Organ weight changes in mice after long-term inhalation exposure to manganese oxides nanoparticles

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Abstract. Recently, it has been proven that manganese from inhaled particles of manganese compounds can accumulate in the internal organs of laboratory animals. Nevertheless, there were only a few researches dealing with changes in body morphology induced by inhalation of these particles, even though results of some studies indicate existence of such changes. The aim of our research was to assess the effect of inhaled manganese oxides nanoparticles on weight of internal organs. For this purpose a long-term inhalation experiment on laboratory mice was performed, during which the mice were exposed to MnO, Mn₂O₃ nanoparticles in concentration 2 × 10⁶ particles/cm³ for 17 weeks, 24 hours a day, 7 days a week. Manganese oxides nanoparticles were synthesized continuously via aerosol route in a hot wall tube flow reactor using thermal decomposition of metal organic precursor manganese(II)acetylacetonate in the flow tube reactor at temperature 750 °C in the presence of 30 vol% of oxygen. It was proven that inhaled nanoparticles can influence the weight of internal organs of mice. Moreover, it was discovered that the resulting change in weight of selected organs is disproportional. The mice from the experimental group had statistically significantly lighter kidneys, liver and spleen and heavier pancreas compared to the mice from the control group.

1. Introduction
Toxic effects of manganese were probably firstly described by prof. John Couper of the University of Glasgow in 1837 [1]. He observed gradual development of paraplegia in five workers who crushed manganese oxides in a factory producing bleaching powder. In two of them, who were exposed to manganese for the longest time, these symptoms did not disappear even after discontinuation of exposure and subsequent treatment. Since then a number of similar cases of manganese neurotoxicity have been described. Based on these cases a condition known as manganism (Mn-induced Parkinson-like disease) was described. Manganism is an irreversible progressive brain disease with similar symptoms like idiopathic Parkinson's disease, connected with accumulation of manganese in the brain tissue. The first stage of the disease is usually manifested by impaired fine motor coordination, fatigue, headache, loss of appetite, apathy, insomnia and diminished libido [2, 3]. In the next stage follow:
psychotic behaviour, slurred speech, slow and clumsy gait, rigidity of facial muscles, speech disorder, tremor, dystonia, balance disorders and severe muscle stiffness, which can even lead to immobility. Neurotoxicity of manganese is probably associated with damage of basal ganglia, especially in the areas of globus pallidus and corpus striatum [4]. Neither the exact mechanism of manganese action nor the reason for damage of the basal ganglia is known. However, it is assumed that this could be a consequence of oxidative stress or inflammatory processes [2, 5, 6, 7]. It seems probable that high doses of manganese also induce changes in the levels of the neurotransmitters dopamine, glutamate (glutamic acid) and γ-aminobutyric acid, which may serve as another explanation for neurotoxicity of manganese [8].

Idiopathic Parkinson's disease and manganism are considered different diseases with different symptoms and aetiologies [9]. Unlike patients with Parkinson's disease patients suffering from manganism do not respond to treatment by dopamine substitution, exhibit abnormalities in the areas of globus pallidus and corpus striatum, where manganese selectively accumulates, and also have some different symptoms [2, 9, 10, 11]. Recently, however, it has been shown that exposure to manganese may also contribute to occurrence of idiopathic Parkinson's disease itself, or at least it can accelerate its progression [12]. Racetech et al. [13] came up with the hypothesis that high doses of manganese appear as manganism, while long-term excessive intake of lower doses of manganese can be one of the previously unknown causes triggering idiopathic Parkinson's disease. This statement was supported by the research during which they found out that the symptoms of welders affected by parkinsonism and idiopathic Parkinson's disease patients did not differ significantly. In welders, however, these symptoms occurred on average 17 years earlier than in patients with idiopathic Parkinson's disease. Lucchini et al. [14] also came to a similar result when they demonstrated dependence of parkinsonism incidence in the province of Brescia in northern Italy on the amount of manganese in settled dust collected in different villages, as well as an increased incidence of parkinsonism in people who lived near the ferroalloy plants. There are also indications that long-term chronic exposure to manganese can negatively affect intellectual and motor skills of children [15, 16, 17].

Manganese gets into the bloodstream primarily by ingestion and inhalation [18] provided that the ingestion is usually considered less dangerous way of exposure [9]. Inhaled particles of manganese compounds are partially trapped in the mucus of the lower respiratory tract, partly penetrate the alveoli and from there they get into the blood [19]. The possibility of the passage of manganese compounds nanoparticles from the nasal cavity directly to the brain through the olfactory nerve is also often discussed [9]. In the past, it had been proven that manganese from inhaled particles of manganese compounds can accumulate in the internal organs of laboratory animals, especially in the lungs, liver, pancreas and gall bladder [10, 20, 21, 22, 23]. Results of some studies [3, 23, 24] additionally suggest that inhaled manganese compounds particles may also influence the constitution of animals, but conclusive evidence has not been found yet. Most toxic effects were observed after the inhalation exposure to higher concentrations of manganese [9]. In the past neurotoxicity due to inhalation of manganese compounds particles was observed in some specific occupations, particularly in miners in manganese ore mines, workers in the steel mills and foundries, especially those where ferromanganese and steel are melted, and factories producing "dry cells". Also welders belong among highly exposed occupation groups [19]. Welding electrodes usually contain manganese which then forms a part of the welding fume [25]. In addition, it provably contains ultrafine particles of size <100 nm, which easily penetrate the respiratory system [21]. The welding fume contains manganese primarily in the form of Mn²⁺ and Mn³⁺ [22, 23, 25].

The aim of our research was to extend current knowledge on the effects of inhaled nanoparticles of MnO·Mn₂O₃ on the constitution of mice. We conducted a long-term inhalation experiment, during which laboratory mice were exposed to nanoparticles of MnO·Mn₂O₃ for 17 weeks, 24 hours a day, 7 days a week. On previously chosen days, mice were collected from the cages and autopsied. During the autopsy selected internal organs were weighed.
2. Materials and Methods

2.1. Experimental design
All mice (adult males, ICR line, weighing about 24 g at the beginning of the experiment) were allowed to acclimate to laboratory conditions for at least 1 week before the experiment began. The mice were provided with a commercial diet and water ad libitum. The experimental work was performed in accordance with the ethical approval of the Institute of Animal Physiology and Genetics (no. 081/2010). The experiment started with 80 laboratory mice which were randomly divided into 2 equally sized groups: a fresh-air control and the experimental group exposed to nanoparticles of MnO. Mn2O3. Both groups were placed in identical inhalation chambers and received identical feeding. During the experiment 2 mice from the control group had to be euthanized for health reasons and those were not further analyzed. At chosen time intervals (figure 1) mice were taken out of the cage, euthanized and autopsied one by one. During the autopsy the brain, lungs, heart, liver, kidneys, spleen, pancreas, testes and thymus were weighed.

![Figure 1](image)

Figure 1. Overview of the days when mice were collected from cages during the inhalation experiment, n marks the number of mice taken from experimental group | control group.

2.2. Nanoparticles generation and measurement
Manganese oxides (MnO. Mn2O3) nanoparticles (MnONPs) were synthesized continuously via aerosol route in a hot wall tube flow reactor using a thermal decomposition of metal organic precursor manganese(II)acetylacetonate in the flow tube reactor (in vertical position) at temperature 750 °C in the presence of 30 vol% of oxygen. The vapours of manganese(II)acetylacetonate were generated from solid form of acetylacetonate in saturator at temperature 160 °C and released vapours were transported by nitrogen (purity 5.5, flow rate 0.5 l/min) into the flow reactor. The total flow rate of nitrogen/oxygen mixture through the flow reactor was 2 l/min. At the output from the reactor, MnONPs transported in nitrogen flow were mixed with air (1 l/min). The concentration of produced MnONPs at the reactor output was in the range 1-3 × 10^7 particles/cm^3 and the size of generated nanoparticles MnO. Mn2O3 was in the range 7-50 nm. Before entering the inhalation chamber MnONPs in the mixture of N2, O2 and air (at total flow rate of 3 LPM) were further diluted using filtrated, humidified air (20 L/min) at temperature 21 °C resulting in the MnONPs concentration 2 × 10^6 particles/cm^3. Concentration of nanoparticles and particle size distribution in the inhalation chamber were continuously measured by SMPS (model 3936L72, TSI).

2.3. Statistical analysis
Differences between the group exposed to MnONPs and the control group in organ weight were statistically evaluated. Statistical testing was carried out in two successive steps. First, the exact p-value was computed separately for each day when the mice were taken out and autopsied using one-tailed Wilcoxon Rank Sum test. In case that the exposed and the control group do not differ in organ weight, these p-values computed for different collection days should be uniformly distributed over the interval [0, 1] with median being equal to 0.5 [26]. In the second step, this null hypothesis was tested at the 0.05 significance level using two-tailed Wilcoxon Sign Rank test. Statistically significant results indicate that organ weight in both groups differs at least in one collection day. Software R [27] was used for all statistical computations.
3. Results
Statistically significant differences in organ weight of the liver, kidneys, spleen and pancreas were found (table 1). The liver, kidneys and spleen were considerably lighter in the mice from the group exposed to MnONPs compared to the mice from the control group (figure 2). Pancreas was also affected by MnONPs inhalation, but with the opposite effect. It was proven that the mice from the exposed group had heavier pancreas with higher manganese weight than the mice from the control group.

| organ    | result       | p-value |
|----------|--------------|---------|
| brain    | n.s.         | -       |
| lungs    | n.s.         | -       |
| heart    | n.s.         | -       |
| liver    | C > E        | < 0.01  |
| kidneys  | C > E        | < 0.05  |
| spleen   | C > E        | < 0.05  |
| pancreas | E > C        | < 0.05  |
| testes   | n.s.         | -       |
| thymus   | n.s.         | -       |

Table 2. Comparison of organ weight in mice from the exposed group and the control group for assessed internal organs, n.s. - statistically not significant result, C - control group, E - exposed group.

4. Discussion
Only few researches dealing with the influence of manganese on body weight have been carried out so far [7, 24, 28]. During the experiment performed by Santos et al. [7], two groups of rats were treated intraperitoneally with 25 mg of MnCl$_2$.4H$_2$O/kg every 48 hours. The first group received a total of 4 doses, the second group of 8 doses. The authors tested whether the exposed groups differed from the control groups in body weight, but did not find any statistically significant differences. However, it was not an inhalation experiment, nor nanoparticles were used, so this comparison has only a limited information value.

Comparable long-term inhalation experiment was conducted by Dorman et al. [28] who studied changes in organ weight in macaques due to exposure to inhaled MnSO$_4$. Even they did not find any statistically significant differences in the weights of these organs between the exposed groups and the control group in the monkeys which were euthanized immediately after the end of the experiment. They found only a relative reduction of about 17% in heart weight in the monkeys which were left alive for 90 days after the end of exposure. Nevertheless, the monkeys from the control group were euthanized immediately after the end of the inhalation experiment and were thus of different age. As noted by the authors themselves, the monkeys were still growing during the experiment, so their result cannot be considered decisive.

Torrente et al. [24] gave rats 275 and 550 mg of MnCl$_2$/kg/day in drinking water for 19 weeks, during which the rats were weighed regularly. In the rats from the exposed groups a lower body weight and a lower weight gain together with less food and water intake were discovered in comparison with the control group. Regarding the cause of lower body weight of exposed rats, Torrente et al. suggested that this could be a consequence of aphagia due to lesions in the mesolimbic
dopaminergic system induced by excessive doses of manganese. Under these conditions, reduced food intake would undoubtedly influence the weight of internal organs, but it is not clear whether these changes would be as disproportionate as we observed in our sample.

Figure 2. Development of median weight of the liver, kidneys, spleen and pancreas in mice during the 119 days of the experiment; green point marks the median of the control group; red point marks the median of the group exposed to MnONPs; dashed line connects adjacent medians of the control group; solid line connects adjacent medians of the exposed group.

5. Conclusion
A long-term inhalation experiment was carried out. During the experiment laboratory mice were exposed to MnO/$\text{Mn}_2\text{O}_3$ nanoparticles in concentration $2 \times 10^6$ particles/cm$^3$ for 17 weeks, 24 hours a day, 7 days a week. It was proven that the manganese oxides nanoparticles cause changes in the weights of some organs. The observed disproportionality of these changes is a very interesting phenomenon. While the weight of the liver, kidney and spleen in the exposed group was lower than in the control group, in the pancreas it was the contrary. However, clarification of the cause of this effect is not possible based on our experiment. For this purpose, it would be appropriate to carry out a new experiment, during which some other variables would be monitored, in particular the amount of consumed food and the level of physical activity of mice.
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