans, toxicity due to Mn overexposure is more prevalent (Crossgrove and Zheng, 2004). Mn toxicity is most often found in mining and welding industry workers who are chronically exposed to Mn-containing aerosols or dust (Bowler et al., 2006), in individuals consuming contaminated well-water, or in liver disease patients receiving parenteral nutrition (Pal et al., 1999; Aschner et al., 2005). Experimental studies showed that Mn exposure resulted in accumulation in the brain (Liccione and Maines, 1988) and liver (Shukla and Chandra, 1987). Further, Mn concentrations in brain tissues and other organs increased over time in a dose-dependent manner (David et al., 2006). Accumulation of Mn in the brain can result in a syndrome similar to that of Parkinson's disease (Michael et al., 2007), while accumulation in the liver can result in liver damage and thereby restrict the rate of Mn excretion (Crossgrove and Zheng, 2004; Michael et al., 2009).

Oxidative stress occurs due to either the over production of reactive oxygen species (ROS) or the decrease of cellular antioxidant levels (Ding et al., 1998). As a metal ion, Mn is toxic due to its enhancement of ROS formation and catecholamine oxidation byproducts (Parenti et al., 1988). ROS generated in tissues and subcellular compartments are efficiently scavenged by the antioxidant defense system, which is comprised of antioxidants like glutathione (GSH) and antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Filho, 1996). These antioxidant defenses can protect cells from DNA damage, protein oxidation, and lipid peroxidation (LPO). In rat brain and liver samples, Mn exposure enhanced the rate of ROS generation in mitochondria, increased single strand breaks of mitochondrial DNA and decreased GSH levels in mitochondria and brain homogenates in a dose-dependent manner (Jiao et al., 2008). Monkeys exposed to the 1.5 mg Mn/m^3 had lower GSH levels in the caudate after 15 and 33 days of exposure, and this effect persisted despite 45 and 90 days of recovery (Erikson et al., 2008). Recently, a follow-up study of Mn-alloy workers suggested that former workers continued to have symptoms many years after exposure (Bouchard et al., 2008). However, little data exist on antioxidant levels and enzyme

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doi:10.1016/j.ecoenv.2010.08.011

activities after in vivo Mn exposure. Such information is critical for more fully evaluating the hepatotoxicity of Mn exposure.

In previous studies, we assessed Mn effects on oxidative hepatic damage and membrane fluidity in rats. Results showed that Mn exposure inhibited SOD and GPx activity as well as decreased GSH levels and increased malondialdehyde (MDA) levels in liver tissues. Mn also inhibited SOD activity and increased MDA levels in hepatocyte nuclei in addition to reducing Na^+,K^+-ATPase activity, increasing MDA levels, and decreasing plasma membrane fluidity in hepatocytes. In this study, we evaluated exposure and time-dependent changes as well as recovery after subchronic Mn exposure in liver tissue and hepatocyte mitochondria, membranes, and nuclei. We focused on the mitochondria because they function as the proverbial powerhouses of the cell by facilitating the fundamental biochemical processes that produce energy from nutrients in the presence of oxygen. They are also the key intracellular targets for different stressors, including metal ions. We also focused on the cell membranes, which are complex, fluid mosaic structures. The maintenance of membrane fluidity is a prerequisite for cell function, viability, and growth. Succinate dehydrogenase (SDH) is an enzyme complex bound to the inner mitochondrial membrane. It is the only enzyme that participates in both the citric acid cycle and the electron transport chain. Na^+,K^+-ATPase, Ca^{2+},Mg^{2+}-ATPase, and Cu^{2+}-ATPase are integral membrane proteins (anchored within biological membranes) that move solutes across the membrane and typically against their concentration gradient. ATPase activity has been shown to be directly related to membrane fluidity (Kimelberg and Paphadjopoulos, 1974). In addition, we concentrated on nucleus because it is the most important cellular organelle given its role in the transmission of genetic information, gene expression, macromolecular synthesis, and metabolism. Consequently, in this paper, we evaluated exposure and time-dependent changes in concentrations of Mn and a few essential metals in liver tissues. In addition, we evaluated the activity of SDH, Na^+,K^+-ATPase, Ca^{2+},Mg^{2+}-ATPase, Cu^{2+}-ATPase, and GPx in hepatocyte mitochondria and membranes. Finally, we measured levels of GSH and *MDA* in liver tissues and hepatocyte mitochondria, membranes, and nuclei following subacute and subchronic Mn exposure and subchronic exposure with a recovery period.

2. Methods

2.1. Materials

Manganese chloride ($MnCl_2$, MW 125.84) and other chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise stated and were of the highest possible quality. Ultrapure water was used throughout and was obtained from a Milli-Q Academic System apparatus (Millipore, Bedford, MA, USA). Mn solution was prepared by dissolving $MnCl_2$ in sterile saline at a concentration of 6.0 mg Mn/ml. The solution was prepared on a weekly basis and stored at room temperature.

2.2. Experimental protocols

Male Sprague–Dawley rats weighing 110–120 g were purchased from Beijing (Vital River Lab Animal Technology Co., Ltd.). Upon arrival, the rats were housed in a temperature-controlled room under a 12-hour-light/12-hour-dark cycle and were allowed to acclimate for one week prior to experimentation with food and water provided ad libitum. Rats were randomly divided into six groups with eight rats in each group. The groups were: 1) 30-days group in which $MnCl_2$ solution was administered to rats once daily for 30 days by intraperitoneal injection at 6.0 mg Mn/kg b.w., 2) 90-days group in which $MnCl_2$ solution was administered to rats once daily for 90 days by intraperitoneal injection at 6.0 mg Mn/kg b.w., and 3) 90+30-days group in which $MnCl_2$ solution was administered via intraperitoneal injection once daily at 6.0 mg Mn/kg b.w. for 90 days followed by a 30-day post-exposure recovery period. In addition, there were three control groups, namely control group I (control group for 30-days group), control group II (control group for 90-days group), and control group III (control group for 90+30-days group). We chose the dose based on a previous study by Wang et al. (2008). An equivalent volume of sterile saline was given to the animals in the control groups. The study was conducted in compliance with animal welfare guidelines and was approved by the Capital Medical University Animal Care and Use Committee. Twenty-four hours after the last injection, the rats were killed by decapitation, and their livers were rapidly removed, weighed. Liver weight was recorded and liver/body weight ratio (relative liver weight) of each animal was calculated.

2.3. Hematology and clinical chemistry assay

The levels of white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), and platelets (PLT) were measured with a hematology analyzer (MEK-6318K, Nihon-kohden, Tokyo, Japan). Serum albumin (ALB), total protein (TP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phoshpatase (ALP), and gamma-glytamyl transpeptidase (GGT) were assayed using a chemistry analyzer (7606, Hitachi, Tokyo, Japan).

2.4. Histopathology

Liver samples fixed in formalin were dehydrated by a graded ethanol series, embedded in paraffin, sliced into 5.0-μm sections, and stained with hematoxylin and eosin. These sections were examined using a light microscope (BX51, Olympus, Tokyo, Japan), and the histopathological features of different groups were compared.

2.5. Isolation of mitochondria, membranes, and nuclei

Pure mitochondrial pellets were obtained by differential centrifugation as previously described (Fernández-Vizarra et al., 2002). To obtain liver membrane-enriched fractions, hepatocyte membranes were prepared using a discontinuous Percoll/sucrose gradient as described by Nagy and Delgado-Escueta (1984) with minor modifications as described by Vieira et al. (2001). Hepatocyte nuclei were prepared as described by Blobel and Potter (1966). To ensure the purity of the nuclei, the nuclear suspension was loaded on a second sucrose cushion (2.5 M sucrose, 50 mM Tris–HCl, pH 7.4, 0.2 mM EGTA, 10 mM $MgCl_2$, 5 mM DTT ,and 1 mM PMSF) and ultracentrifuged at 73,000*g* for 1 h. The white nuclear pellet was washed twice and resuspended in homogenization buffer. Protein concentrations in the mitochondria, membranes, and nucleus were estimated using the biuret method (Bradford, 1976) with bovine serum albumin as the standard. The final protein concentration was adjusted to approximately 0.8–1.0 mg/ml.

2.6. Metal estimation

Liver tissues (0.2–0.3 g) were digested with nitric acid using a hot plate. The digested sample was diluted with 2% HNO_3 solution. Scandium (Sc) and germanium (Ge) were used as internal standards. The concentrations of manganese (Mn), calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), and zinc (Zn) were measured by ICP-MS (7500ce, Agilent, Palo Alto, CA, USA). The contents of Mn, Ca, Cu, Fe, Mg, and Zn were calculated using a calibration curve obtained with standard solutions prepared with $Mn(NO_3)_2$, $Ca(NO_3)_2$, $Cu(NO_3)_2$, $Fe(NO_3)_2$, $MgCl_2$, and $ZnCl_2$ in 2% HNO_3.

2.7. Biochemical assays

The activities of GPx, Na^+,K^+-ATPase, Ca^{2+},Mg^{2+}-ATPase, Cu^{2+}-ATPase, and SDH as well as GSH and MDA levels were determined using the appropriate assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturers' instructions. GPx activity was measured by quantifying the rate of oxidation of reduced GSH to oxidized glutathione at 412 nm. GPx activity is expressed as U/mg protein. The activities of Na^+, K^+-ATPase, Ca^{2+},Mg^{2+}-ATPase, and Cu^{2+}-ATPase were measured by quantifying inorganic phosphorus (Pi) production from the conversion of ATP to ADP at 636, 636, and 660 nm using the molybdenum blue spectrophotometric method and are expressed in U/mg protein. SDH activity was measured by quantifying the rate of 2,6-DPIP reduction at 600 nm and is expressed as U/mg protein. GSH levels were measured using the DTNB spectrophotometric method at 422 nm and are expressed as μg/mg protein. The level of MDA was determined using thiobarbituric acid reactive substances (TBARS) measured at 532 nm and is expressed as nmol/g protein.

2.8. Statistical analysis

All data are expressed as the means ± SE. Statistical analysis was performed by two-way ANOVA using SPSS 13.0 statistic package for Windows (SPSS, Inc., Chicago, IL, USA). Post hoc tests were performed using Tukey's HSD test for the comparison between treatment groups and their respective control groups or for the comparison between the different treatment groups. Differences between two means were considered significant if p values were equal to or less than 0.05.