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ORIGINAL ARTICLE

Long term exposure to low levels of Manganese Chloride improves the activity and expression of antioxidant enzymes in adrenal gland of adult rats

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ABSTRACT

Human contamination with Manganese is associated with neurological symptoms similar to observed in Parkinson Disease. Cathecolaminergic system has been considered an important target of Manganese toxicity. Our study aimed to assess the possible damages induced by long term exposure to low levels of Manganese on Adrenal Gland. For this, adult male Wistar rats were treated for the period of 90 days with $MnCl_2$ diluted in drinking water (120 mg/L). Ambulatory behavior was conducted and Adrenal Gland was used for determination of biochemical parameters related to antioxidant status. Our results demonstrated that Manganese increased the activity of Glutathione Reductase and Catalase as well as the expression of Glutathione Peroxidase , and NADP(H) Quinone Oxidoreductase in Adrenal Gland. Overall, the treatment with Manganese did not induce prominent cell damage in Adrenal Gland as evaluated by poly ADP ribose polymerase cleavage, an apoptotic cell death marker. Our study provides a first time description of biochemical alterations in Adrenal Gland when submitted to long term treatment with Manganese, increasing the expression of NADP(H) Quinone Oxidoreductase and Glutathione Peroxidase 1. Taken together, results of our work may represent a biochemical adaptation of Adrenal Gland to prolonged oxidative stress induced by exposure to the metal.

Key words: catecholamine, manganese, long-term treatment, antioxidant status, glutathione peroxidase.

1. Introduction

Manganese (Mn) is an ubiquitous transition metal present in a range of industrial process as making steel alloys, drycell batteries, electrical coils, welding rods among others. Mn is considered an essential nutrient for humans and animals, participating as co-factor in several enzymatic reactions, such as pyruvate kinase, mitochondrial superoxide dismutase (SOD), glycosyl transferase and fatty acid synthesis (1, 2).

Long-term exposure to Mn are related with occupational exposure and is associated with neurological symptoms which may be indistinguishable from idiopathic Parkinsons Disease including fixed gaze, bradykinesia, postural difficulties, rigidity, tremor, dystonia and decreased mental status characterizing a syndrome known as Manganism (3). It has been reported that chronic consumption of drinking-water containing Mn in levels ranging from 81 to 2300 ug/l were associated with progressively higher prevalence of neurological signal in older persons (4). In this aspect, factors as source and duration of exposure as well as nutritional status can interfere in the intensity and incidence of Mn neurological symptoms.

A variety of biochemical changes are induced in response to Mn exposure, with includes glutathione (GSH) and dopamine (DA) depletion, increased oxidative stress (OS), impairment of energy metabolism and antioxidant systems (5-7). Mn exposure both *in vivo* an *in vitro* is associated with alteration of mitogen-activated protein kinase (MAPK) signaling transduction pathways; this family of proteins are implicated in regulation of multiple cellular events including differentiation, proliferation, cell death, adaptive and immune responses (8). Activation of these kinases may occur in response to hyperosmotic stress, cytokine exposure, and toxic injury, including OS (9, 10) and metals, such as mercury (Hg), lead (Pb), and Mn (11-13).

Previous data has evidenced the catecholaminergic system as an important target of Mn intoxication. Studies carried out in culture cells, demonstrated a depletion of dopamine (DA)

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in nigrostriatal neurons during Mn exposure (14,15). Other evidenced that dopaminergic mesencephalic culture cells were more susceptible to Mn toxicity than other neuron population while that in *vivo* administration of Mn caused loss of Tyrosine Hydroxylase (TH) positive cells in Substantia Nigra (16).

Concerning dopaminergic system, the adrenal medulla is the place where chromaffin cells are most commonly located. These cells secrete and collect noradrenaline, adrenaline and dopamine (DA) (17) which influences the activity of almost all tissues and organs and plays an important role in the protective response to physiological stresses such as hypoxia, hemorrhage and hypoglycemia (18, 19). Previously, our group demonstrated than pheocromacitoma cell line (PC12) was affected by Manganese Chloride ($MnCl_2$) exposure, inducing phosphorylation of MAPKs and cell death; such effects were mostly observed in higher concentrations and longer periods (13).

Thus, this work aimed to investigate the effects of long-term Mn exposure on enzymatic antioxidant cellular defense and modulation of expression and phosphorylation of stress responsive proteins on Adrenal Gland (AG) of adult rats.

2. Material and Methods

2.1. Materials

Manganese chloride (CAS 13446-34-9), Quercetin (Q4951) (CAS 117-39-5), 5,5′-Dithiobis(2-nitrobenzoic acid) (D8130) (CAS 69-78-3), Acetylthiocholine iodide (A5751) (CAS 1866-15-5), 1-Chloro-2,4-dinitrobenzene (237329) (CAS 97-00-7), 2′,7′-Dichlorofluorescein diacetate (DCHF-DA, 35845) (CAS 2044-85-1), 2-Mercaptoethanol (M6250) (CAS 60-24-2), and anti-rabbit immunoglobulin (alkaline phosphatase- linked antibody, A-3687) were obtained from Sigma Aldrich (St Louis, MO). Anti-phospho-p38 (Thr180/ Tyr182) and anti-total-p38, anti-phospho JNK1/2 (Thr183/ Tyr185) and anti-total-JNK1/2, anti-phospho ERK1/2 (Thr202/Tyr204) and anti-total-ERK1/2 and β-actin antibodies were purchased from Cell Signaling Technology. SDS (CAS 151-21-3), Acrylamide (CAS 79-06-1), Bis-acrylamide (CAS 110-26-9), hybond nitrocellulose were obtained from GE Healthcare Life Division. Anti-PARP, anti-GPx1 and GPx4, anti-TrxR, anti-NQO1, anti-metalothioneine, anti NRF-2 and anti HSP-70 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were commercial products of the highest purity grade available.

2.2. Animal Treatment

Ten male Wistar rats (180–200 g) were obtained from Universidade Federal de Santa Maria and maintained in an air conditioned room (20-25ºC) under natural lighting conditions with water and food (Guabi-RS, Brazil) *ad libitum*. All experiments were performed in accordance with the Guiding Principles of the Committee of Animal Care of Universidade Federal de Santa Maria (0089.0.243.000-07). Animal were caged in two groups of five; after two weeks of acclimatization, one group was used as control while the second one was treated with $MnCl_2$ 120mg/L diluted in the drinking water for the period of 90 days. At the end of treatment the locomotor ability of animals was tested in the open field dome and subsequently, animals were decapitated and the AGs were immediately removed for biochemical analysis. The concentration of Mn selected for this study was approximately four times lower than that previously reported in the literature for similar models of exposure, and closer to that observed in epidemiological studies (20).

2.3. Open Field Test

The ambulatory behavior was assessed in an open-field test as described previously (13). The apparatus consisted of a wooden box measuring 40 × 60 × 50 cm. The floor of the arena was divided into 12 equal squares. The number of squares crossed with all paws (crossing) was counted in a 6 min session.

2.4. Sample Preparation and Enzyme Assays

For enzymes activity, whole AG were mechanically homogenized with a tissue homogenizer (Marconi, Brazil) in 1 mL 0.1 M phosphate buffer, pH 7.0, and centrifuged at 1000 g for 5 min at 4ºC. The remaining supernatant was then centrifuged at 20.000 g for 30 min. The resulted supernatant was used for determination of glutathione S-transferase (GST) (21), catalase (CAT) (22), Glutathione Peroxidase (GPx) (23), glutathione reductase (GR) (24), thioredoxin reductase (TrxR) (25) and SOD (26). Protein concentration was determined by the method of Bradford et al. (27) using bovine serum albumin as the standard.

2.5. Western Blotting

Quantification of Glutathione Peroxidase 1 and 4 (GPx1/4), NADP(H) Quinone Oxidoreductase (NQO1) and nuclear factor erythroid 2 (NFE2)-related factor 2 (NRF-2) expression, MAPKs phosphorylation and expression, metallothionein (MT), Heat shock 70 kDa protein (HSP70) and TH expression and poly (ADP)-ribose polymerase (PARP) cleavage was performed using Western blotting based on protocols previously described (13) with minor modifications. Whole AGs were mechanically homogenized at 4ºC in 200 µL of buffer (pH 7.0) containing 50 mM Tris, 1 mM EDTA, 0.1 mM phenylmethyl sulfonyl fluoride, 20 mM Na_3VO_4, 100 mM sodium fluoride and protease inhibitor cocktail. The homogenates were centrifuged at 1000 g for 10 min at 4ºC and the supernatants (S1) collected. After protein determination (27) using bovine serum albumin as standard, β-mercaptoethanol, SDS and glycerol was added to samples to a final concentration of 8 and 25%, respectively, and the samples frozen until further analysis.

Proteins were separated using SDS–PAGE (10%), and then electrotransferred to nitrocellulose membranes as previously described (13). Membranes were washed in tris-buffered saline with Tween (100 mM tris – HCl, 0.9% NaCl and 0.1% Tween-20, pH 7.5) and incubated overnight (4ºC) with different primary antibodies, all produced in rabbit (anti-ERK1/2, anti-p38MAPK, anti-JNK1/2 total and phosphorylated forms, anti-β-actin and anti-PARP, anti-Nrf2, anti-TrxR, anti-HSP70 and anti-NQO1).

Following incubation, membranes were washed in tris-buffered saline with Tween and incubated for 1 h at 25ºC with alkaline phosphatase-linked anti rabbit-IgG secondary specific antibodies. Antibody binding was visualized using the NBT-BCIP kit (KPL, MD, USA). Band staining density was quantified using the Scion Image (Scioncorp ver. Beta 4.0.2) software and expressed as a fold change of the mean relative to control group. The loading controls were performed by analysis of β-actin using specific antibodies.

2.6. Statistical Analysis

Statistical analysis was performed using the Student t-test. Differences were considered to be significant at the $p<0.05$ level.

3. Results

Mn treatment (120mg/L) did not alter the number of crossings and rearing frequency when compared with the control group (figure 1A and 1B).

Animals treated with Mn had 43%, 30% and 28% higher activity of GPx, GR and CAT respectively, than those that did not receive treatment (table 1).

Analysis of imunoblotting demonstrated an increase in expression of GPx1 and NQO1 in 36 and 30% respectively (Figure 2). In counterpart, Nrf2 transcriptional factor, GPx4, HSP70, MT and TrxR expression was not altered. Proteins member of MAPK family ERK1/2, $p38^{MAPK}$ and JNK1/2 expression and phosphorylation were not modified by Mn treatment in AG. Moreover, only the full length form of PARP (116 kDa) was detected indicating that Mn long term exposure did not induce apoptotic cell death in AG. In accordance, no loss of cathecolaminergic cells was observed in response to Mn, since TH expression was unaltered. All these data are represented in the blottings of figure 3.

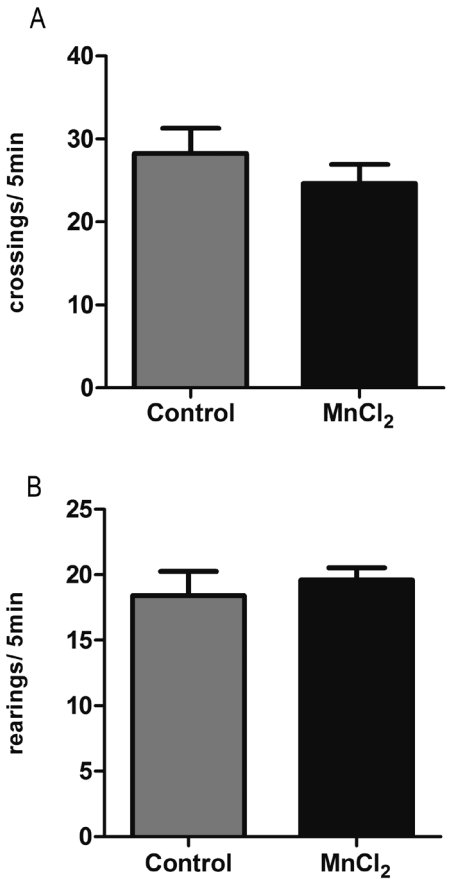

Figure 1. Behavioral analysis of rats exposed to 90 days with 120 mg/L with Mn in drinking water. (A) Number of crossings; (B) rearing activity. Data were analyzed by Student t-test and expressed as mean ± S.E.M. N=5. No differences between groups were observed.

4. Discussion

The present work aimed to investigate the effects caused by long term exposure to low levels of Mn through drinking water on AG taking in consideration possible alterations in antioxidant status, phosphorylation and expression of stress responsive proteins, and possible damage to catecholaminergic cells of this organ. It is important to consider that the concentration of Mn which the animals were exposed was approximately 100 times lower than that used in previous work (28), where it was observed decrease in number of crossings and oxidative stress induction in striatum following 30 days exposure.

AG is a structure important for maintenance of body homeostasis. In adrenal cortex, different steroids, as cortisone, corticosterone, 11-dehydrocorticosterone has been isolated (29). Adrenal medulla is recognized by the presence of chromaffin cells, which secret catecholamines including epinephrine and

Table 1. Antioxidant enzymes activity.

	GPx	GR	CAT	SOD	GST	TrxR
Control	88.6 ± 7.9	52.4 ± 8.1	2.5 ± 0.4	33.0 ± 7.7	44.4 ± 11.3	56.8 ± 6.9
Manganese	127.4 ± 24.2***	68.3 ± 9.5**	3.2 ± 0.3***	33.1 ± 4.6	49.9 ± 6.6	60.2 ± 12.4

Values are expressed as mean (mU/mg protein) ± SEM, n = 5.
** p < 0.01 Control *vs* Mn.
*** p < 0.001 Control *vs* Mn.

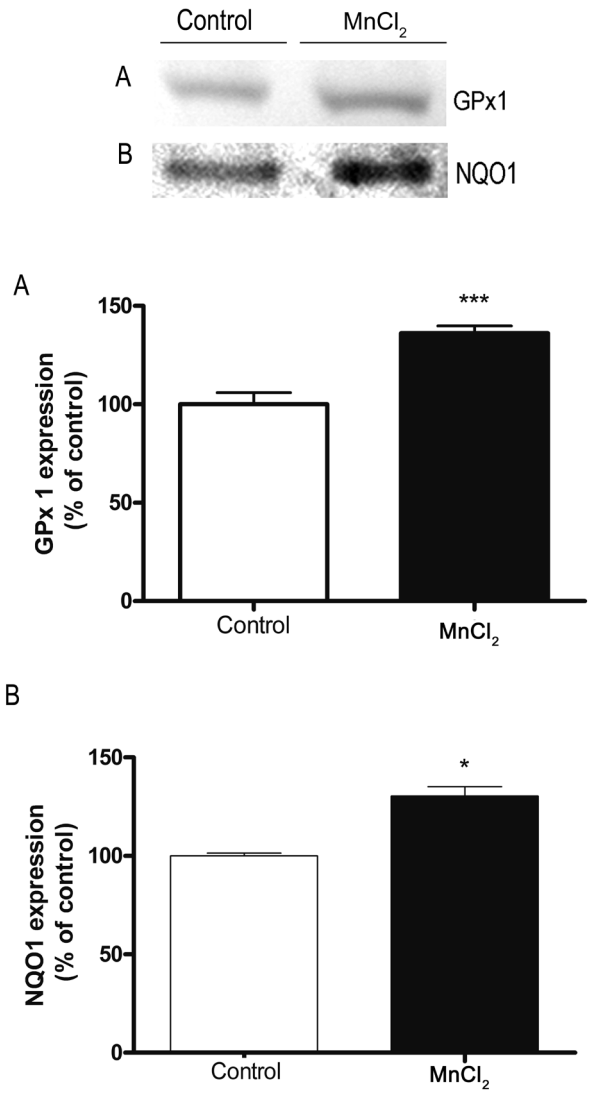

Figure 2. Modulation of GPx1 and NQO1 expression in response to long term exposure to Mn (120 mg/L) in drinking water. Adult male rats were treated for 90 days with $MnCl_2$ diluted in drinking water, AGs were removed and proteins separated by SDS-PAGE, and transferred to nitrocellulose membrane, which was incubated with specific primary antibodies to detection of GPx1 and NQO1. (A) GPx1 expression. (B) NQO1 expression. Expression of proteins was quantified by densitometric analysis. The data are expressed as percentages of the control (considered 100%). The values are means of three to five experiments ± SD. Statistical significance: *p<0,05; **p<0.001 compared to control group.

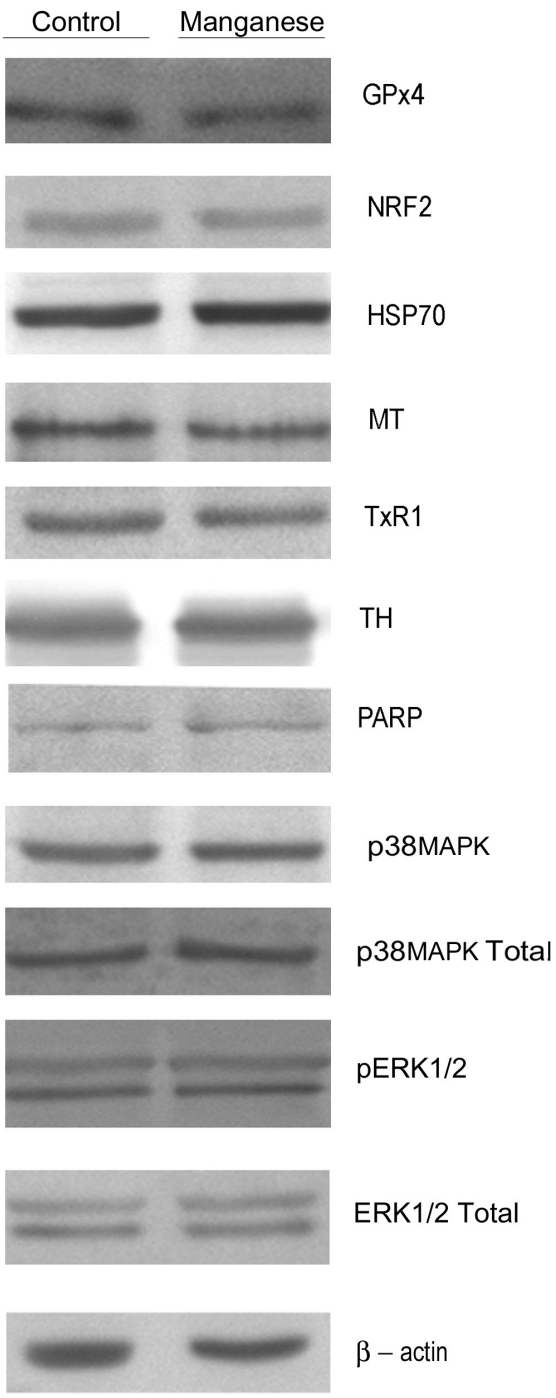

Figure 3. Analysis of proteins expression and/or phosphorylation in response to long term exposure to Mn (120 mg/L) in drink water. Adult male rats were treated for 90 days with $MnCl_2$ diluted in drinking water, AGs were removed and proteins separated by SDS-PAGE, and transferred to nitrocellulose membrane, which was incubated with different specific primary antibodies. Total content (expression) and phosphorylation of protein described in the picture was quantified. The panel is a Western Blotting showing expression of GPx4, Nrf2, HSP70, MT, TRx1, TH, PARP, and MAPKs (p38MAPK and ERK1/2) total and phosphorylated forms and β-actin expression. Each blotting is representative of three independent experiments.

norepinephrine, important hormones in the 'fight and flight' response influencing the activity of almost every tissue (30,31). In this aspect, until now, to our knowledge, no study has been focused on toxicological effects associated to long term exposure to Mn in AG. So far, the most import finding of the present work was that even concentrations unable to cause locomotor alterations affects the biochemistry of AG leading to changes in expression of antioxidant enzymes.

Free radicals play a key role in Mn-induced neurotoxicity (32). Such aspect is related with the ability of Mn to enhance reactive oxygen species (ROS) generation through catalysis of dopamine (DA) autoxidation and quinones formation leading to oxidative damage and overcoming cell death (2). However, it is unknown if Mn can cause oxidative damage or modulating signaling proteins related with cellular stress in AG, an important site for catecholamine synthesis and release. Previous studies demonstrate that Mn enhanced autoxidation of DA increasing the generation of ROS O_2^-, H_2O_2 and OH^- and cytotoxic DA-o-quinone (33, 34). Our group has previously demonstrated a considerable increase in H_2O_2 production in pheocromacitoma cell line (PC12) exposed for 24h to Mn (13), in parallel to augmented activity of TH, the step limiting enzyme in catecholamine biosynthesis. In this study, Mn stimulated the activity of enzymes GPx, GR and CAT in AG in parallel with increased expression of NQO1 and GPx1 isoform without altering GPx4 isoform expression. NQO1 metabolizes dopamine-derived quinones (DAQ) (35) and GPx-1 is necessary for H_2O_2 reduction, thereby the augmented expression of these enzymes by Mn contributes to the antioxidant capacity of the cell (36).

The Nrf2 mediate the induction of a set of drugmetabolizing enzymes such as GST and NQO1 (37). Nrf2 binds to Antioxidant Response Elements (ARE) transcribing multitude of antioxidant genes. Disruption of protein Keap-1- Nrf2 interaction or genetic overexpression of Nrf2 can increase the endogenous antioxidant capacity of brain thus representing protection against OS in neurodegenerative diseases (38). Nrf2 regulates the expression of antioxidant enzymes including GR, peroxiredoxin, thioredoxin and TrxR, CAT, SOD and GPx (39).

Experiments conducted by (40), showed that Mn inducted Nrf2 mRNA expression and cytosolic/nuclear migration in pheocromacitoma cell line (PC12). In the present study, long term exposure to Mn did not alter the expression of Nrf2, however, increase the expression of GPx1 isoform and NQO1. Concerning this data, it is recognized that regulation of Nrf2 that responds for its activation also involves mechanisms of phosphorylation/dephosphorylation, acetylation/deacetylation, disrupting Nrf2/Keap1 complex and translocation of Nrf2 to the nucleus promoting transcriptional activation of Nfr2 dependent genes in response to inducing signals (37) indicating activation of this factor. Other mechanisms acting on antioxidant defense system regulated by Nrf2 includes metal-chelation by MT and induction of stress response proteins. In this aspect, we did not observed induction in expression of chaperone HSP70 and MTs, possibly these proteins were initially stimulated and returned to basal levels at the end of treatment since they are mostly responsive to initial stress response to metals (41).

MAPKs phosphorylation and expression, represented by proteins $p38^{MAPK}$, ERK and JNK1/2, as well as chaperone HSP70 and MTs expression were not affected by Mn treatment in AG. Our research group showed previously that prolonged treatment of the PC12 clone with Mn, which was developed from a pheochromocytoma tumor of the rat adrenal medulla, induced phosphorylation of JNK1/2 and $p38^{MAPK}$ and decreases cell viability only in very high concentrations of Mn (from 500 μM) (13). In this aspect this work is in accordance with data obtained from cell culture studies, since the concentration of Mn used here is four times or even lower than concentrations able to cause neurological symptoms in similar model of exposition (20, 28).

Overall, this study shows that prolonged exposure of adult rats to relatively low concentrations of Mn induces expression of at least two antioxidant enzymes (GPx1 and NQO1) in AG, without causing cell death or activation of cell stress activated proteins. Our result point out to an adaptive mechanism linked to long term exposure to Mn, inducing expression of GPx1 and NQO1 counteracting the overproduction of ROS and protecting cells against cell damage associated with OS.

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Declaration of interest

The authors declare that there are no conflicts of interest.

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