Research Article

Toxicity Assessment Due to Chronic Exposure to Manganese in Male Wistar Rat

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Abstract | Manganese (Mn) is present everywhere: in rocks, soil, water, food, and in various forms. Our bodies utilize it to contribute to energy metabolism and perform many other functions, but prolonged exposure can lead to toxicity in the organism. This study aimed to evaluate the effects of chronic exposure to Mn, specifically its impact on the general condition of male Wistar rats, by recording all clinical symptoms associated with this intoxication. Different doses ranging from the lowest to the highest (6 mg/kg, 25 mg/kg, 30 mg/kg, and 40 mg/kg i.p.) were administered intraperitoneally over a period of 12 weeks. The objective of the study was to identify the lethal dose and the tolerated toxic dose in order to establish a toxicity model. Weight gain was monitored weekly, and, at the end of the exposure period, the rats were euthanized and their organ weights (liver and kidneys) were recorded. Our results indicate signs of intoxication resulting from chronic exposure to Mn. Specifically, doses of 25 mg/kg and 30 mg/kg were found to be toxic and adversely affect the general condition of the rats. In contrast, the 6 mg/kg dose showed no adverse effects when compared to the 25 mg/kg and 30 mg/kg doses. Additionally, the 40 mg/kg dose proved to be lethal. Weight changes were observed in rats injected with the highest doses of Mn, along with alterations in organ weights. This study led us to conclude that chronic exposure to Mn induces dose-dependent toxic effects, as evidenced by the observed clinical signs of toxicity.

Keywords | Manganese, Chronic exposition, Toxicity, Clinical symptom, Dose-dependent, Rat

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INTRODUCTION

Manganese (Mn) ranks as the 12th most abundant element and the 5th most abundant metal on Earth. It is widely distributed in the environment, and found in water, soil, and food sources. This metallic element is essential for cell survival (Santamaria, 2008; Lucchini and Tieu, 2023), playing a crucial role in various physiological processes. Mn acts as a trace element vital for the normal functioning of numerous bodily processes, including the metabolism of sugars, amino acids, and lipids. Additionally, it plays pivotal roles in the nervous and immune systems, cellular energy regulation, the formation of bone and connective tissue, and the activation of specific enzymes (Gillet *et al.*, 2010). The primary pathway for Mn absorption is via the gastrointestinal tract. However, absorption can also occur through the lungs upon inhalation exposure (O'Neal and Zheng, 2015; Haynes *et al.*, 2015). Prolonged exposure to Mn can be detrimental to health, particularly notable among mining and welding workers who experience chronic exposure to aerosols or dust containing Mn (Bowler *et al.*, 2006), as well as individuals with chronic oral exposure to

contaminated water (Lao *et al*., 2017). Moreover, genetic disorders may impact or alter the distribution and function of Mn within the organism (Baj *et al*., 2023).

The primary target organ of Mn is the brain, as evidenced by previous studies indicating that exposure to Mn leads to its accumulation in both the brain and liver. Moreover, it has been observed that the longer the duration of exposure, the higher the concentration of Mn in these organs (Huang *et al*., 2011). Numerous studies have reported that exposure to high doses of Mn can lead to neurodegenerative diseases including Parkinson's disease, Alzheimer's disease, Huntington's disease, and prion diseases (Harischandra *et al*., 2019). The neurotoxic impacts of this metal are similar to those provoked by other heavy metals (Naïla *et al*., 2021; El-Brouzi *et al*., 2021; Zghari *et al*., 2023a, b). On the other hand, the liver serves as the primary organ responsible for regulating body Mn levels by facilitating endogenous Mn losses in the intestine (Davis *et al*., 1993). Liver cells express a variety of Mn transporters on their cell membranes (Nam *et al*., 2013), including Mn exporters, which regulate the efflux of excess Mn (Quadri *et al*., 2012). Consequently, the liver plays a crucial role in storing, redistributing, and eliminating Mn from the body. However, accumulation of Mn in the liver can lead to liver damage by interfering with its excretory functions (Milatovic *et al*., 2009). In addition, Mn absorption in the kidney is regulated by several Mn transporters, including ZIP8, ZIP14, and DMT1, located in renal proximal tubule epithelial cells (Fujishiro *et al*., 2012). It has also been reported that the kidneys were affected in rats exposed to Mn through oral ingestion (Ponnapakkam *et al*., 2003). In a study, Mn-treated rats exhibited impaired kidney function, as evidenced by elevated serum urea and creatinine levels. This indicates that any increase in these levels in serum or plasma is a marker of kidney damage. The kidney tissue showed numerous atrophied and shrunken glomeruli, and the renal tubules were deformed with signs of cell apoptosis and necrosis (Mostafa *et al*., 2021). At high doses, this metal disrupts the overall health of the organism, manifesting visible signs of toxicity such as weight loss or slowed weight gain (Tian *et al*., 2018). Additionally, a study by Sárközi *et al*. (2009) reported similar effects of Mn on body weight (Sárközi *et al*., 2009). *In vivo* studies investigating Mn toxicity in rats have indicated that high doses of Mn can be lethal (Tian *et al*., 2018). Hence, determining the LD50 (dose at which 50% of the animals tested died) is crucial. This determination is typically the initial step in evaluating the toxic properties of a substance and is a critical aspect of any experiment.

This study aims to evaluate the dose-dependent effects of Mn on rats. Over 12 weeks, we will record the changes and alterations resulting from chronic Mn intoxication. Additionally, we aim to identify the lethal dose and establish a model dose that can be used to induce Mn toxicity.

MATERIALS AND METHODS

Conditioning and constitution of rat batches for manganese toxicity assessment

A total of 35 male Wistar rats from the breeding of the Faculty of Life Sciences at the University of Ibn Tofaïl were used in the current work. All rats were maintained under LD 12/12 (12 h light/12 h darkness) and at a standard temperature (21±1 °C), during which water and food were provided. The experimental procedures were carried out by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Ethics Committee (Local Institutional Research Committee). All efforts were made to minimize the number of rats and their suffering.

The rats were divided into four groups of seven animals each (7 males). The different groups of rats are distributed as follows:

Control group: Rats were injected with physiological saline buffer (0.9% NaCl).

Mn-6 group: Injected with 6 mg/kg body weight (Huang *et al*., 2011).

Mn-25 group: Injected with 25 mg/kg body weight (Tian *et al*., 2018).

Mn-30 group: Injected with 30 mg/kg body weight.

Mn-40 group: Injected with 40 mg/kg body weight.

Mn doses were administered daily during the 3-month treatment period by intraperitoneal injection. The rats were weighed every week, and their weights were noted. At the end of the 12 weeks, the rats were sacrificed, and their livers and kidneys were collected and weighed in a precision balance. The relative organ weights were calculated using the formula: Relative Organ Weight = (Organ Weight (g)/ Body Weight (g)) × 100. This value is expressed as a percentage of the total body weight and allows for comparisons between treated and control groups, regardless of variations in overall body weight.

Manganese poisoning protocol

The Mn solution is prepared from a commercial form called manganese chloride tetrahydrate ($MnCl_2 4H_2O$). We selected four doses to determine the most toxic and lethal dose: 6 mg/kg and 25 mg/kg, as per the literature cited in the referenced articles, using a 0.9% NaCl solution.

The saline solution or $MnCl_2 4H_2O$ (obtained from Sigma-Aldrich, St. Louis, MO, USA) used in this study was injected intraperitoneally and chronically, once daily for 12 weeks. The doses of 30 mg/kg and 40 mg/kg were tested for the first time in this study by our team.

Groups not treated with Mn (control) received intraperitoneal injections of 0.9% NaCl. The injection

volume of Mn was 0.2 ml/100 g body weight each week. The control group was injected with an equal volume of physiological saline solution as the group treated with Mn.

Observation of symptomatic disorders

After treatment with Mn at different doses, the animals were returned to their cages, where they could access food and water again. They were observed immediately, and then daily for 12 weeks starting from day one, and thereafter once a day. Symptomatic disorders such as aggressiveness, lack of appetite, motor difficulties, changes in hair color, etc., were noted in animals injected with different doses. In addition, to assess the deterioration of olfaction, a piece of cheese was hidden in the cage, and the rat was allowed to search for it. The time it took for the rat to find the cheese was recorded and compared with the time taken by control rats.

Evaluation of toxicological parameters: Determination of DMT, LD100 and LD50

The number of deceased animals was recorded in each batch of this toxicity experiment, which aimed to determine the following toxicological parameters:

- The 50% lethal dose (LD50), defined as a dose that kills 50% of the animals,
- The 100% lethal dose (LD100), a dose that kills all animals
- The maximum tolerated dose (MTD), representing the maximum dose that kills no animals when Mn is administered

In theory, the average lethal dose, or LD50, provides information on the amount of a substance required to cause adverse effects in the body. Moreover, there are various methods to assess effects, including physiological, biochemical, and behavioral measurements. However, toxicity remains one of the most extensively examined indicators (Gómez-Ariza *et al.*, 2000), and one of the methods used to calculate the LD50 is the Behren-Karber method, which is a non-parametric approach. It involves observing equal spacing of dose intervals and an equal number of subjects at each dose from 0% to 100%.

It is calculated as follows:

- LD50 = LD100 - Σ (a x b) / n
- LD100: 100 % mortality dose
- LD50: 50% mortality dose
- n: Number of animals in each group
- a: The difference between two consecutive doses
- b: The arithmetic mean of the deaths from two consecutive doses.

Statistical analysis

IBM's SPSS version 23 was used for all statistical analysis (IBM Corp., Armonk, NY, United States). Body weight and organ weight data were statistically analyzed using ANOVA (One-way). The values were expressed as mean ± SEM. A $p < 0.05$ was interpreted as a significant difference.

RESULTS AND DISCUSSION

Clinical signs and mortality

Clinical signs

During the treatment period, rats exhibited symptoms following exposure to different doses of Mn. Rats treated with a single dose (25 mg/kg or 30 mg/kg) of Mn displayed signs of Mn intoxication as presented in Table 1. Additionally, rats treated with both doses showed changes in coat quality, characterized by yellowish hair, hair loss, and piloerection, as well as weight loss. Some rats in the 30 mg/kg group exhibited abdominal and testicular swelling, while abdominal lesions were observed in both groups. Asthenia, bradykinesia, weakness of muscle tone, deterioration of olfaction, and digestive tract diarrhea were also observed. Signs of toxicity in the 6 mg/kg group were minimal. Not all signs were observed in rats treated with the 40 mg/kg dose, as they succumbed within the first few weeks. These observations for the different groups are illustrated in Figures 1, 2, and 3.

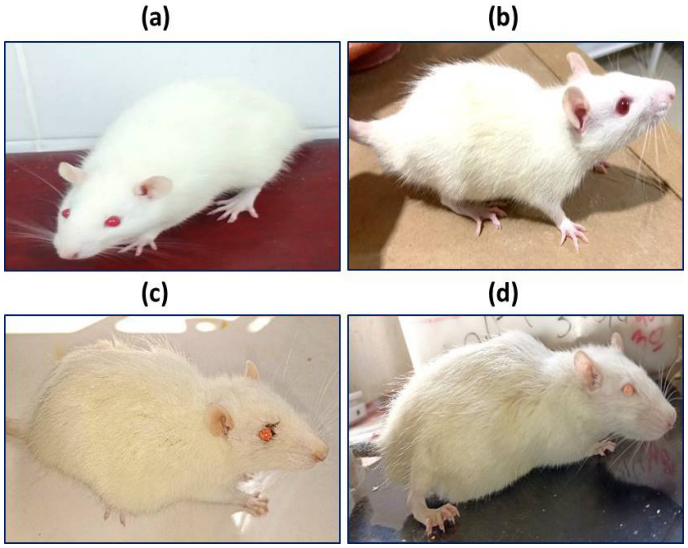

Figure 1: Hunched back with flattened limbs in control group (a), rat treated with 6 mg/kg (b), rat treated with 25 mg/kg (c), rat treated with 30 mg/kg (d).

Mortality

Following intraperitoneal injection of Mn at different doses, the mortality rate was recorded for each dose administered. An increase in animal mortality was observed with increasing doses, indicating a dose-dependent effect (Figure 4). The highest dose resulting in mortality for all animals was 40 mg/kg, while the MTD was determined to be 30 mg/kg. The LD50 of the metal was calculated to be approximately 32 mg/kg, as detailed in Table 2.

Table 1: The observed symptoms of toxicity associated with different doses treated by manganese and the melatonin effect in Rat at 1, 2, 3, 4, 5, 6,7, 8, 9, 10, 11, and 12 weeks after exposure, respectively.

Observations	Control	6 mg/kg Mn	25 mg/kg Mn	30 mg/kg Mn	40 mg/kg Mn
Weight fluctuation	____	____	+	+++	+++
Depression/anxiety	____	+	++	+++	+++
Hair color	White	White	Yellowish	Yellowish	Yellowish
Olfactory impairment (Hyposmia)	____	____	++	+++	+++
Hair loss	____	____	+	++	+++
Piloerection	____	____	+	+++	+++
anorexia	____	____	+	+++	+++
Diarrhea	____	+	+	++	+++
Aggressiveness	____	____	+	++	____
Normal gait	+++	+++	++	+	+
Abdominal ulcers	____	____	+	+++	+++
Testicular swelling	____	____	____	+	____
Abdominal swelling	____	____	____	+	____
Asthenia	____	____	++	+++	++
Stiffness	____	____	+++	+++	+++
Slow movement	____	+++	++	+++	+++
Sens of exploration	+++	+++	++	____	____
Eye color	Red	Red	Light red	Light red	Light red
Mortality	____	____	____	++	+++
Hunched back	____	____	+	+++	+++

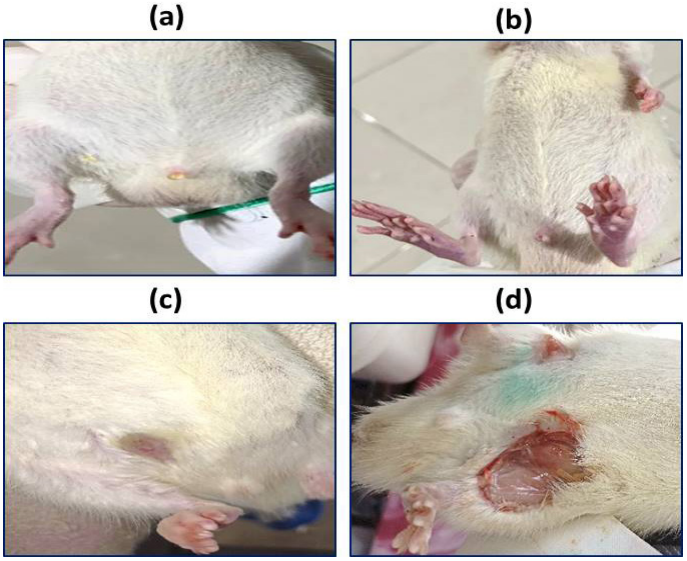

Figure 2: Abdominal lesions in control group (a), rat treated with 6 mg/kg (b), rat treated with 25 mg/kg (c), rat treated with 30 mg/kg (d).

Table 2: Determination of the LD50 according to arithmetic and Behrens and Kerber methods.

Mn doses (mg/kg)	Number of rats	Number of dead rats	Mortality %	a	b	a*b
6	7	0	0	19	0	0
25	7	0	0	5	1.5	7.5
30	7	3	42.85	10	5	50
40	7	7	100			

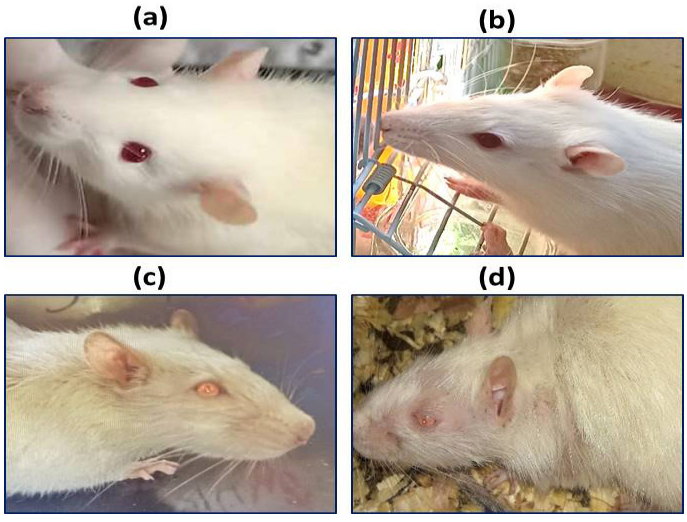

Figure 3: Hair color, piloerection, hair loss, and eyes color and paleness of the mucous membranes of the eyes in control group (a), rat treated with 6 mg/kg (b), rat treated with 25 mg/kg (c), and rat treated with 30 mg/kg (d).

Effect on body weight gain and organ weight of rats

Effect of Mn on body weight gain

Figure 5 shows the evolution of the average body weight of rats throughout the Mn treatment period. Analysis ofdaily body weight measurements showed a slow variation in body weight compared with the other groups, with a significant decrease in the 30 and 40 mg/kg dose groups during the last weeks of treatment (from week 11 onwards).

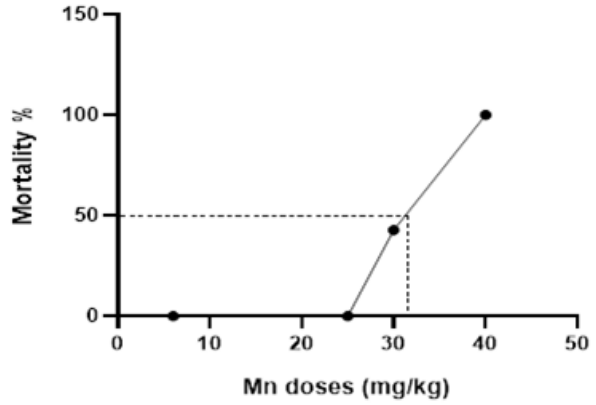

Figure 4: Dose-lethality curve after intraperitoneal injection of Mn at different doses.

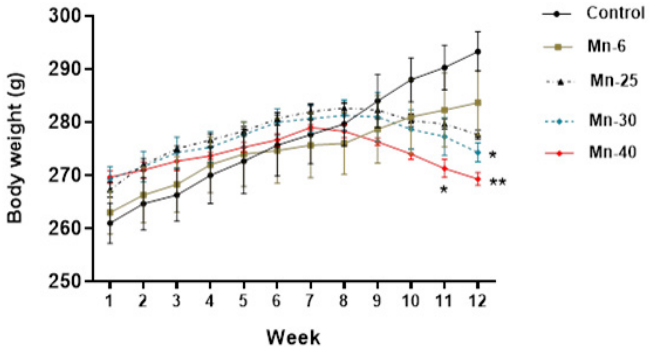

Figure 5: Mean body weight (Mean ± S.E.M) of male rats treated with Mn repeated intraperitoneal doses (6, 25, 30, and 40 mg/kg) for 12 weeks. Significantly different from vehicle control: *p < 0.05, **p < 0.01, respectively.

In addition, compared with the control group, rats treated with 25 mg/kg showed slow weight growth and a decrease in body weight from week 9, and rats treated with 6 mg/kg showed almost similar weight growth to the controls.

Effect of Mn on the organ weight of rats

Statistical analysis has revealed that Mn-6, Mn-25, Mn-30, and Mn-40 significantly increased the relative liver weights in a dose-dependent fashion when compared to the control group ($p < 0.01$, $p < 0.001$, $p < 0.001$, and $p < 0.001$, respectively). In addition, the groups treated with Mn at the doses of 25, 30, and 40 mg/kg had increased relative kidney weights (in a dose-dependent manner) in comparison to the control group ($p < 0.01$), while Mn-0.6 non-significantly increased the relative kidneys weight ($p > 0.05$) (Figure 6).

Mn is essential for both humans and animals when consumed within the limits of their daily dietary requirements. However, exposure to high doses can be detrimental, and contamination of air and water sources poses a risk of Mn poisoning to the general population. Despite its necessity for various physiological functions, excessive accumulation of Mn in the human body can result in severe toxicity, impacting the entire organism. Our study aims to investigate the toxic effects of Mn at varying doses on the rat organism by documenting visible symptoms and monitoring weight fluctuations relative to the injected dose. Additionally, we aim to calculate the lethal dose and establish the appropriate dose for an animal model of Mn poisoning.

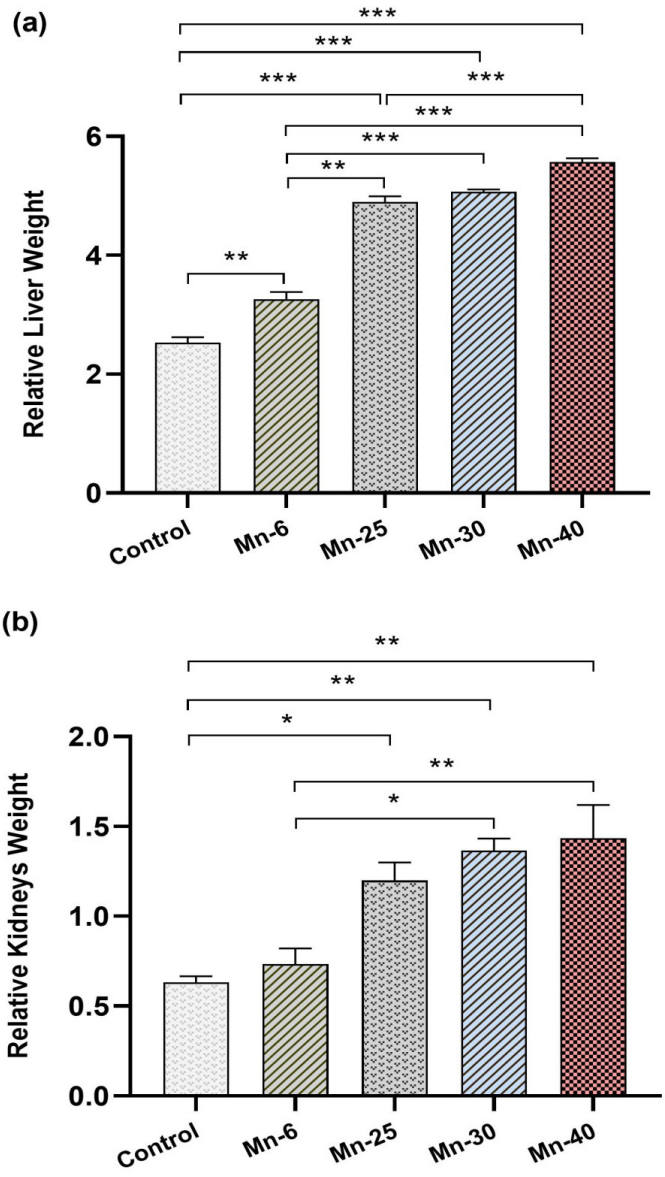

Figure 6: Relative weights of liver and kidneys (Mean ± SEM) of male rats treated with Mn repeated intraperitoneal doses (6, 25, 30, and 40 mg/kg) for 12 weeks. Significantly different from vehicle control: *p < 0.05.

In our study, the intraperitoneal injection of Mn at various doses into rats and their observation for 12 weeks revealed differing effects depending on the injected dose. Chronic exposure to Mn resulted in notable consequences, manifested as visible alterations in the general condition of the rats. These alterations were characterized by the emergence of signs of intoxication stemming from toxic effects. It was observed that in rats treated with doses of

25 mg/kg and 30 mg/kg, symptoms of toxicity occurred at these levels and were characterized by external signs that increased in severity with the dose injected. These signs included yellowing and loss of hair, as well as bristling. Research suggests that environmental stress can alter skin structure, predisposing to stress-related skin diseases (Alexopoulos and Chrousos, 2016). In addition, it has been noted that hair growth can slow due to growth inhibition (Peters *et al*., 2006). Chronic stress related to strain modifies the active hair cycle (Liu *et al*., 2013). We also observed the appearance of ascites in some rats at the doses of 25 mg/kg and 30 mg/kg, which is in line with findings reported in another study (Tian *et al*., 2018). Additionally, abdominal lesions and diarrhea were noted, along with paleness of the mucous membranes of the eyes and pads of the extremities in the high-dose Mn group. Other symptoms, such as reduced exploratory behavior, increased fatigue, and decreased activity, were also observed. In this sense, research reporting the observation of some of these symptoms in rats after exposure to Mn includes the study conducted by O'Neal *et al*., which found that Mn treatment reduced exploratory behavior (O'Neal *et al*., 2014). The study also noted that with longer durations of exposure, rats exhibited reduced activity, hair loss, diarrhea, and other symptoms. Another study reported that muscle and joint weakness, hunched posture, and kinetic tremors were observed in Mn-intoxicated rats (Bowman *et al*., 2011), which is consistent with our observations in rats treated with doses of 25 mg/kg and 30 mg/kg. Moreover, our results align with the study conducted by El-Fari *et al*. (2019) which affirmed that Mn intoxication decreases spontaneous locomotor behavior and muscle strength (El-Fari *et al*., 2019). In addition, in our study, the Mn dose of 6 mg/kg showed low toxicity compared to Mn doses of 25 mg/kg, 30 mg/kg, and 40 mg/kg, which exhibited signs of toxicity. The significant gap between the doses is chosen to establish a dose-response relationship, grounded in scientific rationale, supported by previous research, and aligned with regulatory requirements. The Estimated Safe and Adequate Daily Dietary Intake (ESADDI) for adults for manganese is 5 mg Mn/day. The LOAEL (lowest observable adverse effect level) for manganese is 4.2 mg Mn/day for a 70-kg individual (Greger, 1998).

Observing the mortality rate is crucial in toxicity studies (Teo *et al*., 2002). In addition to detecting abnormalities and behavioral signs caused by Mn, throughout the 90-day observation period, rats in this study exhibited mortality. The finding of our study showed that intraperitoneal administration of Mn induces toxicity in rats, with a dose-response effect evident as a dose of 40 mg/kg resulted in the mortality of all rats. In a study by Tian *et al*. (2018) the dose lethal to all rats was reported as 50 mg/kg (Tian *et al*., 2018). However, the toxicological parameters in our study were as follows: The MTD was 25 mg/kg (ip), and the LD50 of the rats was estimated to be 32 mg/kg ip. The LD50 was calculated using the Behren-Karber method. Based on the results of our study and their alignment with previous findings, we can conclude that 25 mg/kg serves as a suitable preliminary dose for establishing a model of Mn poisoning.

Body and organ weights were used to assess the general state of health and toxic effects on the rat organism following chronic exposure to Mn. The results showed a significant reduction in weight gain during the last weeks of exposure in the groups exposed to 25 mg/kg and 30 mg/kg Mn. Our results are consistent with those of (Finkelstein *et al*., 2007; Bouabid *et al*., 2014), who reported a decrease in body weight in adult rats following chronic exposure to Mn (Finkelstein *et al*., 2007; Bouabid *et al*., 2014). This decrease in body weight gain may result from a generalized disruption of normal physiological processes, such as a deficiency in energy metabolism and functional alterations in the hypothalamic nuclei that contribute to body weight control (Bouabid *et al*., 2014; O'Neal and Zheng, 2015). Additionally, a study indicates that the reduction in body weight gain could be attributed to a lack of food intake caused by loss of appetite or gastrointestinal disorders provoked by this metal (Misselwitz *et al*., 1995).

The relative organ weight in toxicology studies is also an important indicator of the harmful effects of the tested compound (Lazic *et al*., 2020; Rhaimi *et al*., 2023). Importantly, an increase in kidney and liver volume was observed in rats treated with Mn in a dose-dependent manner compared to the control group. Our results are consistent with a study reporting that Mn administration caused a significant increase in relative liver weight in treated rats (Ismail, 2019); another study also observed this increase (Huang *et al*., 2011). As known, the liver is the primary organ responsible for storing, redistributing, and eliminating Mn to maintain Mn homeostasis in the body. Consequently, exposure to excessive levels of Mn can lead to severe liver damage, resulting in various chronic liver diseases (Gandhi *et al*., 2022). Other works suggest that this increase in liver weight could be attributed to high-fat accumulation in hepatocytes. Additionally, the mechanisms by which Mn exposure affects kidney weight may involve oxidative stress, inflammation, and disruption of normal cellular functions. Mn-induced oxidative stress can damage renal tissue, leading to compensatory hypertrophy, where the kidney tissue increases in size in response to injury or stress (Niknahad *et al*., 2020). These alterations in the kidneys and liver may explain the changes in weight and volume observed in our study.

CONCLUSIONS AND RECOMMENDATIONS

This study revealed the toxic effects on the general health of rats due to prolonged exposure to high doses of Mn, including effects on weight, liver, and kidneys. In addition, it enabled us to select an assay for a toxicity model to be explored in our future studies, focusing specifically on the neurotoxic effects of Mn on neuroaffective, locomotor, and olfactory functions. These results underline the importance of continuing research into the mechanisms underlying Mn-induced toxicity. Continued analysis and research are crucial for gaining a comprehensive understanding of these symptoms and for the development of potential treatments.

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NOVELTY STATEMENT

This study aims to present a clear representation of the clinical symptoms resulting from chronic exposure to Mn and to determine the lethal dose, thus enabling researchers interested in studying Mn to visualize its effects at different doses in rats.

AUTHOR'S CONTRIBUTION

All authors contributed equally to the manuscript.

Data availability

The data sets analyzed in this study are available from the corresponding author on reasonable request.

Conflict of interest

The authors have declared no conflict of interest.

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